Screening of metabolites and the antimicrobial potential of endophytic Actinobacteria isolated from medicinal plants

By

S Pradeep
30EE21A26008

Thesis Submitted to the

Academy of Scientific & Innovative Research

For the award of the degree of

MASTER OF TECHNOLOGY

In

Computational Biology and Bioengineering

Under the Supervision of
Dr. Syed G. Dastager

CSIR-National Chemical Laboratory (NCL), Pune 411008, India

Academy of Scientific and Innovative Research AcSIR Headquarters,

CSIR-HRDC campus Sector 19, Kamla Nehru Nagar,

Ghaziabad, Uttar Pradesh. – 201002, India

May 2023

Table of contents

Certificate

This is to certify that the work incorporated in this Thesis entitled, " **Screening of metabolites and the antimicrobial potential of endophytic Actinobacteria isolated from medicinal plants**)," submitted by S Pradeep, to the Academy of Scientific and Innovative Research (AcSIR), in partial fulfillment of the requirements for the award of the Degree of Master of Technology in (COMPUTATIONAL BIOLOGY AND BIOENGINEERING), embodies original research work carried out by the student. We certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources and used in this research work has been duly acknowledged in the thesis. Image, illustration, figure, table, etc., used in the thesis from other sources, have also been duly cited and acknowledged.

It is also certified that this work done by the student, under my supervision, is plagiarism free.

(Signature of Student)

Mr. S Pradeep

15.05.2023

(Signature of Supervisor)

Dr. Syed G. Dastager

15.05.2023

Declaration

I hereby declare that the work of the thesis entitled, "**Screening of metabolites and the antimicrobial potential of endophytic Actinobacteria isolated from medicinal plants**", submitted for the degree of M. Tech in Computational Biology and Bioengineering to AcSIR has been carried out by me at NCIM-resource centre, CSIR-National Chemical Laboratory, Pune-411008, Maharashtra, India. Under the supervision of Dr. Syed G. Dastager and my DAC members Dr. Mahesh S. Dharne, Dr. V. Koteswara Rao and DAC chairperson Dr. V.V Bokade.

This work is original and has not been submitted in part or full by me for any other degree or diploma to any other university or institute.

Date: - 15.03.2023

S Pradeep

(Research fellow AcSIR)

Acknowledgement

I am immensely grateful to my supervisor Dr. Syed G. Dastager, for his constant guidance and support throughout my M. Tech coursework as well as projects. He has constantly encouraged me to focus on achieving my goal and move forward with the investigation in-depth.

My special thanks to Dr. Mahesh S Dharne, Head of NCIM, for supporting and motivating me to carry out research at NCIM.

I thank my DAC members Dr. Mahesh S. Dharne, Dr. V. Koteswara Rao, and Dr. V.V Bokade, for their guidance and suggestions towards the progress of work. I would like to thank AcSIR for giving me the opportunity to pursue M. Tech in CSIR- NCL.I want to thank AcSIR engineering coordinator, Dr. Ram Rup Sarkar for his constant support during M. Tech degree.

I would like to thank Director, CSIR- NCL, for providing all the facilities and making the laboratory environment safe and healthy. I thank the Head of, the Biochemical sciences division for all the support required during my course work of M. Tech and I also Thank Library personnel and staff for their support during my coursework in issuing books.

I want to thank the Student academic office (SAO) office for their continuous efforts for all my AcSIR and other academic-related documentation and processing.

I thank Mr. Abujunaid Khan for his timely suggestions to complete my M. Tech thesis.

I also thank colleagues and the NCIM staff for their support.

I am grateful to God and my parents for allowing me to carry out distant study.

Keywords

S. aureus - *Staphylococcus aureus*

E. coli - *Escherichia coli*

C. albicans - *Candida albicans*

BCR 10 - *Streptomyces cinereoruber*

YA - *Streptomyces pluricolorescens*

NCIM - National Collection of Industrial Microorganisms

ATCC - American Type Culture Collection

AMR - Antimicrobial resistant strain

LPM - Litres per minute

Fig - Figures

PCR - Polymerase chain reaction

F – Forward primer

R - Reverse primer

min - minute

hrs - hours

Screening of metabolites and the antimicrobial potential of endophytic Actinobacteria isolated from medicinal plants

Abstract

A largely unexplored source of possible endophytic Actinobacteria includes unique bioactive substances associated with medicinal plants. Endophytic Actinobacteria, primarily the members from the *Streptomyces* genus, are known to be the key source of novel antimicrobial compounds since ancient past. The present research primarily focuses on screening and isolation of the endophytes from the roots and tubers of the *Aloe vera* (*Aloe barbadensis miller*) and turmeric plant (*Curcuma longa*). The isolates were identified on the basis of morphology, Gram character and sequencing of the 16S rRNA gene. Of the seven promising isolates, two most potent ones, *viz.* YA and BCR 10 were the major source of inhibition against the test pathogens. In particular, the crude extract of BCR 10 was further explored for its antimicrobial potency toward *Escherichia coli* (NCIM-2065), *Staphylococcus aureus* (NCIM-2127), and *Candida albicans* (NCIM-3102). Additionally, the activity of this crude extract was tested against several standard ATCC antimicrobial resistant (AMR) strains such as *Enterobacter cloacae (BAA-2468)*, *Escherichia coli (*BAA-2469), *Klebsiella pneumoniae* (BAA-2470), *Escherichia coli* (BAA-2471), *Klebsiella pneumoniae (*BAA-2472), and *Klebsiella pneumoniae* (BAA-2473). It was observed that the crude extract was capable of inhibiting BAA-2473, thereby proving its candidacy as a potent antimicrobial compound, especially against drug resistant Gram-negative microbes. Furthermore, to scale-up the yield of this crude extract, fermentation, optimization of the process parameters, and the downstream purification was carried out.

Keywords: - *Streptomyces*, AMR strain, Medicinal plant, Bioactive compound, fermentation

Chapter 1 - Introduction

Actinobacteria also known as Actinomycetes comprise of a phylum of Gram-positive bacteria. These bacteria are found mostly in the terrestrial and aquatic (including marine) habitats as free-living organisms [1], and are characterized by a distinctive filamentous appearance and high DNA guanine and cytosine (G+C) content, and a heterotrophic mode of nutrition [2]. Actinomycetes are considered to be an intermediate link between bacteria and fungi. Many Actinobacteria grow as mycelium, much like filamentous fungus, and many of these mycelial Actinomycetes reproduce through sporulation. These bacteria have displayed an exceptional capacity to produce a variety of bioactive secondary metabolites [3]. Some of the most diverse bacteria ever identified, includes *Streptomyces, Thermobifida,* and *Frankia* are found in the numerous mycelial species of Actinobacteria. For three key reasons, *Streptomyces* has drawn the most interest among the three genera. The first is that *Streptomycetes* are widespread and significant in soil. They play significant roles in the cycling of carbon locked in insoluble organic detritus, mainly from plants and fungi. The creation of several hydrolytic exo-enzymes is what makes this activity possible.

Moreover, the evolutionary range of the genus is rather broad [4]. Finally, *Streptomycetes* are among Nature's best chemists and create an amazing variety of bioactive secondary metabolites. As a result, they are highly valued in business and medicine [5].

Actinobacteria are widely known for producing primary and secondary metabolites that are used in a variety of industries. They are also a prospective source for various crucial enzymes that are mass-produced. Actinomycetes are widely renowned for being an endless source of antibiotics. Most currently used antimicrobials were first discovered in Actinomycetes, particularly in the genus *Streptomyces* [6]. They create immunomodifiers that improve immune response as well as enzyme inhibitors that are helpful in the treatment of cancer [7].

Actinobacteria that reside inside plants and appear to have no obvious effects on them are known as endophytic Actinobacteria. Some *Actinobacteria* have specialized functions, such as guarding the host plants against pests and illnesses. The nitrogen-fixing bacteria of non-leguminous plants, endophytic Actinobacteria, make up a sizable portion of the rhizosphere and are also present inside plants, with the well-investigated species coming from the genus *Frankia* [8]. An effective countermeasure to the growing dangers of medication resistance against human

and plant infections is screening endophytic Actinomycetes for their functional purpose. It is widely known that medicinal plants with a long history of ethnobotany are good candidates for isolating powerful endophytic bacteria since these organisms are crucial for the development of the therapeutic properties of the plants [9]. Endophytic Actinomycetes, particularly those found in healthy, surface-sterilized tissues, are thought to be a possible source for creating secondary metabolites, a variety of organic compounds having antibacterial, antioxidant, and plant growth-promoting properties [10].

As mentioned earlier, According to studies, microbial communities have a high potential for producing new bioactive chemicals that have applications in agriculture, pharmaceuticals, and other fields [11]. Thus, the separation of endophytes from medicinal plants is significant. The production of a wide variety of bioactive metabolites by endophytic Actinobacteria, including antibacterial, anticancer, immunosuppressive, and other medicinal substances, is well recognized.

The current work primarily focuses on the isolation of endophytes from the root and tuber parts of the turmeric plant (*Curcuma longa*) and *Aloe vera* (*Aloe barbadensis miller*). Gram staining, DNA isolation, 16S rRNA gene sequencing, and Phylogeny are used to characterize the isolates. Seven endophytic Actinobacteria (*Streptomyces*. sp) were identified from eight isolates after being evaluated for metabolite production and bioactivity profiling against *Escherichia coli* (NCIM-2065), *Staphylococcus aureus* (NCIM-2127), and *Candida albicans* (NCIM-3102). *Streptomyces cinerreoruber*, a potent endophyte active against *Escherichia coli* (NCIM-2065) with a higher inhibition zone was screened with the antimicrobial resistance strain (AMR), *Enterobacter cloacae* (BAA-2468), *Escherichia coli* (BAA-2469), *Klebsiella pneumoniae* (BAA2470), *Escherichia coli* (BAA-2471), *Klebsiella pneumoniae* (BAA-2472), *Klebsiella pneumoniae* (BAA-2473).

Chapter 2 - Plant collection and isolation of endophytic Actinobacteria

2.1 Plant collection and Surface sterilization

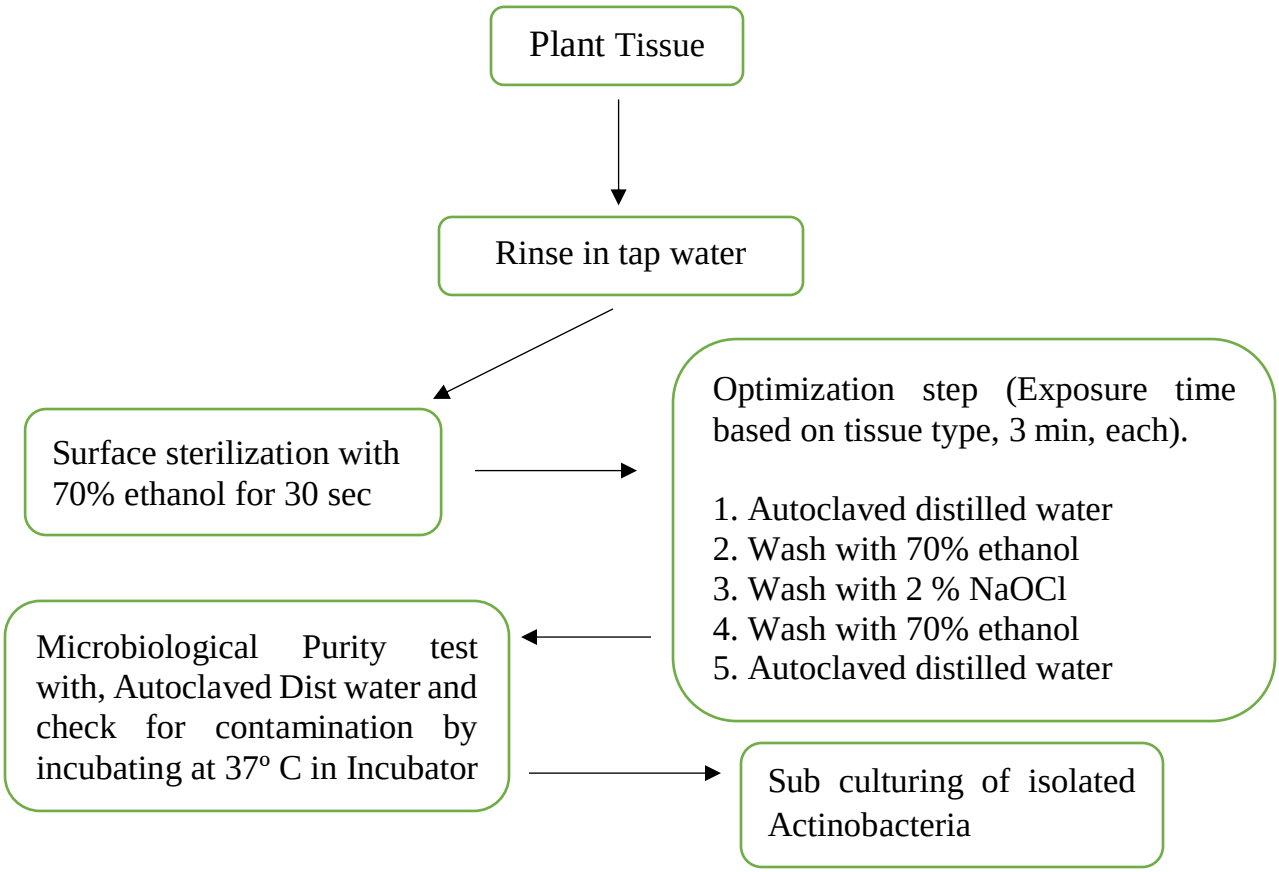
In India, the states of Rajasthan, Gujarat, Tamil Nadu, Andhra Pradesh, and Maharashtra are where *Aloe vera* is most commonly found [12]. It contains ingredients that have the potential to be active, including vitamins, minerals, enzymes, and anthraquinones, which have unique features that qualify it as a therapeutic plant. Turmeric has been employed in several medicinal formulations throughout the years all around the world. Turmeric is said to provide a number of medicinal advantages, including increasing physical energy, lowering gas, getting rid of worms, and improving digestion. The yellow spice turmeric has medicinal and preventative properties, particularly effective against cancer. [13].

Aloe vera plant (*Aloe barbadensis miller*) and the Turmeric plant (*Curcuma longa*). (Fig 1&2) were collected from Vadodara, Gujarat, India. For the isolation of Actinobacteria. Coordinates: 22º 21´ 39.942´´N, 73º 8´ 2.636´´E. Within 24 hours of collecting the plant, the experiment was set up with the root and tuber parts of the plant.

Fig. 1 *Aloe vera* (Leaf, stem and root)

Fig. 2 Turmeric plant (Leaf, stem, root, and tuber)

Flow-chart for the sterilization of the plant's surface for endophyte isolation



2.2 Actinobacteria isolation from Medicinal plants

All endophytic bacteria were isolated from medicinal plants were streaked onto different well-defined agar plates.

