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Searching The Possible Antibiotic Producing Actinomycetes in The Soil of Nagpur City and Its Bioassay

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Abstract

The study aimed at isolating actinomycetes capable of producing antibiotic like agent once sampled from Nagpur city soil. The study involved selective media as starch casein agar, to isolate actinomycetes, then number of morphological, biochemical tests and 16S rRNA gene sequencing used to identify the species level identity of bacteria. Later on, partially purified bioactive agents tested against pathogen to certain antibacterial features in it. The study recorded that Actinomycetes, Streptomyces sp; Amycolatopsis and Saccharothrix sp profoundly available in tested soil with bio-active agent having nature as alcoholic, phenolic or steroidal. These biomolecules found controlling Pseudomonas species but failed to control E. coli, S. aureus, and Klebsiella species. In a conclusive remark, Actinomycetes prevalent in Nagpur soil can be utilized for developing new antibiotics to control multidrug-resistant pathogens especially Pseudomonas species as per the investigation.

Keywords:- Antibiotic, Actinomycetes, soil, Antibacterial activity, 16S rRNA.

Introduction

Actinomycetes remain the transitional form between fungi and bacteria; still it has been grouped into bacterial domain, representing largest taxonomic unit[16]. As per general classification, they are Gram-positive filamentous bacteria containing high guanine-plus-Cytosine (G+C) content of the genome[17]. Actinomycetes generally favour growing in diverse environment of soil as well as in aquatic niches as well as in plants or animal as pathogens. Mainly to survive in such a harsh condition, they remain dependent on semi-dormant spore-forming capability especially when nutrient remains limited[19].

Human certainly exploited the potential of Actinomycetes since two-third of antibiotics commercialized till date originated from them primarily via *Streptomycetes*[2,7,9].

These Streptomycetes contains more than 50 different secondary metabolite gene clusters which stand vital for antibiotic production[8,21,30] .

In today's scenario number of Actinobacteria explored to produce antibiotic featured bioactive compounds such as *Streptomyces orchidaceus* producing Cycloserine[24] ; *Marinispora species* producing Marinomycin[13]; *Streptomyces lindensis* producing Retamycin[22] and *Streptomyces lydicus* producing Streptolydigin[14],conventional we know that *Streptomyces* as actinobacteria accounts for 80% of total antibiotic production to date[9] .

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In a similar view, the present study attempted to isolate antibiotic (bio-active agent) like compound from them which may showcase antibacterial activity against human pathogens by involving microbial, analytical and molecular techniques in together.

Method

Soil collection

The ability of soil harbouring actinomycetes for their antibiotic production has attempted in the present study. Nagpur city agricultural soil (5 locations) selected for the same where black soil collected about 100 g per site from the depth of 25 cm mainly close to the rhizosphere in a plastic bag.

Soil processing

Once soil brought to the laboratory, it was sieved through 250 µm pore size and later on added to the 10 ml physiological saline (NaCl 8.5 g / L) by weighing 1 gram accurately. It was then homogenized by shaking over vortex mixture. This homogenate of soil used as stock culture and further added as 1:9 proportion with sterile distilled water to make dilutions up to 10^{-5}. Here every diluent used as an inoculum to get sorted colonies of Actinomycetes on a given medium.

Inoculation and Isolation of Actinomycetes

Nagpur soil suspension carrying actinomycetes first of all confirmed on starch casein agar medium once long filaments of Actinomycetes appears on them. Direct use of SCA medium proposed since it remains added with Amoxicillin (20 µg / ml) and cyclohexamide (25 µg/ml) which did not allow bacteria and fungi to grow, respectively. Here the composition of SCA medium set as $FeSO_4$. $7H_2O$ (0.01); $CaCO_3$ (0.02); NaCl (2.00); K_2HPO_4 (2.00); $MgSO_4.7H_2O$ (0.05); KNO_3 (2.00); Casein (Vitamin free) 0.30; soluble starch (10) and agar (18.00) grams per litre with pH set at 7.0 ± 0.1. Inoculation carried by spread plate and preparation incubated at 37°C for 48 hours to record colonies.