Isolation Media

Table 1: - Actinomycetes Isolation Agar (AIA)

Ingredients	Gms/Litre
Sodium caseinate	2.0
L-Asparagine	0.1
Sodium propionate	4.0
Dipotassium phosphate	0.5
Magnesium sulphate	0.1
Ferrous sulphate	0.001
Agar	15.0
Final pH (at 25° C)	8.1±0.2

Table 2: - ISP-1 Agar medium

Ingredients	Gms/Litre
HiVeg hydrolysate	5.0
Yeast extract	3.0
Agar	20
Final pH (25°C)	7.0±0.2

Table 3: - Reasoner's 2A Agar (R2A)

Ingredients	Gms/Litre
Casein acid hydrolysate	0.5
Yeast extract	0.5
Proteose peptone	0.5
Dextrose	0.5
Starch, soluble	0.5
Dipotassium phosphate	0.3
Magnesium Sulphate	0.024
Sodium pyruvate	0.3
Agar	20.0
Final pH (at 25°C)	7.2±0.2

Table 4: - ISP-2 Agar medium

Ingredients	Gms/Litre
Peptone	5.0
Yeast extract	3.0
Malt extract	3.0
Dextrose	10.0
Agar	20.0
Final pH (25°C)	6.2±0.2

Table 5: - ISP-3 Agar medium

Ingredients	Gms/Litre
Oat meal	20.0
**Trace salts solution (ml)	1.0 ml
Agar	18
Final pH (25° C)	7.3±0.2

(**Contains in 1 ml of trace salt solution 0.001 gm of each ferric sulphate heptahydrate, Magnesium chloride tetrahydrate, and Zinc sulphate heptahydrate)

Table 6: - ISP-4 Agar medium

Ingredients	Gms/Litre
Starch, soluble	10.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate heptahydrate	1.0
Sodium chloride	1.0
Ammonium sulphate	1.0
Calcium Sulphate	2.0
Ferrous sulphate heptahydrate	0.001
Manganous chloride heptahydrate	0.001
Zinc sulphate Heptahydrate	0.001
Agar	20.0
Final pH (25° C)	7.3±0.2

Table 7: - ISP-5 Agar medium

Ingredients	Gms/Litre
L-Asparagine	1.0
Dipotassium hydrogen phosphate	1.0
**Trace salts solution (ml)	1.0 ml
Agar	20
Final pH (25° C)	7.4±0.2

(**Contains in 1 ml of trace salt solution 00.001 gm of each ferric sulphate heptahydrate, Magnesium chloride tetrahydrate, and Zinc sulphate heptahydrate)

Table 8: - ISP-6 Agar medium

Ingredients	Gms/Litre
HiVeg peptone	15.0
HiVeg peptone No.3	5.0
Yeast extract	1.0
Ferric ammonium citrate	0.5
Dipotassium phosphate	1.0
Sodium thiosulphate	0.08
Agar	15.0
Final pH (25° C)	6.7±0.2

Table 9: - ISP-7 Agar medium

Ingredients	Gms/Litre
L-Asparagine	1.0
L-Tyrosine	0.5
Dipotassium phosphate	0.5
Magnesium sulphate.$7H_2O$	0.5
Sodium chloride	0.5
Ferrous sulphate.$7H_2O$	0.01
Trace solution (ml)	1.0
Agar	20.0
Final pH (25°C)	7.3±0.1

Sub-culturing Media

Table 10: - MGYP- agar medium

Ingredients	Gms/Litre
Malt extract	3.0
Dextrose	10.0
Yeast extract	3.0
Peptone	5.0
Agar	20.0
Final pH (25° C)	7.0±0.2

Parts of the plant were dissected and plated in several well-defined isolations (Fig. 3) conditions after being surface sterilized. As one of the isolation media plates was tested for effectiveness, no microbial growth was seen (Fig.4&5). After a 24-hour incubation at 37 °C, Actinobacterial colonies are chalky and leathery, adhere firmly to the agar surface, and produce hyphae resembling conidia and sporangia in a culture medium [14]. Similar chalky and leathery characteristics were seen in *Aloe vera* root (ISP4, ISP6, and R2A) (Fig. 6) and turmeric plant rhizome (ISP7 and R2A) (Fig.7), following incubation in well-defined media.

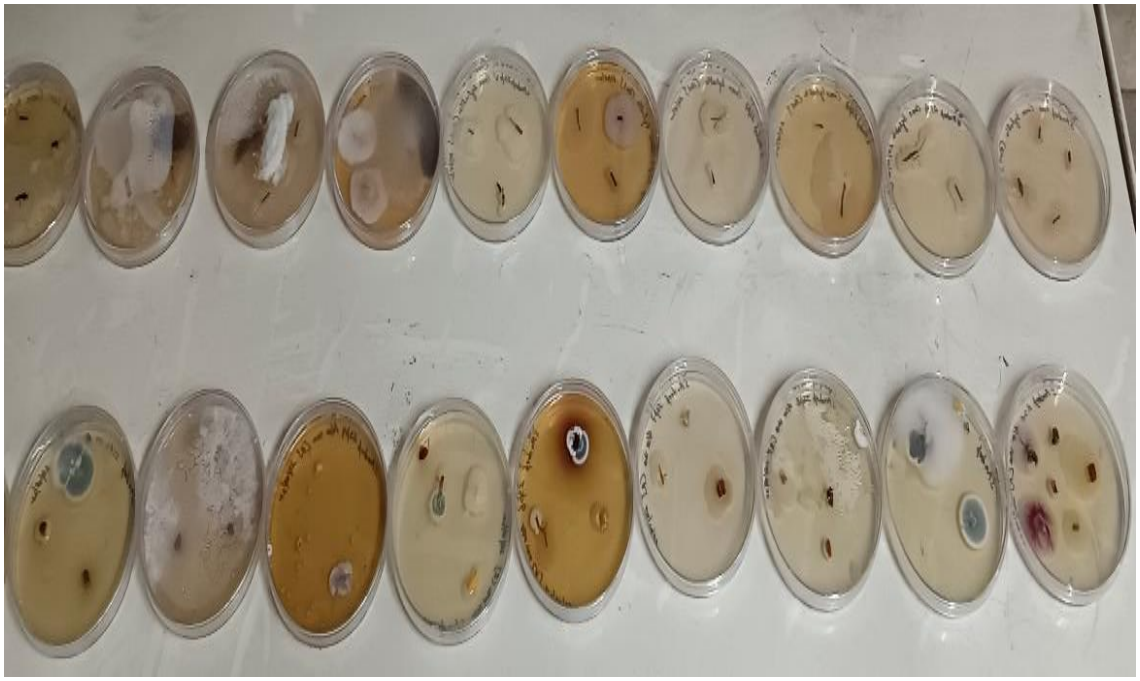


Fig. 3 shows the dissection of a plant (*Aloe vera* and Turmeric) into well-defined media (ISP1, 2, 3, 4, 5, 6, and 7), AIA, and R2A.

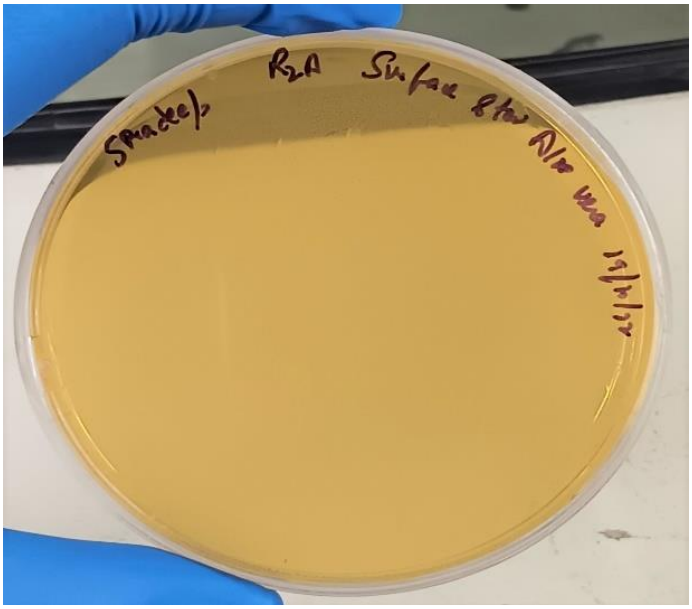


Fig. 4 Effectiveness of microbial growth, dissected *Aloe vera* plant

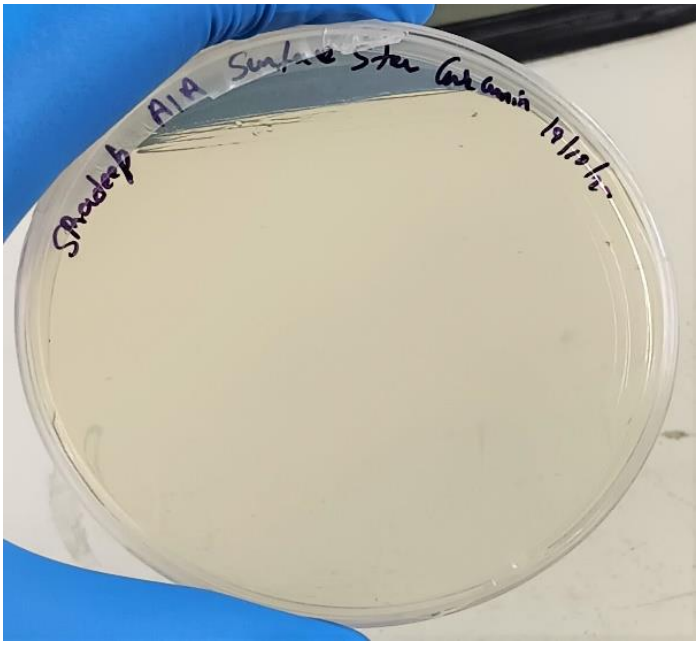


Fig. 5 Effectiveness of microbial growth, dissected Turmeric plant

Fig. 6 Highlighted spot denotes the *Aloe vera* root's leathery and chalky appearance and dissection.

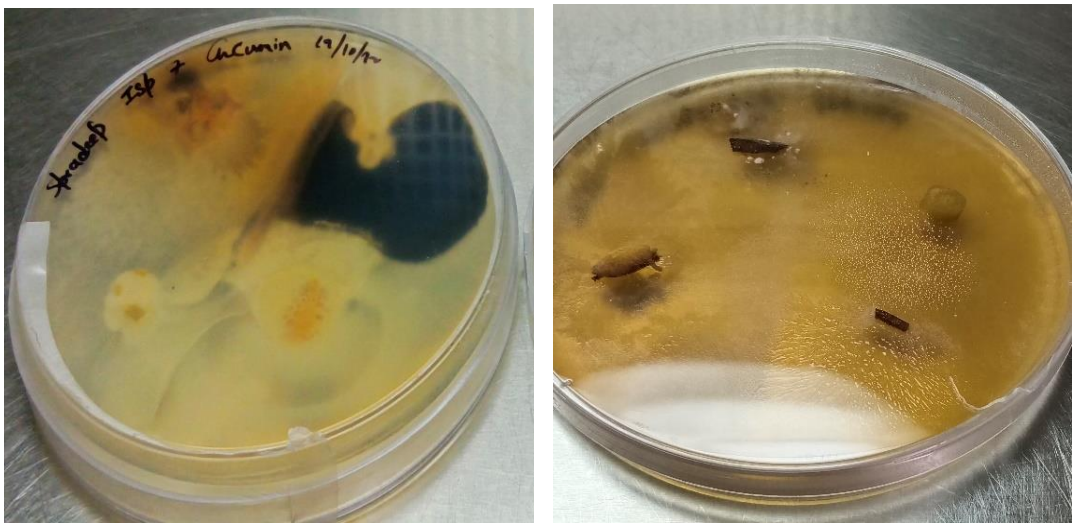


Fig. 7 Chalky and leathery appearance of the ISP7 and R2A rhizomes of the Turmeric plant.

2.3 Differential staining and morphological characteristics of isolates

To differentiate two kinds of microorganisms based on their cell wall nature

Gram Staining: - Technique to distinguish bacteria into Gram-positive and Gram-negative based on peptidoglycan layer, using (crystal violet, iodine, decolorizer, and safranin). Fig (8, 9, 10, 11, 12, 13&14).

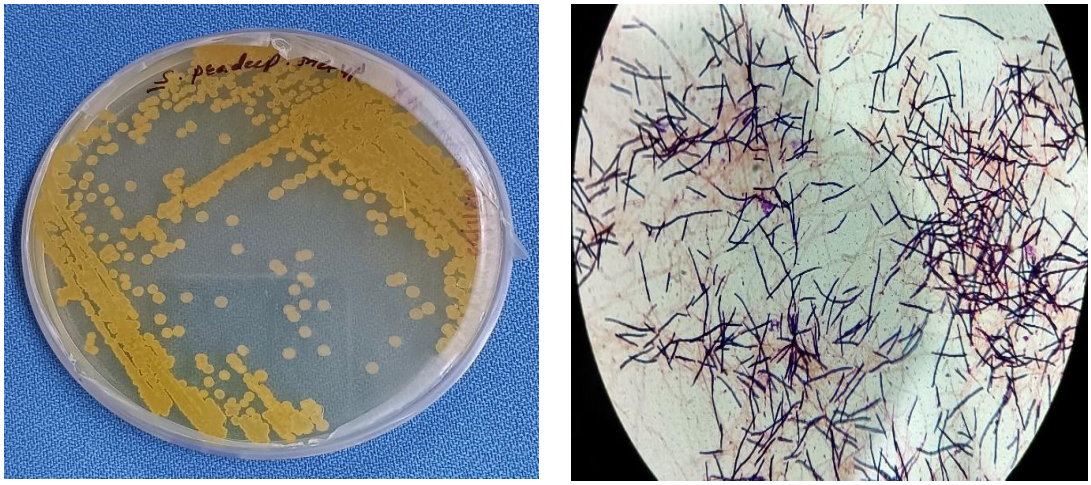


Fig. 8 Gram-positive Filamentous rod-shaped (YA)

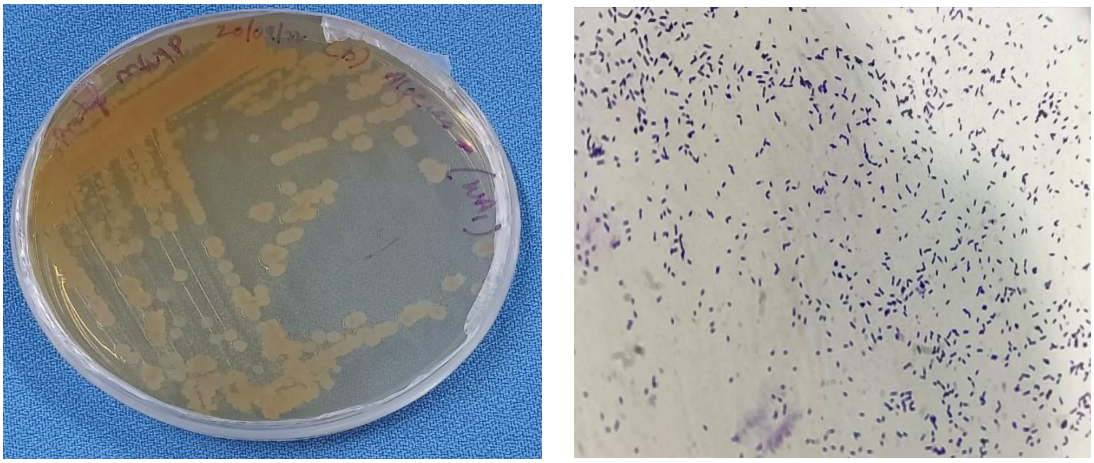


Fig. 9 Gram-positive, short rod-shaped (WA)

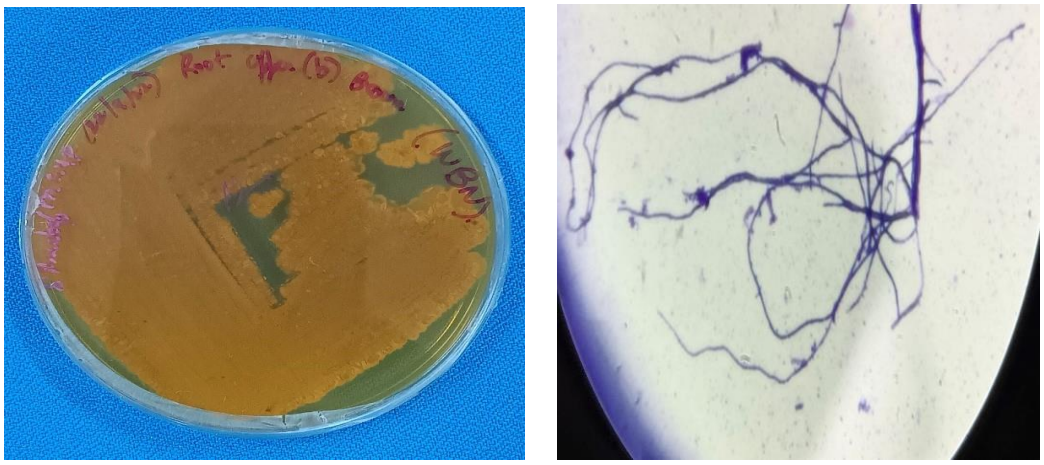


Fig. 10 Gram-positive, long filamentous shaped (WBN)

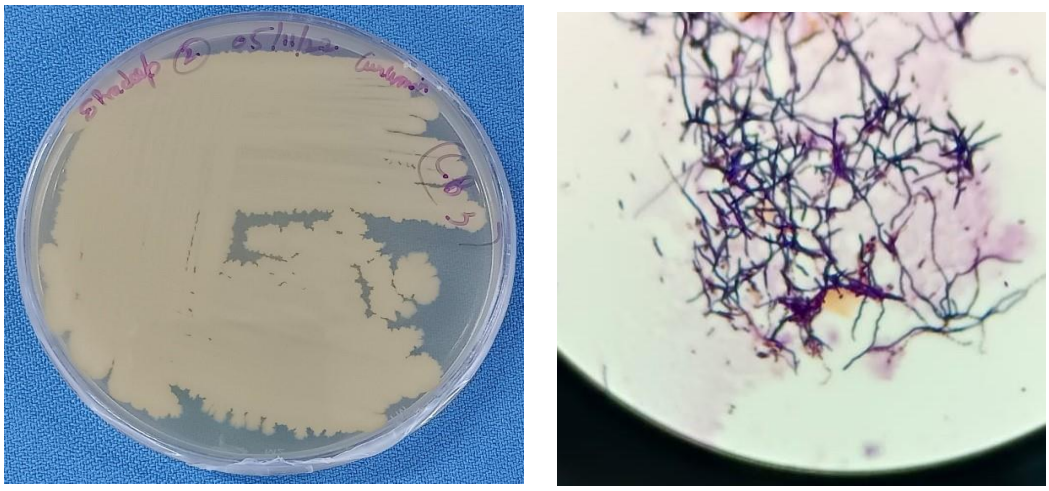


Fig. 11 Gram-positive, rod-shaped filamentous (CB3)

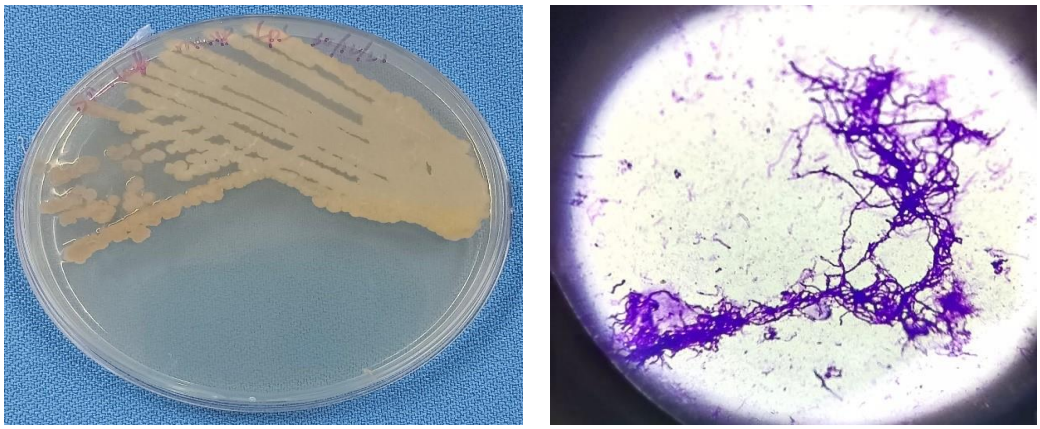


Fig. 12 Gram-positive filamentous shaped (LP6)

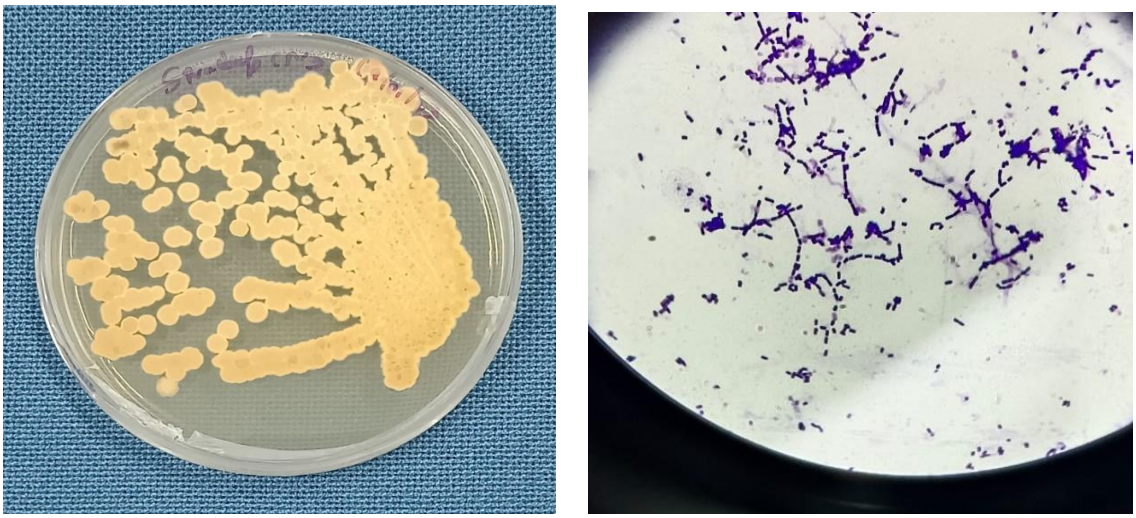


Fig. 13 Gram-positive rod-shaped (CP8)

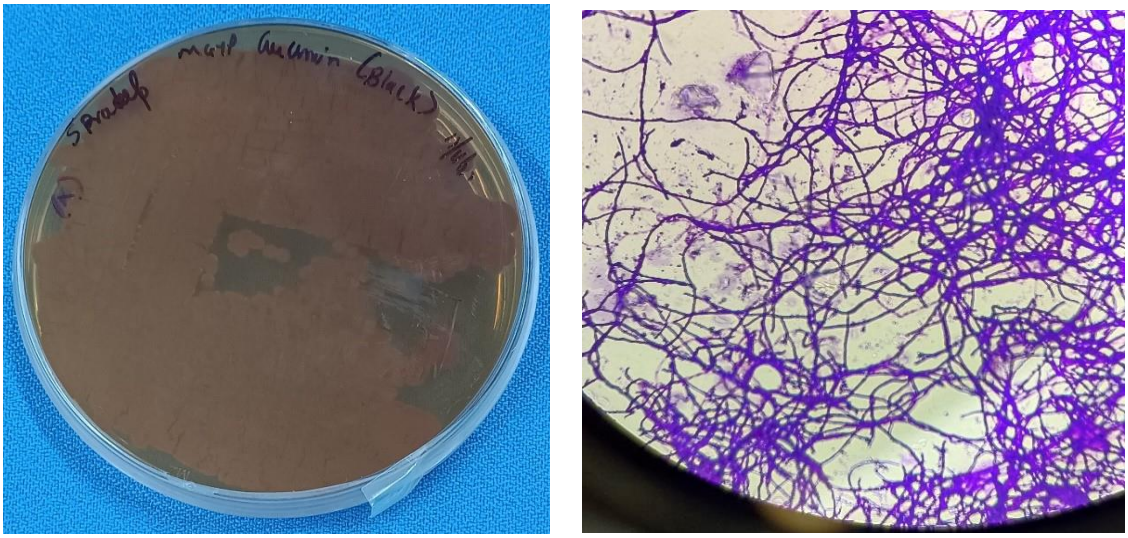


Fig. 14 Gram-positive filamentous shaped (BCR 10)

2.4 Maintenance and Preservation of Culture

Pure culture isolates were next inoculated in MGYP broth and incubated at 28 °C, 150 rpm for four days to achieve pure culture using the streaking technique, as illustrated in (Fig. 15, 16, 17, 18, 19, 20& 21).

Afterward, glycerol stock was made to keep our isolates at -80 °C.

1. Prepare 50 % glycerol stock and autoclave it.
2. Take cryo vials that are sterile for preservation.
3. Add 800 µL of Glycerol stock to the cryovials.
4. Add an equal volume of the incubated cultures to the vials using a micropipette. Gently mix the mixture, then seal the vial's top using parafilm and store at -80 °C.

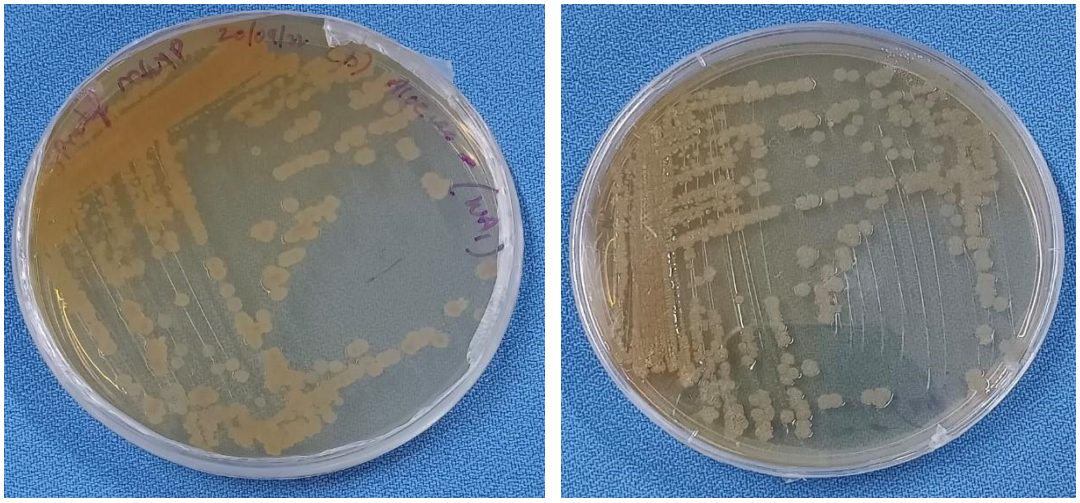


Fig. 15 Isolate from *Aloe vera* root part (WA)

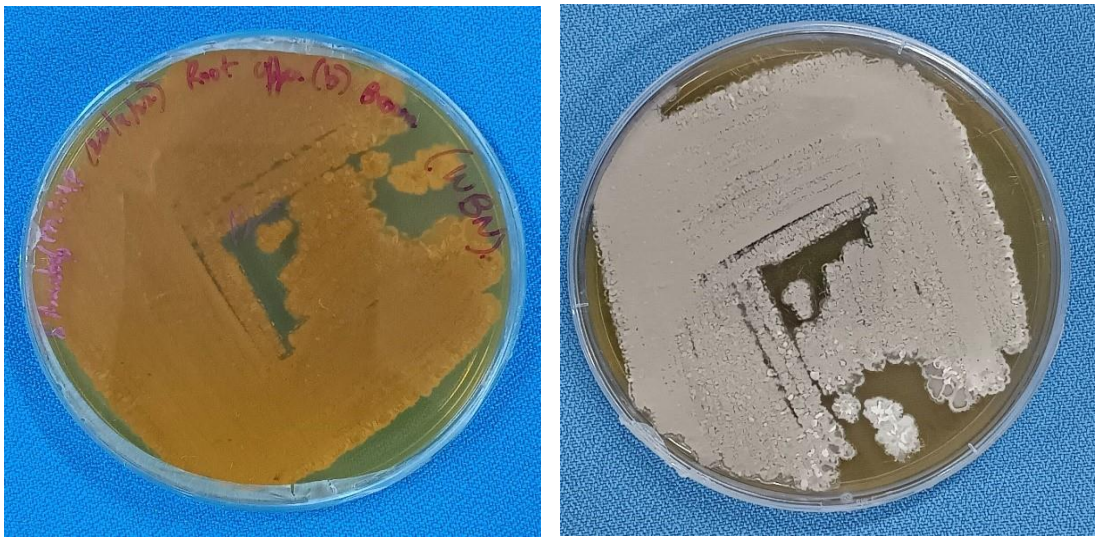


Fig. 16 Isolate from *Aloe vera* root part (WBN)

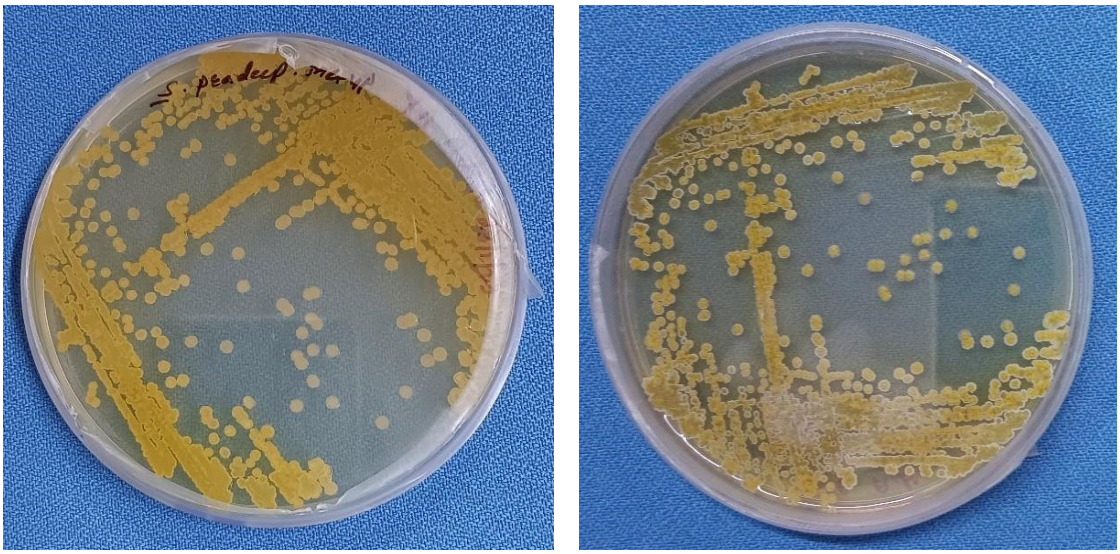


Fig. 17 Isolate from *Aloe vera* root part (YA)