Preliminary Identification.

Upon incubation, Actinomycetes colonies appeared on the SCA medium recorded to distinguish variation in features and based that every colony recorded for salient features such as Aerial mycelia, colony and growth form, spore forms and soluble pigment and then sub cultured on conventional media.

Primary screening of Actinomycetes

Actinomycetes sampled successfully from five soils maintained in the broth and O.D set at absorption 1.0 at 680 nm. In a similar way human multidrug pathogens *Escherichia coli, Klebsiella species, S. aureus, Pseudomonas species,* and *Salmonella species* maintained in a nutrient broth set at 0.1 OD at 680 nm. Further to check the ability of bio-active compounds formation by Actinomycetes which seems to be inhibiting pathogens was carried out once both co-cultured on a nutrient agar medium. Here, first of all, actinomycetes inoculated as a straight line in the centre of the plate and allowed to get absorbed completely. After 15 minutes, perpendicular line crossing the Actinomycetes inoculum made by the pathogen inoculum so that both isolates will remain in close contact once growing in together. This type of inoculation allows the produced bio-molecules to interact directly and ability of

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growth inhibition could ascertain from potential isolate. Plates then incubated at 37°C for 24 - 48 hours and appearing clear zone of growth inhibition of pathogen around actinomycetes indicated particular strain of Actinomycetes able to produce bioactive compound controlling that pathogen successfully. These producing isolates then selected further for investigation.

Secondary Screening

Since in the primary mass screening of Actinomycetes, those able to inhibit several human pathogens found to be few strains screened successfully for the bio-active agent production. These selected actinomycetes were then grown in a nutrient stress condition when maintained on SCA liquid medium as 200 ml. During growth in stress condition, it has assumed that bacteria will produce high level of bioactive compounds and can control pathogen. Once the process initiated 48 hours old, broth with positive growth centrifuged at 15000 rpm for 15 minutes and only supernatant collected then further added with dichloro-ethane, ethyl acetate and n- butanol (1:1:1) solution in equal proportion and nominated as organic solvent. Preparation was then mixed thoroughly so that bio-active compounds get dissolved into an organic solvent. The organic solvent then collected and set to evaporation to concentrate the bio-active compounds. Upon complete dryness, sample once again dissolved in 10 ml sterile distilled water and 100 µl of it used in a well diffusion assay.

In a well diffusion assay, previously sensitive pathogens once again tested by incubating plates at 37°C for 48 hours.

Identification of Bio-active compound

Identification of chemical feature of bio-active compound produced by the actinomycetes detected on thin-layer chromatography (TLC) where silica gel loaded with samples having mobile phase of ethyl acetate: methanol (6:4). Preparation analysed by different staining solvents (E.g. iodine vapours) and resultant retardation value and colour of spot noted to recognise nature of bio-active compound.

16S rRNA gene sequencing

Once the ability of actinomycetes noted to produce bio-active compounds by which controlling human pathogens targeted for their 16S rRNA gene by using polymerase chain reaction along with sequencing methodology suggested by Rai et al., (2013)[25], Here upon successful amplification followed by sequencing BLAST homology and phylogram, the molecular identity of actinomycetes confirmed.

Result

Isolation of Actinomycetes

Since five samples sites investigated in the present study total of thirteen actinomycetes isolated from sites when colonies successfully appeared on SCA medium as in Table 1 and Fig 1.

Morphology of Actinomycetes.

Actinomycetes (n= 13) further checked for their Aerial mycelia, soluble pigment, spore forms successfully as given in Table 2.