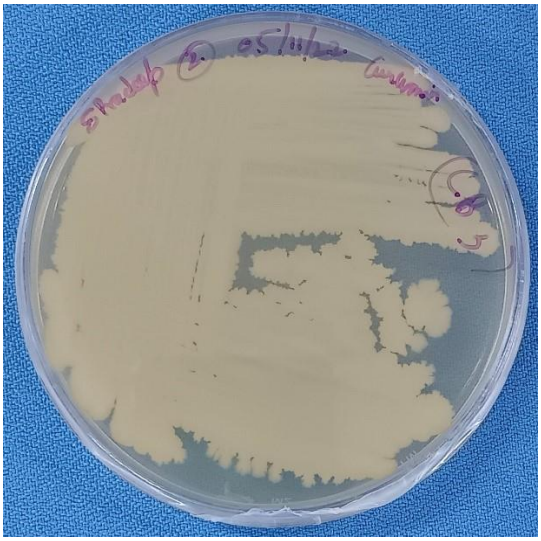


Fig. 18 Isolate from Turmeric plant rhizome part (CB3)

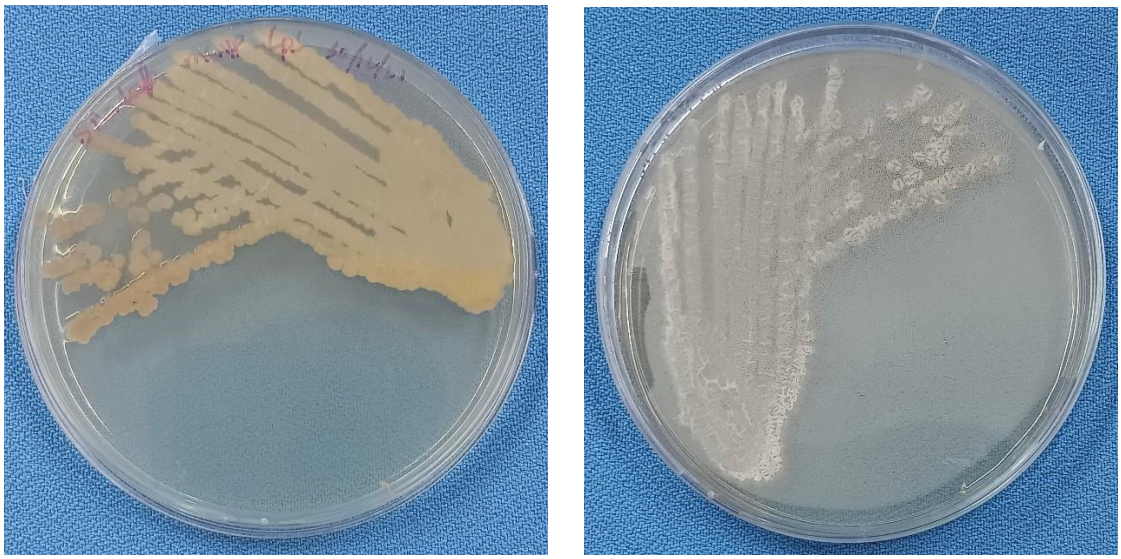


Fig. 19 Isolate from Turmeric plant rhizome part (LP6)

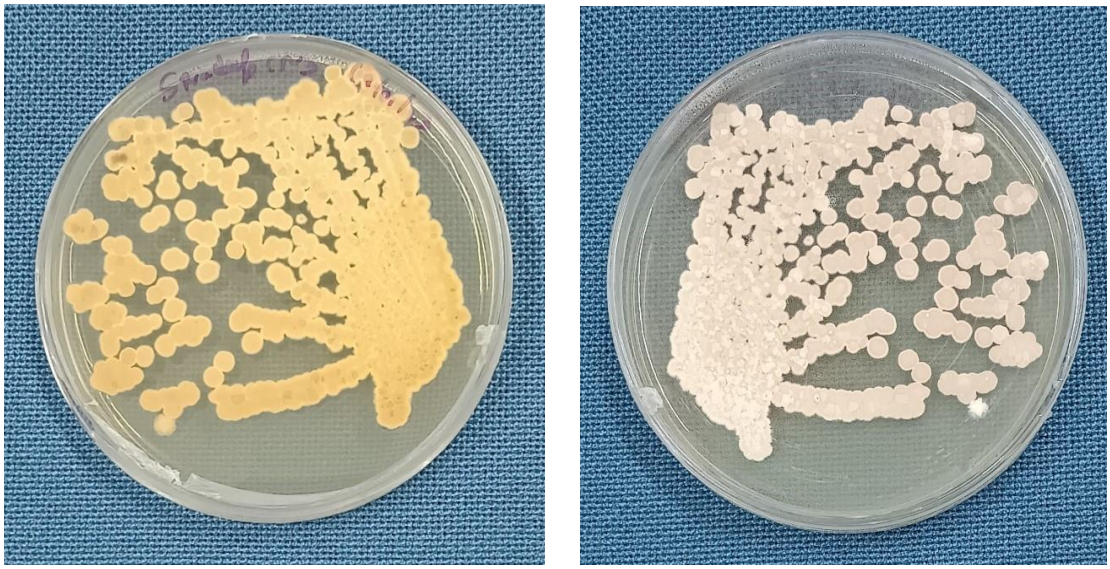


Fig. 20 Isolate from Turmeric plant rhizome part (CP8)

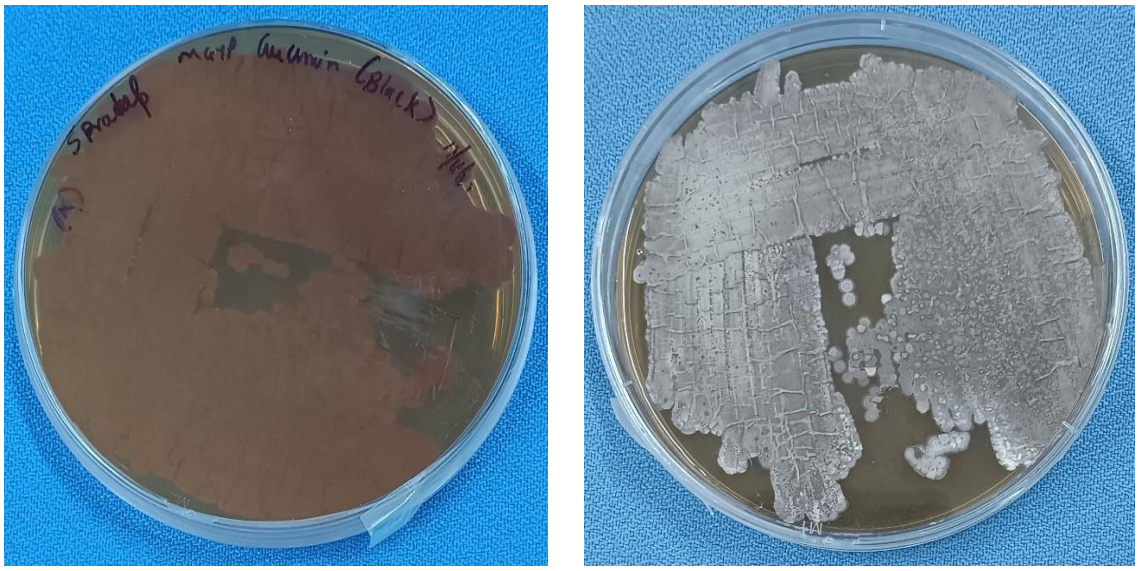


Fig. 21 Isolate from Turmeric plant rhizome part (BCR 10)

Chapter 3 - Antimicrobial screening and molecular identification

3.1 Antimicrobial screening of endophyte isolates

Isolated Actinobacteria which were sub-cultured on MGYP were inoculated in the Liquid Cultivation media (LCM), as a seed culture to carry out the fermentation of the cultures in the volume of 50 ml, and these cultures were incubated at 28 ºC, under shaking condition for four days at 150 RPM.

Table 11: - LCM composition

Ingredients	Gms/Litre
Soya meal	20.0
Mannitol	20.0
Dextrose	4.0
Final pH (25° C)	7.0±0.2

The grown cultures were transferred from LCM to the metabolite production media (50 ml), 10 % of the seed culture, which is 5 ml, was transferred to the production media, and the flasks were incubated at 28ºC, 150 rpm for ten days.

Table 12: - Metabolite production media 5333

Ingredients	Gms/Litre
Starch soluble	15.0
Yeast extract	4.0
Magnesium Sulphate.$7H_2O$	0.5
Dipottasium hydrogen phosphate	1.0
Final pH (25° C)	7.0±0.2

Table 13: - Metabolite production media 5254

Ingredients	Gms/Litre
Dextrose	15.0
Soya meal	15.0
Yeast extract	5.0
Calcium carbonate	2.0
Sodium chloride	5.0
Final pH (25° C)	7.0±0.2

Table 14: - Metabolite production media 5294

Ingredients	Gms/Litre
Starch	10.0
Dextrose	10.0
Glycerol	10.0
Yeast extract	4.5
Peptone	2.0
Calcium carbonate	3.0
Sodium chloride	1.0
Final pH (25° C)	7.0±0.2

Metabolite extraction protocol

1. The metabolite was extracted from *Aloe vera* and turmeric plant isolates on days 4, 7, and 10 of the experiment, which was carried out in duplicates (Fig 22, 23& 24).

2. In 250 ml extraction bottles, 50 ml of fermented culture that was 4, 7, and 10 days old was combined with twice as much ethyl acetate (Fig 25, 26& 27)

3. Extraction bottles were kept inside a shaking incubator at 150 rpm and 28 º C for 30 minutes in the

4. The organic layer was separated for the Extraction at 40 º C, 90 rpm, and 120 mbar.

5. Finally, the extract was dissolved in 1 ml of ethyl acetate and transferred into a sterile micro centrifuge tube.

6. Extract was screened for antimicrobial activity as mentioned in (Table 15, 16, 17& 19)

Below.

7. (Table 18 & 20) includes screening with Antimicrobial-resistant strains.

Fig. 22 Fourth day of culture YA, WBN, and WA extraction in 5333 media

Fig. 23 Seventh day of culture YA, WBN, and WA extraction in 5333 media

Fig. 24 Tenth day of culture YA, WBN, and WA extraction in 5294 media

Fig. 25 Culture BCR10 in 5333, Extraction using ethyl acetate, carried out in duplicates on the fourth day.

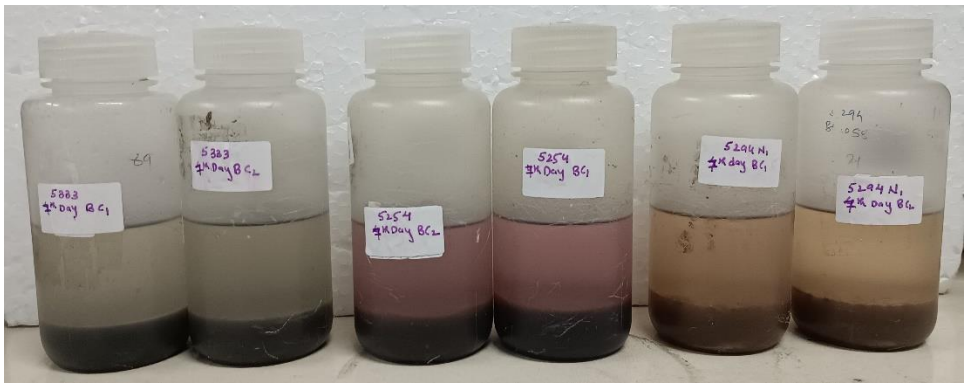


Fig. 26 Culture BCR10 in 5254, Extraction using ethyl acetate, carried out in duplicates on the seventh day.

Fig. 27 Culture BCR10 in 5294, Extraction using ethyl acetate, carried out in duplicates, On the tenth day.

Agar well diffusion method

As determined by zone of inhibition, MGYP and MHA agar petri plates were used to test the efficacy of metabolites from WBN, YA, and WA isolates of Aloe Vera and BCR 10 isolates of Curcuma longa against test strains of *E. coli, S. aureus,* and *C. albicans*.

The Zone of Inhibition implies the test strain's susceptibility or resistance to the BCR 10 extract. Zones of inhibition against *Staphylococcus aureus* (NCIM-2127) of 4th, 7th, and 10th day extracts in 5294 medium appeared to demonstrate increased efficacy of inhibition of more than 20 mm

(Fig. 28, 31, & 35) after the screening of metabolites from BCR 10 (50 µL sample) against test strains and antimicrobial resistant bacteria.

On 4, 7, and 10th day extracts in 5294 medium also seemed to be effective in inhibiting *Candida albicans* (NCIM-3102) by more than 15 mm (Fig. 30, 33, & 37).

On the seventh day, extract in 5333 media showed the maximum potency of more than 40 mm of inhibition zone against *Escherichia coli*, showing bactericidal activity with a clear zone even after three days (Fig. 33), which yielded our interest in studying its efficacy against Gram-negative AMR test strains.

Table 15: - Screening for antimicrobial activity of WBN isolate

Extract day	*S. aureus (*NCIM-2127)			*E. coli* (NCIM-2065)			*C. albicans* (NCIM-3102)		
	5333	5254	5294	5333	5254	5294	5333	5254	5294
4	+++	+	+++	-	-	-	-	-	-
7	++	+++	++	-	-	-	-	+++	-
10	++	+	+++	-	-	-	-	-	+++

(Performed in duplicate, ten percent seed inoculation, zone of inhibition in mm: > 10 mm = +; 10-15 mm = ++, >15 mm = +++, h = hazy)

Table 16: - Screening for antimicrobial activity of YA isolate

Extract day	*S. aureus (*NCIM-2127)			*E. coli* (NCIM-2065)			*C. albicans* (NCIM-3102)		
	5333	5254	5294	5333	5254	5294	5333	5254	5294
4	+	+	+++	+	-	+	+	-	-
7	+++	+++	+++	++ h	-	-	-	+	-
10	+++	+++	+++	+	-	+	+	-	++

(Performed in duplicate, ten percent seed inoculation, zone of inhibition in mm: > 10 mm = +; 10-15 mm = ++, >15 mm = +++, h = hazy)

Table 17: - Screening for antimicrobial activity of YA isolate

Extract day	*S. aureus (*NCIM-2127)			*E. coli* (NCIM-2065)		
	5333	**5294 (W)**	**5294 (N)**	5333	5294 (W)	5294 (N)
4	+++	**+++**	+++	+++ h	+++ h	+++ h
7	+++	+++	+++	+++ h	+++ h	+++ h
10	+++	+++	+++	+++ h	+++ h	+++ h

(Performed in duplicate, twenty percent seed inoculation, zone of inhibition in mm: > 10 mm = +; 10-15 mm = ++, >15 mm = +++, h = hazy. W and N represent similar media composition where in N (Corn strip replaced with Yeast extract))

Table 18: - Antimicrobial strains activity of potent YA isolate from 5294

ATCC Culture	4^{th} day extract	7^{th} day extract	10^{th} day extract
BAA-2468	++	+	+
BAA-2469	**+++**	**+++**	**++**
BAA-2471	++	++	++
BAA-2472	++	+	+
BAA-2473	++	++	+

Zone of inhibition in mm: > 10 mm = +; 10-15 mm = ++, >15 mm = +++

BAA-2468 - *Enterobacter cloacae*

BAA-2469 - *Escherichia coli*

BAA-2471 - *Escherichia coli*

BAA-2472 - *Klebsiella pneumoniae*

BAA-2473 - *Klebsiella pneumoniae*

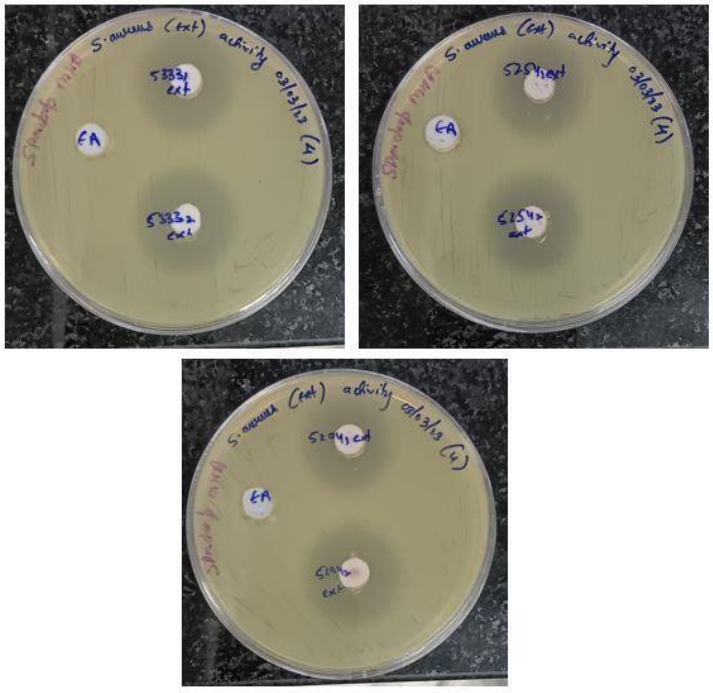


Fig. 28 Zone of inhibition against *S. aureus* (NCIM-2127) of 4th day extract, BCR 10.

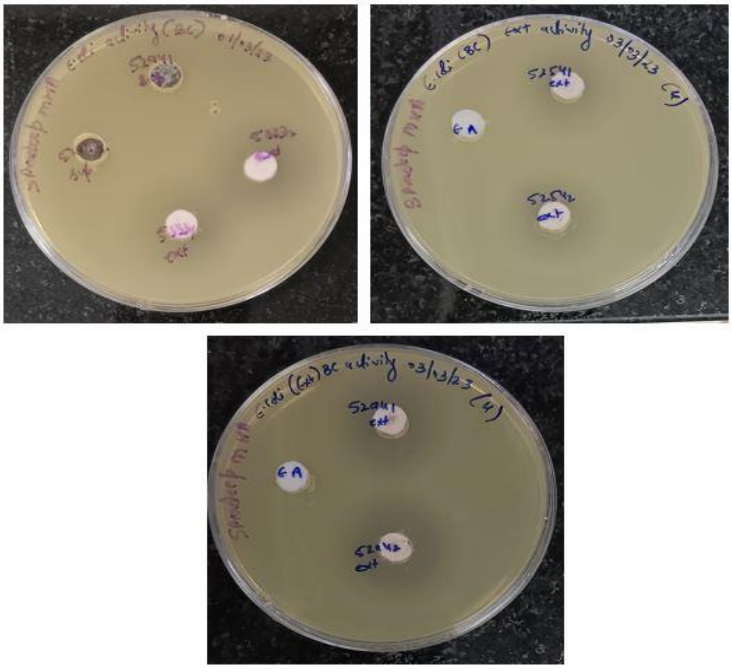


Fig. 29 Zone of inhibition against *E. coli* (NCIM-2065) of 4th day extract, BCR 10.

Fig. 30 Zone of inhibition against *C. albicans* (NCIM-3102) of 4th day extract, BCR 10.

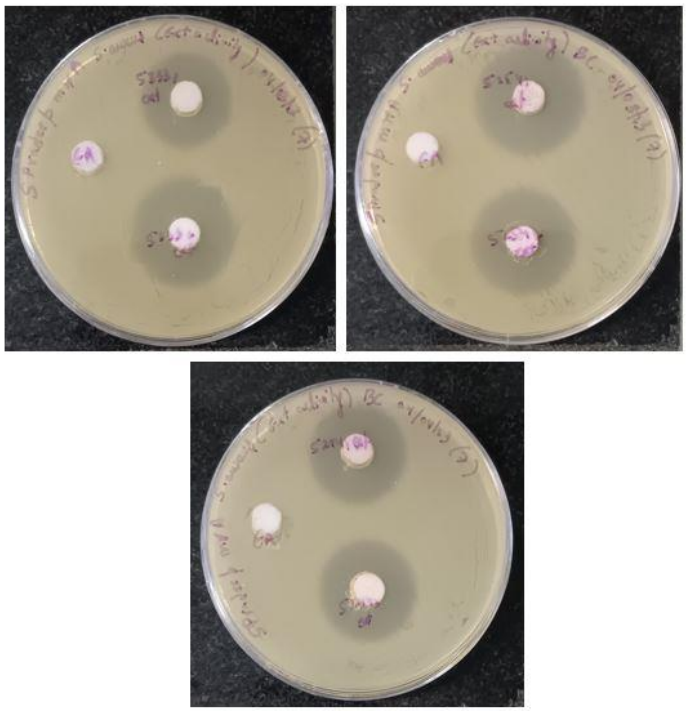


Fig. 31 Zone of inhibition against *S. aureus* (NCIM-2127) of 7th day extract, BCR 10.

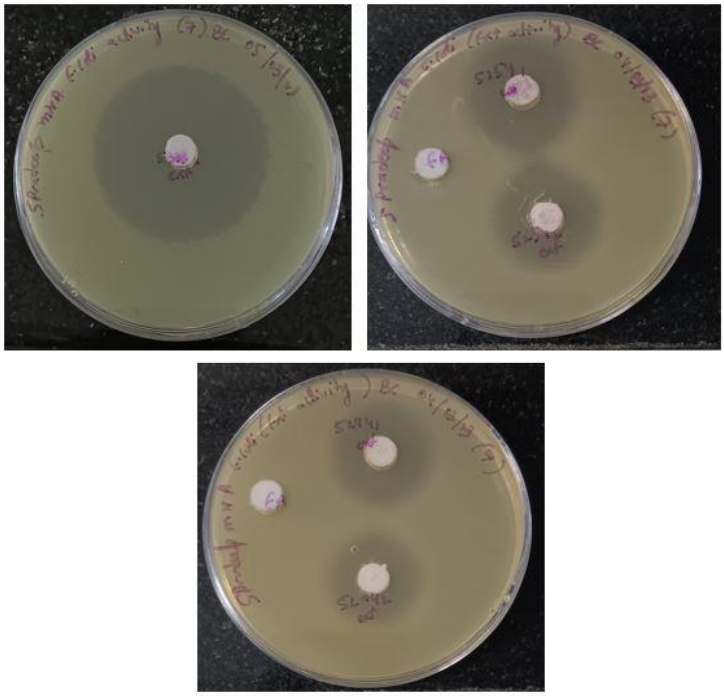


Fig. 32 Zone of inhibition against *E. coli* (NCIM-2065) of 7th day extract, BCR 10.

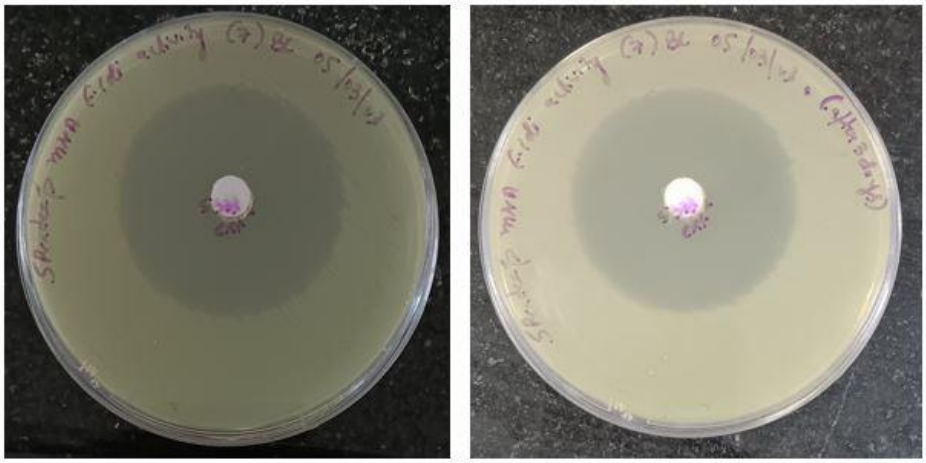


Fig. 33 Comparing the zone of inhibition against *E. coli* (NCIM-2065) of the seventh day's extract with the same plate's results after third day, BCR 10.

Fig. 34 Zone of inhibition against *C. albicans* (NCIM-3102) of 7th day extract, BCR 10

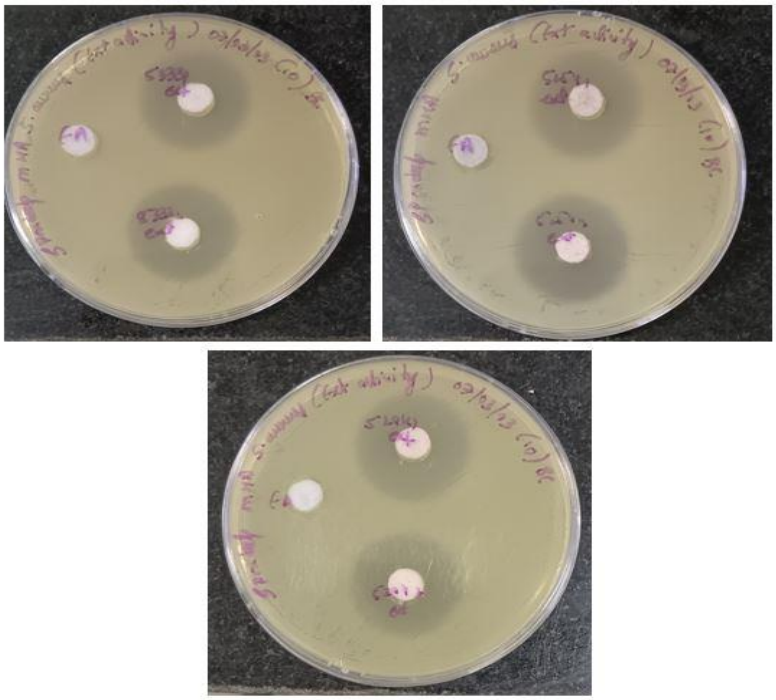


Fig. 35 Zone of inhibition against *S. aureus* (NCIM-2127) BCR of 10th day extract, BCR 10

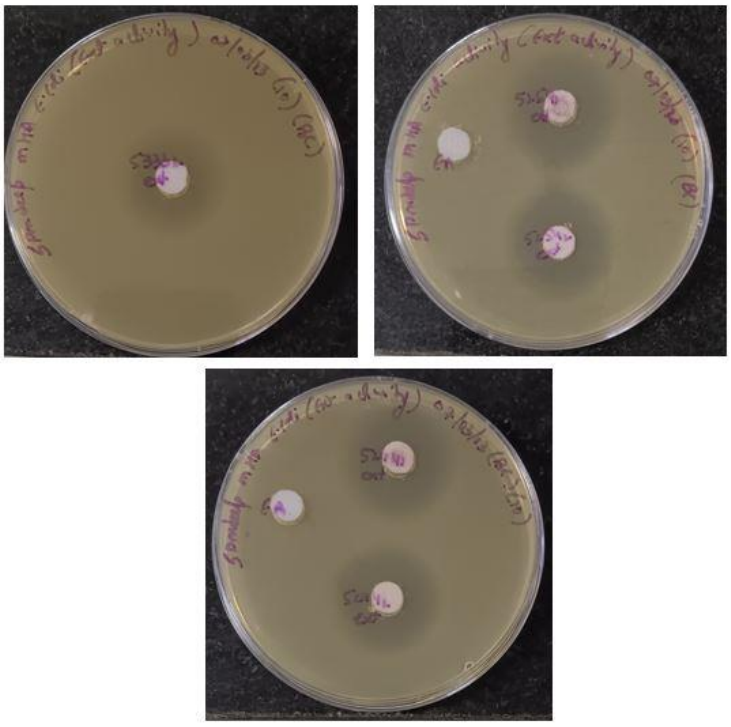


Fig. 36 Zone of inhibition against *E. coli* (NCIM-2065) of 10th day extract, BCR 10

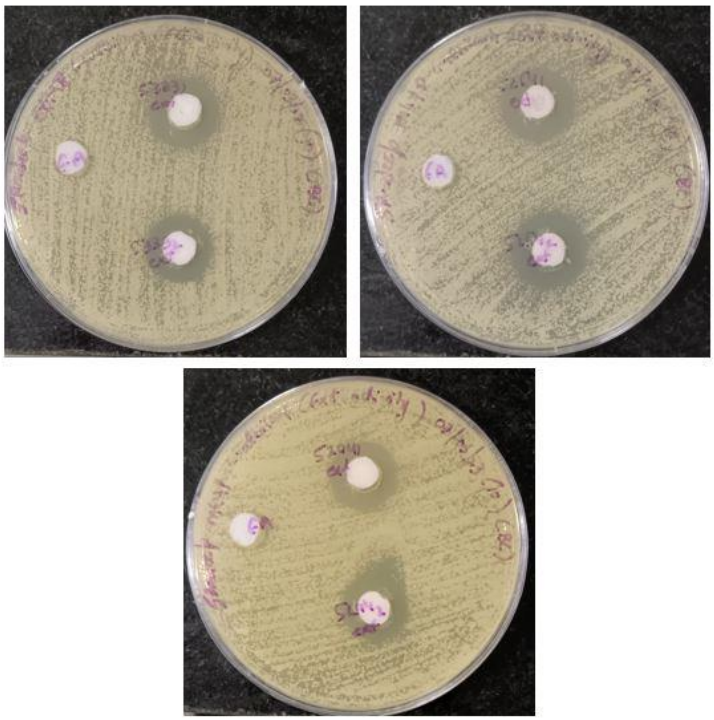


Fig. 37 Zone of inhibition against *C. albicans* (NCIM-3102) of 10th day extract, BCR 10.

Table 19: - Screening for antimicrobial activity of BCR 10 isolate

Extract day	*S. aureus (*NCIM-2127)			*E. coli* (NCIM-2065)			*C. albicans* (NCIM-3102)		
	5333	5254	5294 N	**5333**	5254	5294 N	5333	5254	5294 N
4	+++	+++	+++	++ h	++ h	+++ h	+++	+++	+++
7	+++	+++	++ h	**(+++)**	+++ h	+++ h	+++	+++	+++
10	+++	+++	++ h	+++ h	+++ h	+++	+++	+++	+++

(Performed in duplicate, ten percent seed inoculation, zone of inhibition in mm: > 10 mm = +; 10-15 mm = ++, >15 mm = +++, **(+++)** = highest inhibition zone > 40 mm with bactericidal activity, h = hazy, 5294 N (N represents regular 5294 media with corn strip replaced with yeast extract))

S. aureus - Staphylococcus aureus *C. albicans - Candida albicans*

E. coli - Escherichia coli

Table 20: - Antimicrobial strains activity of potent BCR 10 isolate from 5333, zone of inhibition in mm: > 10 mm = +; 10-15 mm = ++, >15 mm = +++, **(+++)** - highest zone of inhibition.

ATCC Culture	7^{th} day extract
BAA-2468	+++
BAA-2469	+++ h
BAA-2471	+++ h
BAA-2472	+++ h
BAA-2473	**(+++)**

Zone of inhibition in mm: > 10 mm = +; 10-15 mm = ++, >15 mm = +++, (+++) - highest zone of inhibition

BAA-2468 - *Enterobacter cloacae*

BAA-2469 - *Escherichia coli*

BAA-2471 - *Escherichia coli*

BAA-2472 - *Klebsiella pneumoniae*

BAA-2473 - *Klebsiella pneumoniae*

The antimicrobial-resistant strains of Gram-negative bacteria were screened using the seventh-day extract of BCR 10 in 5333 media. Whereby greater than 35 mm clear zone of inhibition was seen against *K. pneumoniae* (BAA-2473) after 24 hours of incubation (Fig.38).