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Primary screening for antibiotic production

As per co-cultured method, pathogens (*E. coli, S. aureus, Pseudomonas species, Klebsiella species,* and *Salmonella species*) when co-cultured with Actinomycetes isolates, the result indicated that *Pseudomonas sp.* found to be the most sensitive towards bio-active compounds produced by five isolates and *Salmonella species* remained growth-sensitive towards three actinomycetes compounds. Out of these isolate 9 and isolate 11 found to be inhibiting both *Pseudomonas sp* and *Salmonella sp.* In contrast, *E. coli, S. aureus* and *Klebsiella sp* remained utterly insensitive to any of the bio-active compounds as in Table 3 and Fig 2.

Secondary screening

As per the primary investigation, *Pseudomonas sp* remain the most sensitive towards bio-active agents of five actinomycetes. After that once again non-MDR and MDR *Pseudomonas species* checked with partially purified bio-active compounds of actinomycetes isolates, once again both sets of *Pseudomonas* found to be positive growth inhibited. Also, Non-MDR strains remain more sensitive for inhibition as compared to MDR strains as in Table 4.

Detection of antibiotic compound

Based on the ability to produce the bio-active compound, these five isolates (1-5) recorded for producing compounds by involving TLC technique. Here very close but variable retention factor recorded in centimetre as in Table 5 and based on Vanillin spraying presence of alcoholic, phenolic or steroid nature has been ascertained as in Fig 3.

16S rRNA sequencing

Based on the 16S rRNA gene sequencing, isolate 1 identified as *Amycolatopsis speibonae*; isolate 2 as *Saccharothrix algeriensis;* isolate 3 as *Streptomyces olivicoloratus ;* isolate 4 as *Streptomyces tanashiensis* and isolate 5 as *Amycolatopsis speibonae* once homology and phylogeny carried out by BLASTN and ClustalW based alignment followed by tree building in MEGA 6 software (Data not shown).

Discussion

Soil represents an enormous opportunity to explore its resources for better living. Human explored it in a definite way to make life on earth livable. In a similar success, we able to isolate soil bearing actinomycetes capable of controlling the growth of human pathogens mainly of *Pseudomonas sp.* in a profound way by their ability to produce bio-active compound. In early time, Matsumoto and Takahasshi (2017)[18] narrated the success of actinomycetes since they able to provide bio-active compound once isolated from root region. Ability to control multidrug-resistant bacteria reported via actinomycetes bio-active compounds once they were separated from soil planted with fruit orchard, oil palm and dipterocarp forest[15].

In the present study, a variety of species like *Streptomyces, Saccharothrix* and *Amycolatopsis species* detected to produce bio-active compounds able to control pathogens. Similarly, Guo et al., (2015)[6] mentioned some 26 genera, ten families and seven orders of the class Actinobacteria remain present in red soil of Southern China having antibiotic features.

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In the present study, selective growth of actinomycetes on SCA medium stands vital for directed research; otherwise, it is tough to isolate only actinomycetes directly from unprocessed soil since it contains uncountable organisms. In a similar pattern, Kizhakedathil and Chandrasekaran (2018)[12] utilised SCA medium to separate α- amylase, protease, chitinase, cellulose producing actinomycetes once sampled from agricultural soil of Kolatur, Tamilnadu, India. Rai et al., (2016)[26] also mentioned an essential role of SCA medium to isolate Actinomycetes BS1-BS20 when sampled from plant rhizosphere.

In a decisive role, ability to control *Pseudomonas species* found to be the main arsenal of actinomycetes sp. strain 1-5 and up to certain extent for *Salmonella species* but on the other hand, those failed to control *E. coli, S. aureus* and *Klebsiella species* at a given concentration.

Srivibool and Sukchotiratana (2006)[29] also mentioned that actinomycetes isolated from coastal soil profoundly control *Pseudomonas aeruginosa* followed by *Streptomyces sp* and *Actinomaclura species.* In a similar success, *Pseudomonas aeruginosa* recorded to be controlled by bio active agents of actinomycetes[23,4,27,1].