Additionally, as a control, we used the antibiotics amoxycillin and tetracycline to check for an inhibitory zone against the AMR test strain BAA-2473. No Inhibition was observed (Fig. 39).

We may infer that our effective extract of BCR 10 exhibits the highest level of inhibition. In contrast, the control exhibits no inhibition, making our extract can play a role as a result.

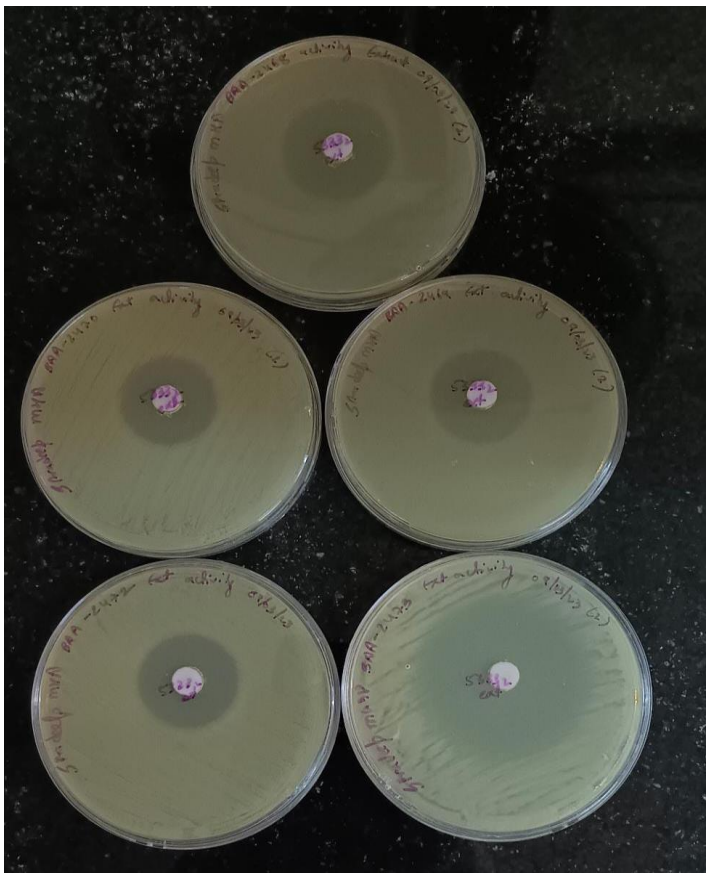


Fig. 38 Efficacy of 7th day extract of BCR 10 against AMR strain.

Fig. 39 Control check for the inhibition.

3.2 Molecular identification of isolates

3.2.1 *Streptomyces* DNA Isolation

1. Transfer four loops of a 48-hour-grown culture to a 1.5 ml centrifuge tube in 200 µL of lysis buffer (LB) for subsequent cultures.

2. Crush the culture before adding another 200 µL of Lysis buffer.

3. The crushed culture should be mixed with 20 µL of RNase before being incubated in a water bath at 65° C for 10 mins.

4. The incubated tubes should be inverted two or three times at room temperature.

5. Add 130 µL of precipitation buffer to the sample, incubate for 5 mins at room temperature, and centrifuge at 13,000 rpm for 5 mins.

6. Transfer the supernatant onto the Hi Shredder tube and centrifuge for 2 mins at 13,000 rpm.

7. Transfer the supernatant onto a 2.0 ml centrifuge tube and add 675 µL of the Binding buffer.

8. Transfer 650 µL of supernatant to the spin column (mm) and centrifuge at 8000 rpm for 1 min.

9. Discard the flow-through, repeat step eight, and discard the flow-through again.

10. Add 500 µL of diluted wash buffer and centrifuge at 13,000 rpm for a min.

11. Run the step ten for an additional 1 min at 13,000 rpm for drying.

12. Transfer only the filter to the new bottom tube, add 50 µL of elution buffer, and incubate for 30 mins at room temperature.

13. Centrifuge at 10,000 rpm for a minute and store the DNA at four degrees

3.2.2 16S rRNA Gene amplification and sequencing

Polymerase chain reaction (PCR) is the technique opted to amplify the nucleic acid sequences with the help of (denaturation, annealing, and extension). In several cycles with 2^n, n denotes the number of amplification cycles.

PCR reaction components:

(i) Reaction buffer - PCR buffer contains $MgCl_2$ salt, whereas in Mg^{2+} divalent cations, stabilizing the enzyme polymerases as cofactors.
(ii) Deoxynucleotide triphosphate (dNTPs) - essential for building the phosphodiester backbone, includes the four nitrogenous bases.
(iii) Template DNA - Is the template DNA sequence to be amplified.
(iv) Primer - PCR uses forward and reverse primers amplifying the forward and reverse strands of the template sequence. 8F and 1542R
(v) Taq DNA polymerase – DNA nucleotidyl transferase helps synthesize DNA sequences obtained from *Thermus aquaticus, a* thermophilic bacterium.

Steps involved in PCR:

(i) Denaturation helps denaturing ds DNA by disrupting hydrogen bonds, resulting in ss DNA at 95 º C.
(ii) Annealing - by using primers that bind to the forward and reverse strands of the template at 55 °C, annealing is accomplished.
(iii) Extension - by adding dNTPs, Taq DNA polymerases synthesize a new DNA strand complementary to the template.

Initial denaturation - 95°C, for 5 min

Denaturation - 95°C, for 30 sec

Annealing - 55 °C, for 1 min Number of cycles is 35

Extension - 72 °C, for 1 min 30 sec 50 µL reaction mixture

Final extension - 72 °C, for 10 min

Table 21: - PCR reaction table

PCR Reagent Mixture	
	For 50 µL reaction
PCR buffer (25mM $MgCl_2$)	5 µL
dNTP's (5mM)	5 µL
Forward primer (pico.mol/µL)	2.5 µL
Reverse primer (pico.mol/µL)	2.5 µL
Taq polymerase (1U/µL)	0.5 µL
DNA template (50-100 ng/µL)	1 µL
PCR water	Adjust to 50 µL

(To ensure the DNA is pure and the concentration is between 50 and 100 ng/µL, a Nanodrop reading is obtained at 260/280 nm to measure absorbance.)

Table 22: - Nanodrop reading to quantify the DNA at 260/280 nm

Isolates	**Absorbance**	**Concentration (ng/µL)**	**Concentration after dilution (ng/µL)**
YA	1.79	630.9	92.4
WA	1.81	277.9	78.3
WBN	1.8	56.3	56.3
CB3	1.78	52.3	52.3
LP6	1.80	489.2	99.6
CP8	1.82	639.9	90
BCR10	1.80	76.5	76.5

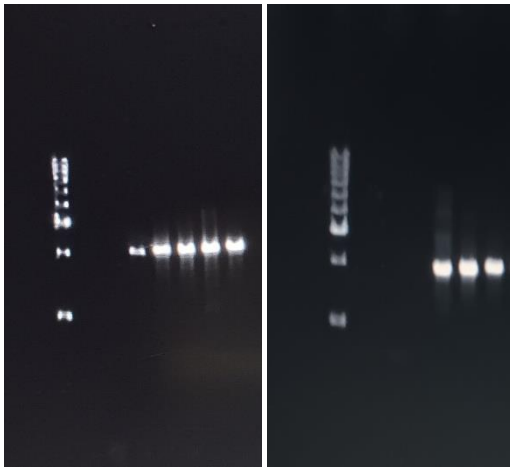
(Following isolates having concentration above the desired range were diluted using Tris EDTA buffer).

Agarose Gel Electrophoresis

It is a molecular biology-based technique to validate the amplified DNA, based on its size and charge, with the help of migration in the presence of the electric field. Migration of DNA depends on molecular weight, wherein low molecular weight migrates faster.

Protocol: -

1. Prepare 0.8% agarose in TBE buffer, cool to 37 ° C, and pour it into the casting tray with the comb.
2. Allow the gel to get solidified, and then the casting tray was transferred to an electrophoretic tank and three fourth filled with TBE buffer.
3. The PCR-amplified product was loaded into the wells with a ladder and negative control.
4. The voltage of 100V was supplied, and bands moved till three fourth portion of the gel.
5. Gel was then visualized under UV light in the gel documentation system (Fig 40).



M 1 2 3 4 5 6 M 1 7 8 9

Fig. 40 Agarose gel, M – DNA ladder 1 Kb, 1 – Negative control, 2&3 are YA, amplified twice.
3-9 are WA, WBN, CB3, LP6, CP8, BCR 10 of 200bp each, and 16S rRNA gene.

PEG-NaCl DNA purification

1. Add an equal amount of PEG NaCl in a 0.5 ml centrifuge tube for a given volume of a PCR reaction.
2. Incubate at 37 ℃ for 30 min in a water bath
3. Centrifuge at 13,000 rpm for 30 min at room temperature
4. Remove the supernatant and discard it with the help of a micropipette
5. Wash the pellet with 125 µL of 70 % cold ethanol
6. Centrifuge for 30 min at room temperature at 10,000 rpm
7. Remove the supernatant and discard it
8. Dry off the pellet to remove traces of 70 % ethanol for 15 min using speed vac.
9. Dissolve the pellet gently in 20 µL of tris EDTA buffer.

16S rRNA gene sequencing

One of the mainstays in the analysis of the genetic makeup of microbial communities has been 16S rRNA gene sequencing. In prokaryotes, the 16S ribosomal RNA has taken on a unique role in the evolution and ecology of microbes. 16S rRNA gene sequence is large enough (1500 bp) to provide information [15] about organisms and is prokaryotes' most prevalent genetic marker.

A DNA sequence known as 16S rRNA codes for the RNA found in bacteria's small ribosomal subunit. All bacteria have the 16S rRNA gene, and all cells, including eukaryotes, have a variant. The 16S rRNA gene of many organisms has been studied. It has been discovered that a region of the molecule rapidly changes genetically, allowing for the differentiation of several species within a single gene.

Amplified PCR (polymerase chain reaction) products of 16S sequences from high-throughput sequencing have frequently been clustered according to similarity to produce operational taxonomic units (OTUs). Representative OTU sequences have been compared with databases referred to determine probable taxonomy. Although valuable and practical, this use of 16S has required some assumptions, such as the now-historical notion that sequences with greater than 95% similarity indicate the same genus and greater than 97% identity represent the same species [16].

First-generation Sanger sequencing was adopted for 16S rRNA gene sequencing. The PCR-amplified target genome was amplified and purified using the PEG-NaCL method and given for Sanger sequencing at NCIM, CSIR-NCL.

(i) DNA isolation
(ii) PCR gene amplification
(iii) PEG-NaCL purification of DNA
(iv) Genetic analyzer (Sanger sequencing) (Thermo), full-length 16S rRNA sequencing

Table 23: - Isolates identified after sequencing and NCBI blast

SL. No	Isolates name	Organism
1	WA	*Streptomyces rubrogriseus* LMG 20318(T)
2	WBN	*Streptomyces violaceorubidus* LMG 20319(T)
3	YA	*Streptomyces pluricolorescens* NBRC 12808(T)
4	CB3	*Priestia filamentosa* SGD-14(T)
5	LP6	*Streptomyces griseoincarnatus* LMG 19316(T)
6	CP8	*Streptomyces roseofulvus* NBRC 13194(T)
7	**BCR10**	***Streptomyces cinereoruber* NBRC 12756(T)**

>F27_1492RC_BCR10 (*Streptomyces cinereoruber*)

CATGCAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAA
CACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACC
GGATACGACCTGCCGAGGCATCTCGGCGGGTGGAAAGCTCCGGCGGTGAAGGATGA
GCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGT
AGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACG
CCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCG
CAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCG
GCTTGTCACGTCGGGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGATACG
GGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCG
CAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGC
TGAGGAGCGAAAGCGTGGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAAC
GCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTG
ACGGGGGGCCCGCACAAGCAGCGGAGCATGTGGGCCCGCACAAGCAGCGGAGCAT
GTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAA
AGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTG
TGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAA
CTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCA
CACGTGCTACAATGGCCGGTACAAAGAGCTGCGATGCCGCGAGGCGGAGCGAATCT
CAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGA
GTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCC
TTGTGGGAGGGA

3.3 Phylogenetic analysis

EzBioCloud database

EzBioCloud is a comprehensive database containing the taxonomic framework of archaea and bacteria, represented by high-quality 16S rRNA gene and genome sequences. With the help of the accompanying bioinformatics tools, this extensive database of the genome, 16S rRNA gene, and taxonomic information could hasten the categorization and identification of archaea and bacteria based on their genomes. One may access the database and associated search tools at http://www.ezbiocloud.net/ [17].

The database's 16S-based ID option was chosen, the 16S rRNA gene sequence was submitted for analysis, and the corresponding hit was found. This database gives us information on top-hit taxon, strain, similarity, and completeness that is clinically important.

MEGA 11

From the ezbiotaxon, to carry out taxonomical analyses, completeness of greater than 95%, full-length 16S sequence [18], and average nucleotide identity greater than 98.65 % [19] is to be considered for making a phylogenetic tree. From the ezbiotaxon, the top ten hit strains (table 24) were considered for making a phylogenetic tree in Fasta format. Multiple sequence alignment using CLUSTAL W and Neighbor-joining, a distance-based methodology, was adopted for constructing a phylogenetic tree [20].

Table 24: - Top hit strains, 16S rRNA gene sequencing by ezBiotaxon

Hit strain name	**Hit strain name**	**Accession**	**Similarity**	**Completeness (%)**
Streptomyces cinereoruber subsp. cinereoruber	NBRC 12756(T)	AB184121	100.00	99.7
Streptomyces violaceorectus	NBRC 13102(T)	AB184314	99.64	99.9
Streptomyces showdoensis	NBRC 13417(T)	AB184389	99.42	99.3
Streptomyces bikiniensis	NRRL B-1049(T)	JNWL01000107	99.35	100.0
Streptomyces viridobrunneus	LMG 20317(T)	AJ781372	99.35	100.0
Streptomyces tanashiensis	LMG 20274(T)	AJ781362	99.06	100.0
Streptomyces nashvillensis	NBRC 13064(T)	AB184286	99.06	99.9
Streptomyces litmocidini	NBRC 12792(T)	AB184149	98.99	99.5
Streptomyces hydrogenans	JCM 4771(T)	BNBS01000241	98.92	100.0
Streptomyces gardneri	NBRC 12865(T)	AB249908	98.92	100.0

Phylogeny

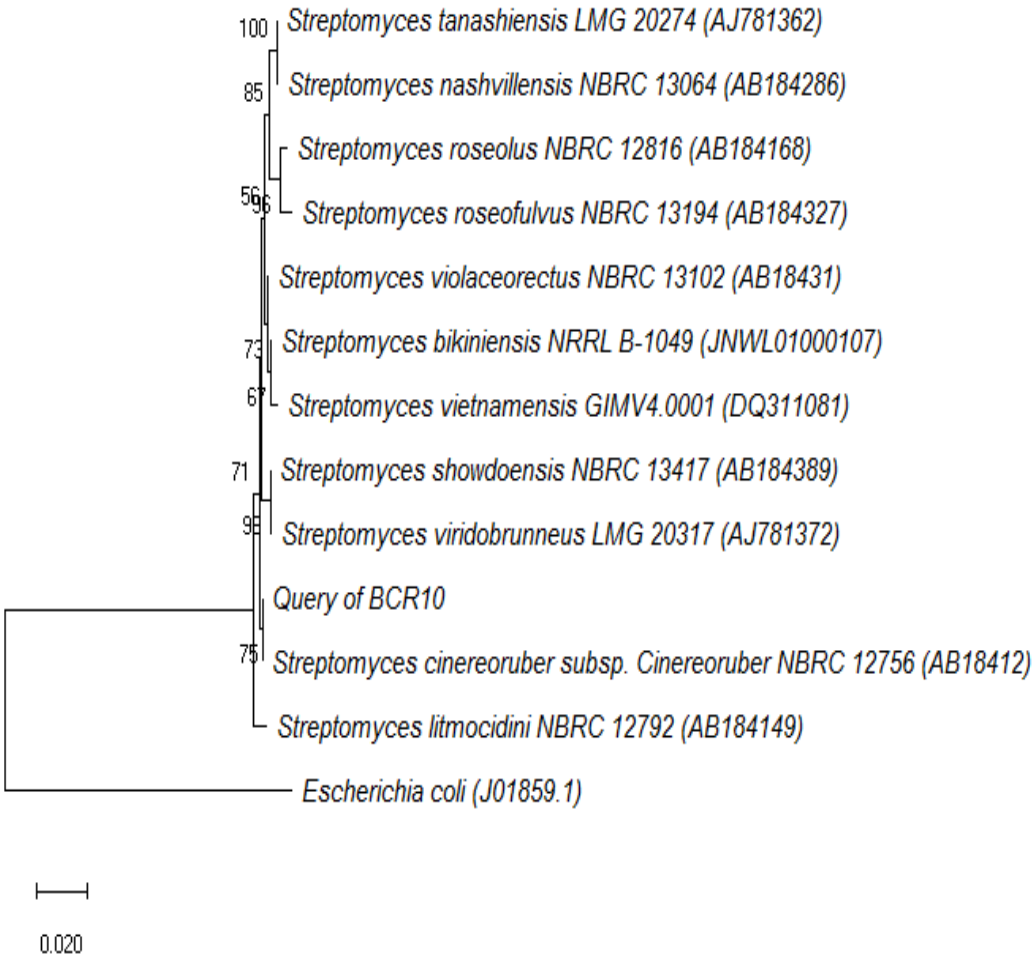


Fig. 41 Phylogenetic tree by Neighbor-joining method for Query BCR10, with its closest reference, along with *Escherichia coli* as an outgroup.

Chapter 4 - Fermentation and partial purification of bioactive compound

4.1 Pilot Scale Fermentation of BCR 10 culture

Scale up from the metabolite screening at Erlenmeyer flask to 14 L fermenter (Brunswick Bioflow ® CelliGen® 115 benchtop fermenter, Eppendorf AG, Germany) (Fig. 42), which is equipped with online monitoring and control dissolved oxygen (DO), pH, temperature, Agitation, and air supply, with a working volume of 10L, in which 9 L of 5333 fermentation media in distilled water and 10 % culture volume, i.e., 1 L of BCR 10 culture.

The application of microorganisms to chemically break down the substrate in the presence of oxygen to create metabolites at the flask level is what a batch bioreactor known as a fermenter does.

Bioreactor and its components

The primary role of a bioreactor is creating an ideal environment for microorganism growth and acquiring the intended product, while the components involved in the fermenter depend on the following factors:

- The microorganism utilized and its nutritional requirements
- The physical conditions demanded like temperature, pH, and agitation speed
- The yield, Nature, and purity of the desired product
- The aeration and agitation mechanisms for uniform distribution of nutrients

Thus, bioreactors are tailored to specific microorganisms and product requirements by adjusting various parameters to optimize fermentation performance.

Temperature control

In a microbial/mammalian system, growth and production are influenced by the environment and the unit's temperature. However, the fermenter's metabolic activity and various components emit heat during the process, which may be eliminated with the help of a cooling loop or to give more heat with the help of a heating jacket.

Aeration and Agitation

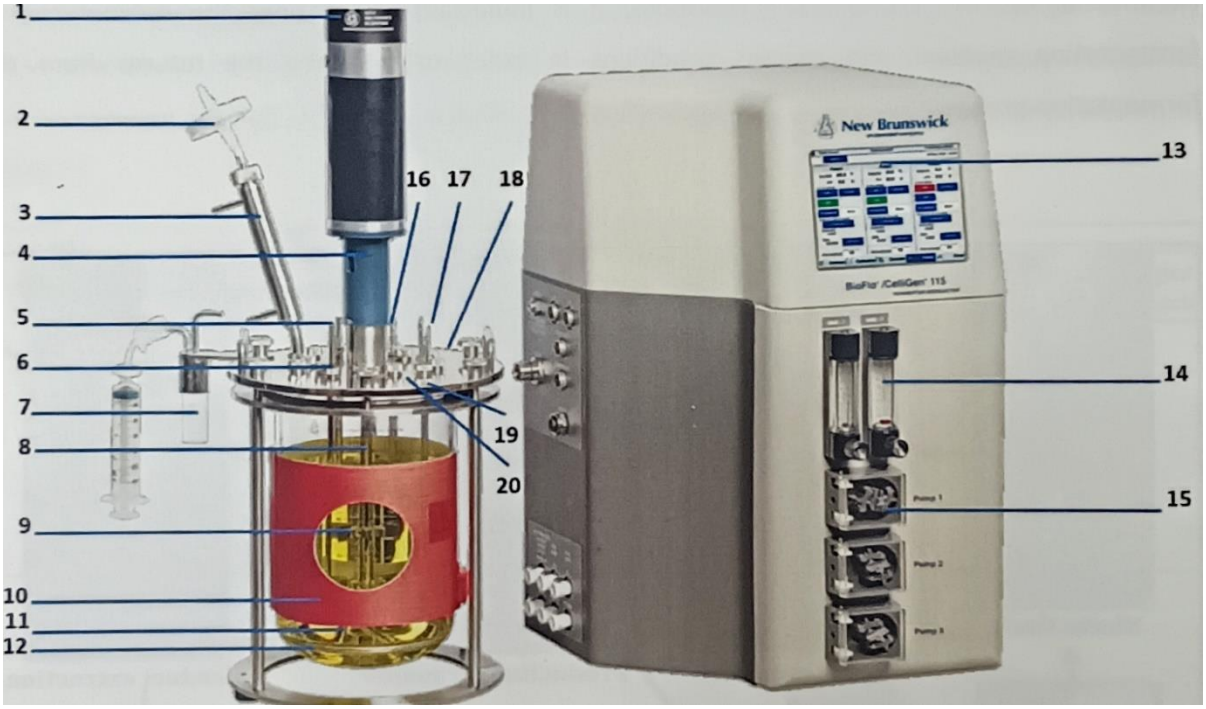
Aeration is to be given in the fermenter at litres per minute (Lpm) to supply microorganisms with enough oxygen for metabolic processes; however, Agitation is provided at rpm to equally distribute the oxygen and nutrients, depending upon the kind of microbe. Usually, for microbial fermentation, macro spargers combined with Ruston turbine-type impellers are employed to provide appropriate mixing and oxygen transport. Moreover, four baffles are inserted to guarantee optimum mixing and minimize vortex formation during Agitation.

Additional ports

Additional ports play a significant role in maintaining the sterility of a fermenter. Applying a positive pressure when using these ports helps maintain the system sanitary. Inoculation port, sample port, and harvest port are just a few ports (Fig. 42). Feed ports are also offered to introduce different components throughout a process, such as an antifoam agent or an acid or base to maintain pH.

Sensor probes

Sensors control pH, temperature, dissolved oxygen, and foam. O-rings are used to secure them in the appropriate ports. Each of these sensors has its features maintained through adjustment. Controlling foam is essential because excessive foaming can contaminate the air and dampen down filters. In our experiment, we employed a 5% silicon-based foam. Safety valves must be installed on all lines operated under pressure to ensure that the pressure stays within the permitted range.



1	Motor	6	Inoculation	11	Sparger	16	harvest
2	Air outlet	7	Sampling	12	Cooling Loop	17	Additional port
3	Condenser	8	Impeller shaft	13	Controlling unit	18	Top lid
4	Mechanical seal	9	Impeller Blade	14	Rota meter	19	DO probe
5	Air inlet	10	Heating Jacket	15	Peristaltic pump	20	Temperature probe

Fig. 42 Production fermenter and its components

Metabolite production in a fermenter

The media component of 10L was made in 9L of distilled water with pH maintained at 7.0 in (5333 media), transferred into the fermenter, and sealed with care to ensure that all valves and ports were closed. The fermenter was cleaned correctly using 70% ethanol. Additionally, 10 mL of Silicon antifoam agent was applied after calibrating the pH probe. Prior to sterilization, a leak test was conducted to make sure there were no leaks. The vessel was then sterilized for 20 minutes at 121°C in a vertical autoclave. Following sterilization, the vessel is pressurized by passing sterile air and maintained under positive pressure throughout the run. Once the medium reached room temperature, prior to inoculation, the sterility was examined, and the DO sensor was calibrated to

100% DO. *Streptomyces cinereoruber* was inoculated aseptically using an inoculation port, and fermentation conditions were applied, as mentioned in (Table 25).

Table 25: - Upstream process parameters optimized for the metabolite production

	Parameters	**Conditions/values**
Inoculum development	Volume	1 L (10 x 500 mL flask with 100 mL LCM media)
	Temperature	28°C
	Shaking	150 RPM (Shaking incubator)
	Duration	Four days
	pH	7± 0.3 (Not maintained during the process)
Metabolite production	Volume	10 L (9 L media + 1 L inoculum)
	Agitation	150 RPM Initially (controlled loop to maintain DO), (Range 150-250 RPM using Ruston turbine impellers was maintained based on DO)
	Aeration	Compressed sterile air of 6 LPM through macro sparger (Adjusted to maintain DO)
	Dissolved oxygen (DO)	Initially saturated to 100% Maintained above 20 % saturation
	Baffles	Yes
	Duration	Two days
	pH	7± 0.3 (Not maintained during the process)

Every 24 hours, Extraction was carried out, followed by testing the bioactive compound's efficacy against the AMR strain (BAA- 2473), and gram staining was used to establish the sample's sterility. When the antibacterial activity of the 24-hour sample was evaluated by loading 50 µL of the sample, it revealed an inhibitory zone identical to that of the flask at a level greater than 35 mm after incubation for 24 hrs at 37 °C.

The same result was obtained after 48 hrs and then employing twice as much ethyl acetate for Extraction in three batches, each producing an inhibition zone greater than 35 mm (Fig.43). For every 500 mL of fermentation broth, 30 mL of extract was obtained.

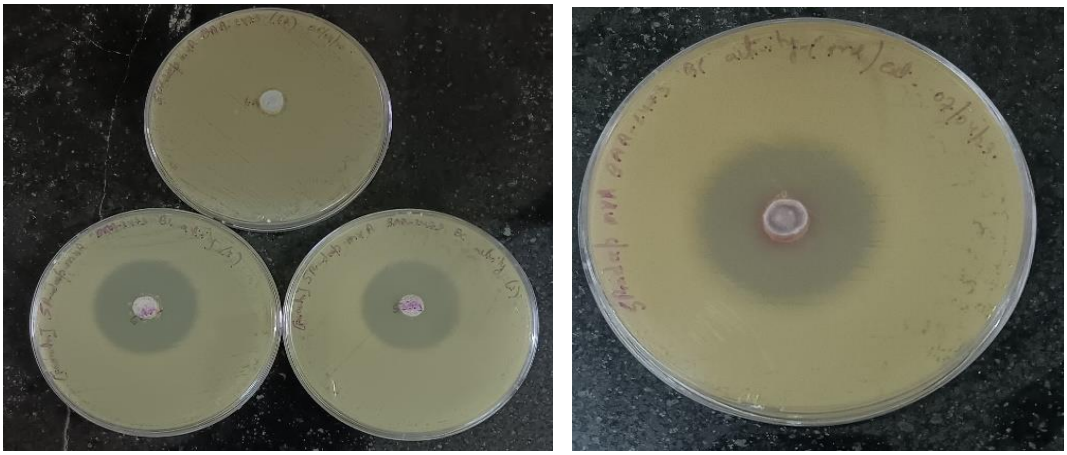


Fig. 43 Inhibition zone of three extraction batches of 48 hrs fermented extract.

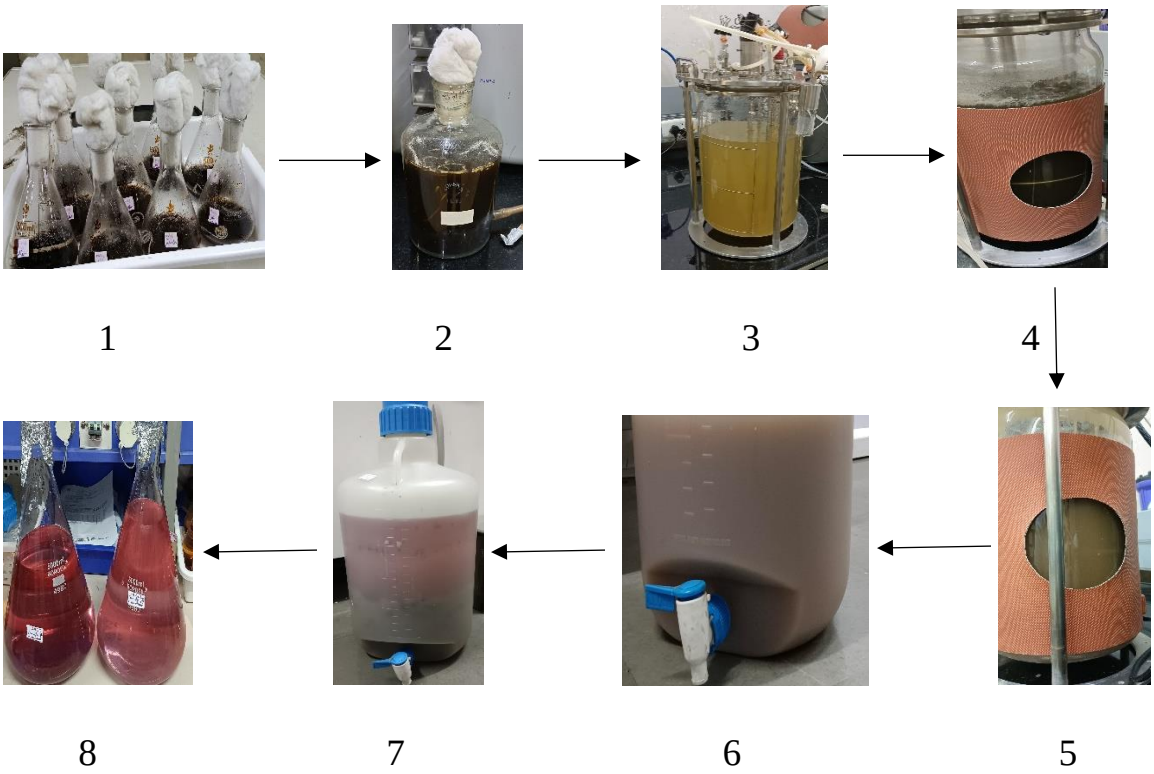
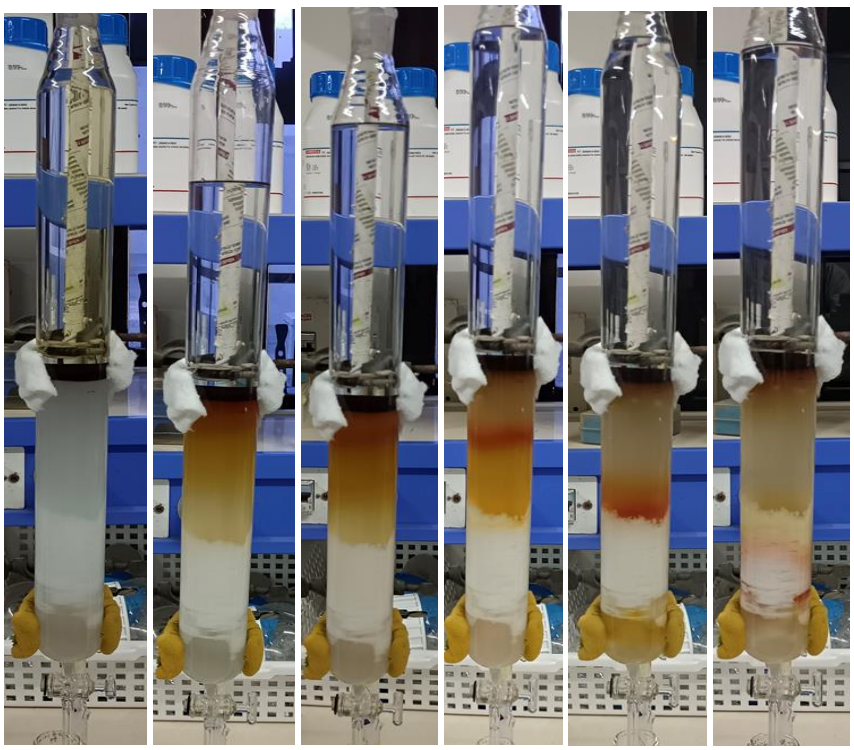


Fig. 44 Representation of pilot scale fermentation, 1 – seed inoculum 10 x 500 mL, 2 – Inoculum bottle 1 L, 3 – Fermenter before seed inoculation, 4- Fermenter after seed inoculation, 5 – Fermenter at 48 hrs. Turning into pale yellow, 6 – Transfer of Biomass for manual shaking, 7 – Ethyl acetate extraction, 8 – Supernatant separation and concentration.