In a compound classification by separation method, the present study determined the retardation factor of bio-active compounds belongs to alcoholic, phenolic or steroid nature having antibacterial features with them when checked in TLC plates.

In a similar record, bio-active of *Streptomyces sp* having RF value as 0.78 very close to our results able to control *Bacillus subtilis, E. coli, S. aureus, Proteus vulgaris,* and *S. typhi.*[11]

Emelda et al., (2012)[5] recognised potential of actinomycetes bio-active compound once ten fractions of them obtained from column chromatography which once again strengthens our finding.

Lastly, in the present study by 16S rRNA identity up to species level recorded having the Nagpur soil dominance with *Amycolatopsis speibonae*, *Saccharothrix algeriensis* and *Streptomyces sp.* Prevalence of these species with ability to produce bio-active compound has reported in number of studies[3,20,10,31,28].

Conclusion

Every creature on earth tries to survive by acquiring available resources with them. The human also doing the same by exploring microbial community of soil since those able to generate 'miraculous compounds' able to control human pathogens. The present study once again concluded that like other soils around the world, Nagpur soil harbours actinomycetes with immense ability to produce antibacterial bio-active compounds. This feature enables them to survive in soil under competitive world and parallel remains useful to explore potential to control human pathogens.

The present study put forward the success of five *Actinomycetes species* able to control *Pseudomonas* no matter those remain in MDR and non-MDR state and further showcase their bio-active compound remains selective in inhibiting the growth of particular pathogen which is much demanded to avoid side effects related with that.

In future, these five actinomycetes could be utilised to explore the real bio-active present in them so that derived therapy could control ever-increasing multidrug-resistant bacteria in the community.

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Table 1: Actinomycetes colonies appeared on the SCA medium when inoculated form agricultural site

Site	1	2	3	4	5
Actinomycetes	6	2	2	2	1

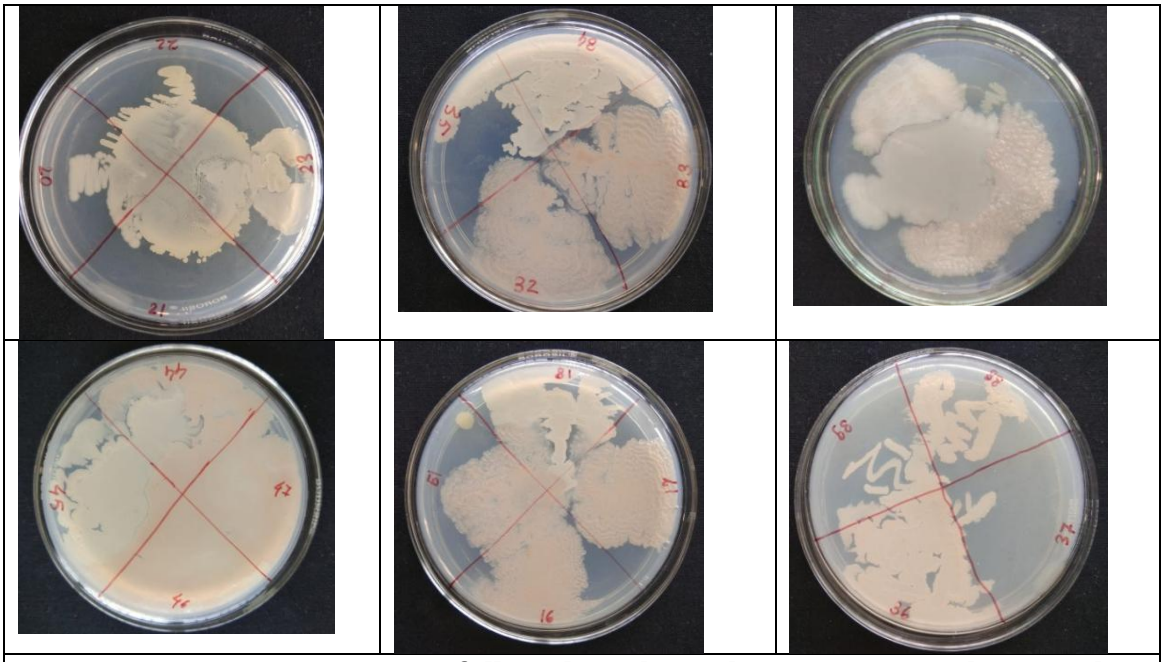

Fig. 1: Actinomycetes successfully adopted to the *in vitro* conditions when inoculated on the nutrient agar medium

Table 2: Morphological features recorded for the actinomycetes grown on SCA and Nutrient agar

Isolates	S8a	S8b	S8c	S8d	S8e
Aerial mycelia	Pale-brown	White	Gray	Pale-brown	White
Growth and colony form	Abundant and rhizoid	Moderate, Oval, tough and leathery	Dark-grey pigment	Abundant and rhizoid	Moderate, Oval, tough and leathery
Soluble pigment	Yellow pigment	None	Dark-grey pigment	Yellow pigment	None
Spore forms	Chainlike in rectiflexous form	Ornamented in open primitivespiral	Oval spores in spiral chains	Chainlike in rectiflexous form	Ornamented in open primitivespiral

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Isolates	S9a	S9b	S9c	S9d	S9e
Aerial mycelia	White	Dark-Gray	Pale-brown	Dark-Gray	White
Growth and colony form	Abundant,	Abundant, rhizoid, and leathery	Abundant and rhizoid	Abundant, rhizoid, and leathery	Moderate, Oval,tough and leathery
Soluble pigment	Yellow pigment	Dark brown pigment	Yellow pigment	Dark brown pigment	None
Spore forms	Smooth and round in spiral	Oval spores in spiral chains	Chainlike in rectiflexous form	Oval spores in spiral chains	Ornamented in open primitive spiral

Table 2 cont… 3 of 3

Isolates	S10a	S10b	S10c
Aerial mycelia	Gray	Pale-brown	Dark-Gray
Growth and colony form	Moderate, complex, tough	Abundant and rhizoid	Abundant, rhizoid, and leathery
Soluble pigment	Dark-grey pigment	Yellow pigment	Dark brown pigment
Spore forms	Oval spores in spiral chains	Chainlike in rectiflexous form	Oval spores in spiral chains

Table 3:- Actinomycetes and pathogen coculturing recorded prominent inhibition of the Pseudomonas species and salmonella species

Sr. No.	*E. coli*	*S. aureus*	*Pseudomonas sp.*	*Klebsiella sp.*	*Salmonella sp.*
1	x	x	x	x	x
2	x	x	x	x	x
3	x	x	x	x	x
4	x	x	Inhibited	x	x
5	x	x	x	x	x
6	x	x	Inhibited	x	x
7	x	x	Inhibited	x	x
8	x	x	x	x	Inhibited
9	x	x	Inhibited	x	Inhibited
10	x	x	x	x	x
11	x	x	Inhibited	x	Inhibited
12	x	x	x	x	x
13	x	x	x	x	x