4.2 Purification using column chromatography

The concentrated extract was then dried using silica gel. A column filled with silica gel (100–200 mesh) was used to separate the mixture using methanol and dichloromethane (DCM), starting with 100% DCM and varying concentrations until 50% of each solvent was used as shown in (Fig.45). All the fractions were tested for antibacterial activity against (BAA-2473), the fifth fraction of 3% methanol and DCM (Fig. 46) was found to exhibit inhibition of more than 35 mm, which was equivalent to the results obtained at shake flask level.



1 2 3 4 **5** 6

Fig. 45 Partial purification using column chromatography, fraction 1- 100% DCM, 2- 0.5% methanol, 3- 1 % methanol, 4- 2 % methanol, **5- 3% methanol**, 6- 4% methanol along with DCM.

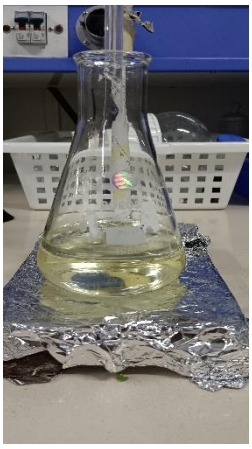


Fig. 46 Collection of the fifth fraction, 3 % methanol, and DCM.

(Utilizing a solvent system of 100% DCM to purify compounds using (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 50, 80 and 100% methanol), DCM was used to make up in volumes up to 500 mL and tested its efficacy against BAA-2473 test strain, showing inhibition as shown in (fig. 47), by loading 30 µL of collected fractions, by agar well diffusion method).

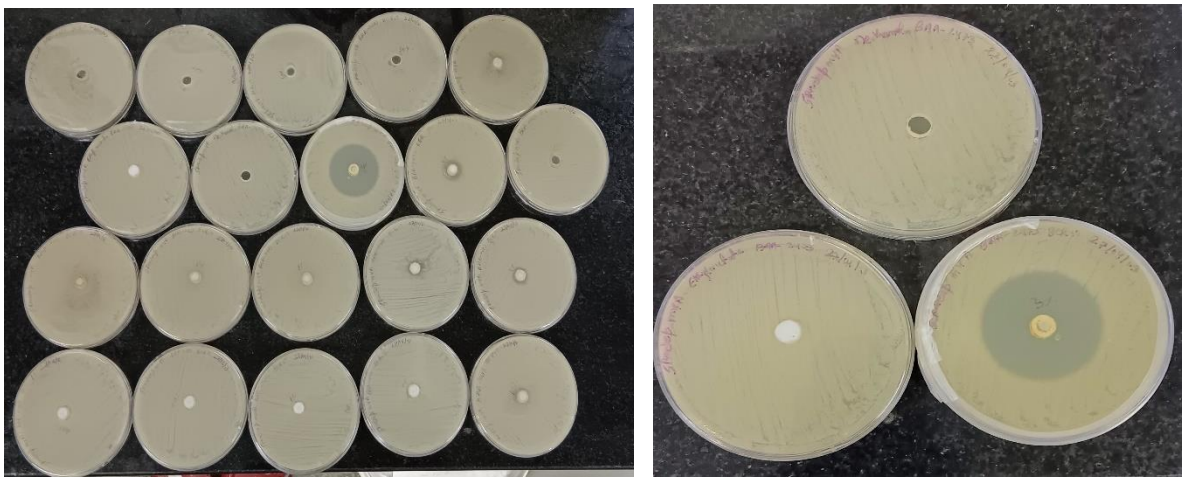
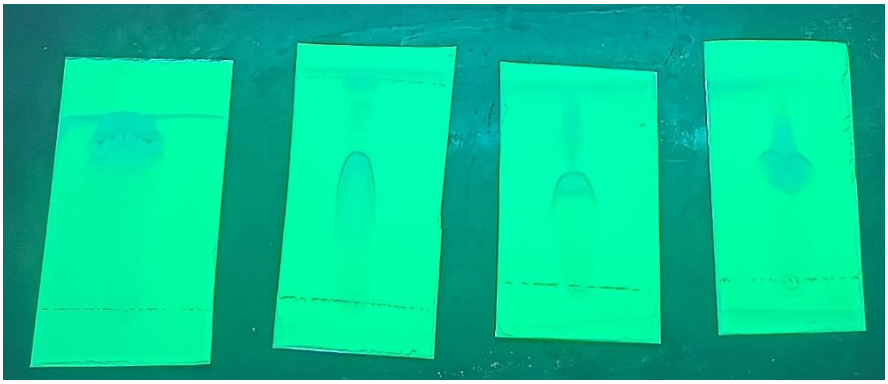


Fig. 47 Comparing the effectiveness of the controls, amoxycillin, and tetracycline, to the pure mixtures from column chromatography fractions.

4.3 Purity analysis by TLC (Thin Layer Chromatography)

The purified mixture is subjected to TLC analysis using polar and non-polar solvents including DCM, methanol, PET ether, and ethyl acetate. Moreover, following a run-up to three-fourths of the TLC, a distinct compound separation is seen under UV exposure (Fig. 48). However, the compounds appear to be quite closely separated from one another, indicating that three compounds from the filtered mixture were present (Fig. 48 (2)).



1 **2** 3 4

Fig. 48 Purity analysis by TLC, 1- 3 % DCM and methanol, **2- 100% DCM**, 3- 25% ethyl acetate and PET ether, 4- 50% ethyl acetate and PET ether.

Chapter 5 - Conclusion

Endophytic Actinobacteria isolates from the medicinal plant, Turmeric (*Curcuma longa*) and *Aloe vera* (*Aloe barbadensis miller*) were tested for their ability to produce metabolites against test strains and antimicrobial strains and a potent BCR 10 (*Streptomyces sp*.). Isolate was identified as a Gram-positive, filamentous-shaped bacterium. Sequence alignment, database similarity searching, and phylogenetic inference closely resemble about 100% similarity with the *Streptomyces cinereoruber subsp. cinereoruber* NBRC (12756), with reference to the outgroup as a control *Escherichia coli*. 16S rRNA gene sequencing confirmed the organism's identity. To optimize the parameters for the *Streptomyces cinereoruber* fermentation, dissolved oxygen had to be altered by introducing 6 LPM of air, and saturation was not allowed to drop below 20% DO saturation. It resulted in the production of the metabolite after 24 hrs of fermentation, as in flask level metabolite was produced on the seventh day, with only Agitation and temperature controlled, which was confirmed by screening against the test and AMR strains, with a clear zone of inhibition greater than 35 mm, and further purification of the extracted compounds using column chromatography, where a similar zone of inhibition was seen in the fifth fraction, and no inhibitions were seen in controls. Additionally, the purity analysis of the compound using TLC revealed the presence of multiple compounds in the purified extract.

References

[1] W. Ludwig *et al.*, "Road map of the phylum Actinobacteria," *Bergey's Manual® of Systematic Bacteriology*, pp. 1–28, 2012, doi: 10.1007/978-0-387-68233-4_1.

[2] D. Macagnan, R. D. S. Romeiro, J. T. De Souza, and A. W. V. Pomella, "Isolation of actinomycetes and endospore-forming bacteria from the cacao pod surface and their antagonistic activity against the witches' broom and black pod pathogens," *Phytoparasitica*, vol. 34, no. 2, pp. 122–132, 2006, doi: 10.1007/BF02981312/METRICS.

[3] G. H. Puttaswamygowda, S. Olakkaran, A. Antony, and A. K. Purayil, "Present Status and Future Perspectives of Marine Actinobacterial Metabolites," *Recent Developments in Applied Microbiology and Biochemistry*, pp. 307–319, Jan. 2019, doi: 10.1016/B978-0-12-816328-3.00022-2.

[4] A. Aderem, "Systems biology: Its practice and challenges," *Cell*, vol. 121, no. 4, pp. 511–513, May 2005, doi: 10.1016/j.cell.2005.04.020.

[5] "Streptomyces in Nature and Medicine: The Antibiotic Makers - D. A. Hopwood - Google Books." https://books.google.co.in/books?hl=en&lr=&id=zPURDAAAQBAJ&oi=fnd&pg=PR9&ots=0S1TLFiDYe&sig=oYrUhNIeHov70ILvvWK42OGHv78&redir_esc=y#v=onepage&q&f=false (accessed Mar. 30, 2023).

[6] Y. Mast and E. Stegmann, "Actinomycetes: The Antibiotics Producers," *Antibiotics*, vol. 8, no. 3, p. 105, Sep. 2019, doi: 10.3390/ANTIBIOTICS8030105.

[7] "Actinomycetes: Role in Biotechnology and Medicine | Hindawi." https://www.hindawi.com/journals/bmri/si/782901/ (accessed Mar. 31, 2023).

[8] D. R. Benson and W. B. Silvester, "Biology of Frankia strains, actinomycete symbionts of actinorhizal plants," *Microbiol Rev*, vol. 57, no. 2, pp. 293–319, Jun. 1993, doi: 10.1128/MR.57.2.293-319.1993.

[9] R. X. Tan and W. X. Zou, "Endophytes: a rich source of functional metabolites," *Nat Prod Rep*, vol. 18, no. 4, pp. 448–459, Jan. 2001, doi: 10.1039/B100918O.

[10] P. Nimnoi, N. Pongsilp, and S. Lumyong, "Endophytic actinomycetes isolated from Aquilaria crassna Pierre ex Lec and screening of plant growth promoters production," *World J Microbiol Biotechnol*, vol. 26, no. 2, pp. 193–203, Feb. 2010, doi: 10.1007/S11274-009-0159-3/TABLES/3.

[11] A. K. Passari *et al.*, "Insights into the functionality of endophytic Actinobacteria with a focus on their biosynthetic potential and secondary metabolites production," *Sci Rep*, vol. 7, no. 1, Dec. 2017, doi: 10.1038/S41598-017-12235-4.

[12] A. Surjushe, R. Vasani, and D. Saple, "ALOE VERA: A SHORT REVIEW," *Indian J Dermatol*, vol. 53, no. 4, p. 163, Oct. 2008, doi: 10.4103/0019-5154.44785.

[13] S. Prasad and B. B. Aggarwal, "Turmeric, the Golden Spice," *Herbal Medicine: Biomolecular and Clinical Aspects: Second Edition*, pp. 263–288, Mar. 2011, Accessed: May 01, 2023. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK92752/

[14] R. Anandan, D. Dharumadurai, G. P. Manogaran, R. Anandan, D. Dharumadurai, and G. P. Manogaran, "An Introduction to Actinobacteria," *Actinobacteria - Basics and Biotechnological Applications*, Feb. 2016, doi: 10.5772/62329.

[15] P. JB, "16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory," *Mol Diagn*, vol. 6, no. 4, pp. 313–321, Dec. 2001, doi: 10.1054/MODI.2001.29158.

[16] P. D. Schloss and J. Handelsman, "Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness," *Appl Environ Microbiol*, vol. 71, no. 3, pp. 1501–1506, Mar. 2005, doi: 10.1128/AEM.71.3.1501-1506.2005.

[17] S. H. Yoon *et al.*, "Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies," *Int J Syst Evol Microbiol*, vol. 67, no. 5, pp. 1613–1617, May 2017, doi: 10.1099/IJSEM.0.001755/CITE/REFWORKS.

[18] O. S. Kim *et al.*, "Introducing EzTaxon-e: A prokaryotic 16s rRNA gene sequence database with phylotypes that represent uncultured species," *Int J Syst Evol Microbiol*, vol. 62, no. PART 3, pp. 716–721, Mar. 2012, doi: 10.1099/IJS.0.038075-0/CITE/REFWORKS.

[19] M. Kim, H. S. Oh, S. C. Park, and J. Chun, "Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes," *Int J Syst Evol Microbiol*, vol. 64, no. PART 2, pp. 346–351, Feb. 2014, doi: 10.1099/IJS.0.059774-0/CITE/REFWORKS.

[20] N. Saitou and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees," *Mol Biol Evol*, vol. 4, no. 4, pp. 406–425, 1987, doi: 10.1093/OXFORDJOURNALS.MOLBEV.A040454.

M.Tech Thesis

ORIGINALITY REPORT

4%
SIMILARITY INDEX

PRIMARY SOURCES

1	uni-mysore.ac.in Internet	26 words —	1%
2	www.coursehero.com Internet	24 words —	1%
3	repository-tnmgrmu.ac.in Internet	22 words —	1%
4	Mengdi Jin, Xiaojing Zhu, Yaoyao Sun, Zhijun Li, Xinwei Li, Yang He, Yane Liu, Ningning Jia, Guoyan Hu, Xingyao Cui, Qiong Yu. "Alterations in the Expression Levels of miR-9-5p and miR-4467 in Peripheral Blood of Patients with First-Episode Schizophrenia", Research Square Platform LLC, 2021 Crossref Posted Content	16 words —	< 1%
5	www.origene.com Internet	15 words —	< 1%
6	link.springer.com Internet	12 words —	< 1%
7	www.bartleby.com Internet	12 words —	< 1%
8	issuu.com Internet	11 words —	< 1%

9	www.mdpi.com Internet	11 words — < 1%
10	Heran Getachew, Blanca Chinchilla, Rosario Fernandez-Godino. "Chapter 409 Genome Editing of Induced Pluripotent Stem Cells Using CRISPR/Cas9 Ribonucleoprotein Complexes to Model Genetic Ocular Diseases", Springer Science and Business Media LLC, 2021 Crossref	10 words — < 1%
11	dx.doi.org Internet	10 words — < 1%
12	etd.lib.metu.edu.tr Internet	10 words — < 1%

EXCLUDE QUOTES	OFF	EXCLUDE SOURCES	< 10 WORDS
EXCLUDE BIBLIOGRAPHY	ON	EXCLUDE MATCHES	< 10 WORDS