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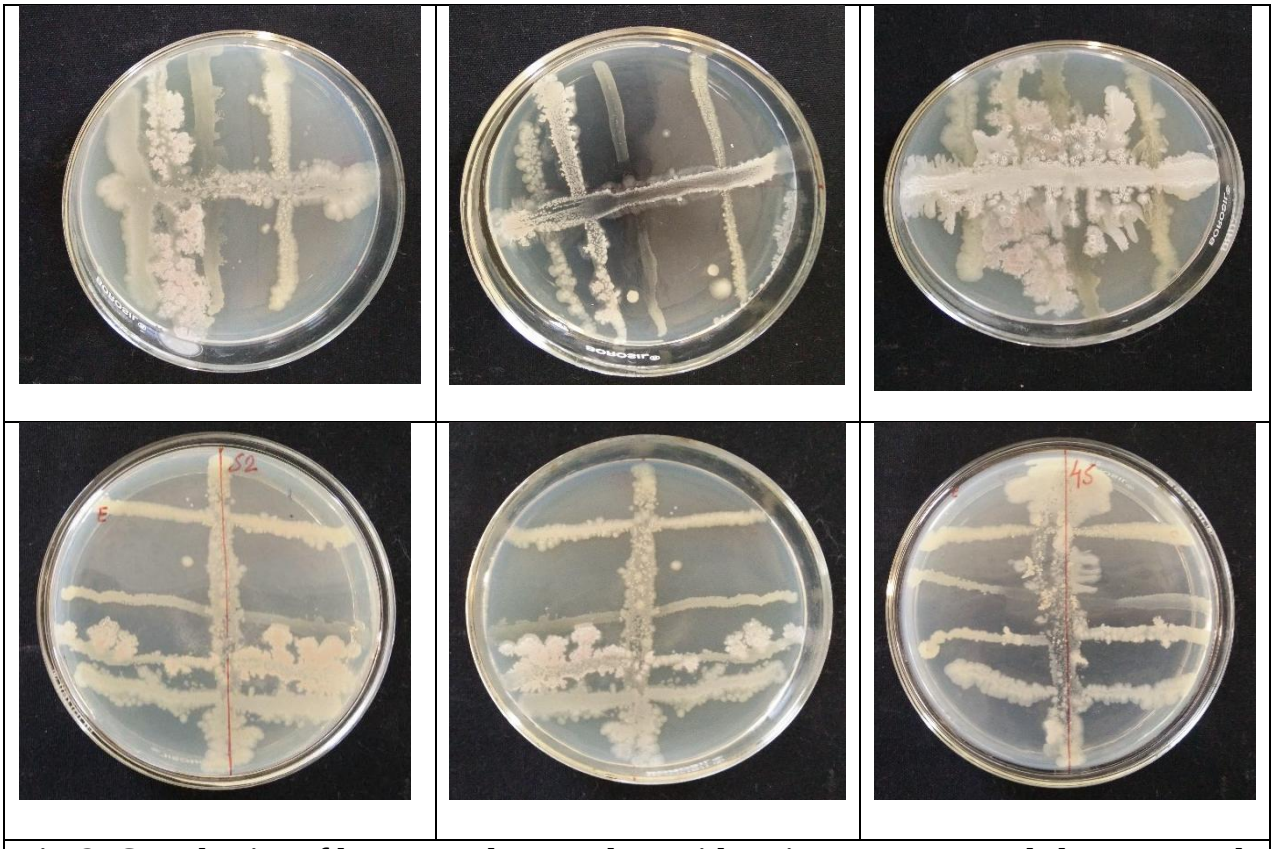

Fig. 2: Co-culturing of human pathogens along with actinomycetes recorded some growth inhibition close to the actinomycetes colonies indicated the presence of growth inhibitory molecules and considered positive

Table 4: - Actinomycetes purified bio-active compound able to control both MDR and non MDR *Pseudomonas* species.

Actinomycetes	MDR zone of inhibition in mm					Non-MDR zone of inhibition in mm					MDR Avg ± std. dev	Non - MDR	P-value
	1	2	3	4	5	1	2	3	4	5			
1	12	18	19	12	15	16	17	20	21	22	15 ±3	19±2.5	0.0644 ns
2	10	10	11	15	18	14	12	13	15	16	12.80 ± 3.56	14.00 ± 1.58	0.5180
3	18	17	08	13	12	14	14	12	09	09	13.60 ± 4.03	11.60 ± 2.5	0.3744
4	08	07	06	08	08	12	12	13	12	09	7.4 ± 0.89	11.60 ± 1.5	0.0007
5	16	15	14	14	14	14	11	14	14	14	14.60 ± 0.89	13.40 ± 1.34	0.1347

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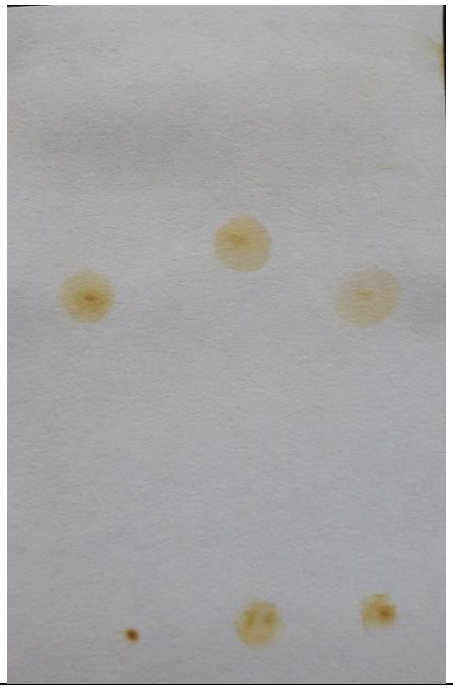
Table 5: Variable retention factor recorded for the Actinomycetes bio-active compounds based on the TLC protocol

Isolate	1	2	3	4	5
Retention factor Rf value in cm	0.75	0.67	0.59	0.75	0.80



Isolate 1

Isolate 2

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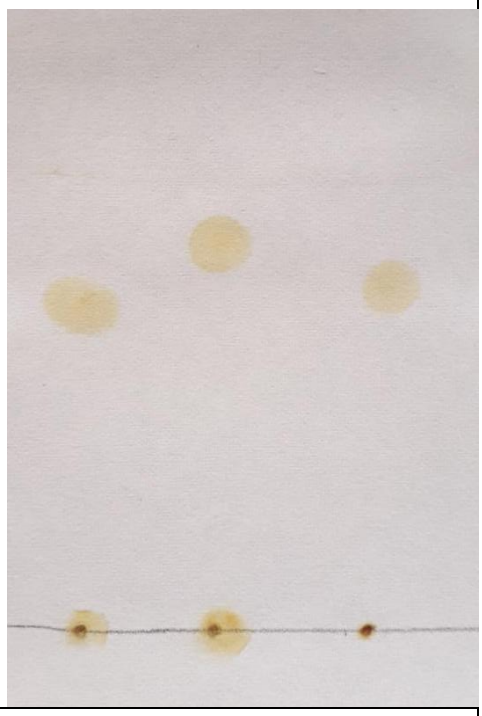
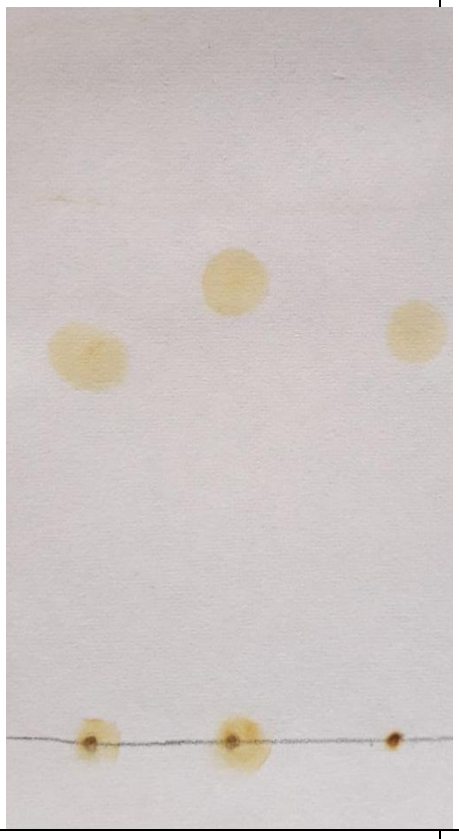
Isolate 3



Isolate 4



Isolate 5

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Fig. 3: As per TLC analysis, bio-active compounds successfully spotted on the gel for all five actinomycetes partially purified samples.