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Identification of Novel Bioactive Gene Producing Actinomycetes: An Introductory Study

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Abstract

This work was initiated with an objective that would screen for bioactive specifically antibacterial molecules secreted by actinomycetes growing in fresh water pond soil. Actinomycetes being treasure trove of thousands of bioactive molecules. It is recommended that either novel or present day bio molecule derivatives should be detected and pertinent drug development process be carried out with this background this study was proceeded to identify strains of *Microbacterium barkeri* (LMA4), *Corynebacterium argetoratense* (LMA5) and *Streptomyces shenzhenensis* (LMA6) form the local marshy pond soil. The actinomycetes was with Arial mycelia was gram positive with profuse branching. In a test considering MDR strain of *Escherichia coli* (BME4) and *Staphylococcus aureus* (BMS4) as test bacteria. The 16s rRNA sequences and the BLASTn analysis helped out to predict the sequence identification as the actinomycetes isolates.

Keywords: Bioactive; Actinomycetes; 16s rRNA Sequence; BLASTn.

1. INTRODUCTION

Antibiotics can truly be considered the epitome of the 20th century's "wonder drugs." This term was widely used in the 1950s (Bud, 2007). At present, treatment options are limited and overused; thus, the search for new antibiotics with novel chemistry using methods less costly than traditional analytical chemistry techniques alone is critical (O'Rourke, 2020). Evidence is accumulating that these interactions control the expression of biosynthetic gene clusters and have played a major role in the evolution of the high chemical diversity of actinomycetes produced secondary metabolites (Meij *et al*., 2017).

India has a unique asset of biodiversity, which can be used as a treasure for the search of novel isolates. With the variation of type of soil, according to the geographical changes, soil provide very complex habitat to the microbes residing in it (Singh *et al*., 2016).

1.1 Actinobacteria/Actinomycetes as Sources of Antibiotics

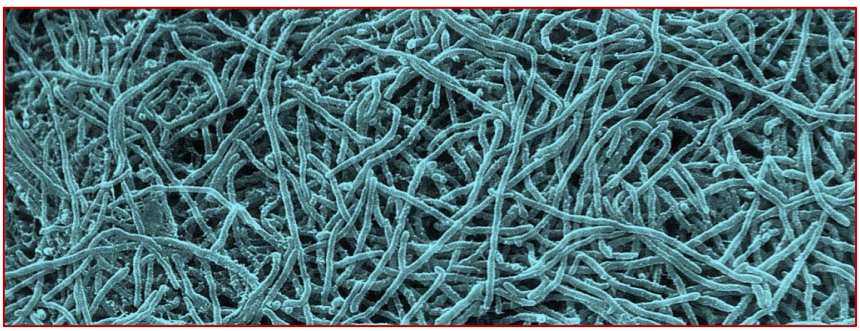

Figure 1: Typical Actinomycetes interwoven mycelia (Source: www.labmedica.com)

Morphologically, the Actinomycetes (Figure 1) are dimorphic dependent upon cultural condition. They may be called Actinomycetes, if the organism maintains the mycelial form, while Actinobacteria, if it is as like as bacteria. But in contrast to fungal and bacterial strains, the Actinomycetes are with high GC content, although they are Gram positives. They bear like various types of spores, spherical, echinus, rugged etc. The Streptomyces are considered to be largest antibiotic producing class followed by Nocardia, Micromonospora etc. The organisms are potential source of antibiotic molecules which are secreted during their antibiosis against antagonizing bacteria. Since the discovery of Penicillin, an array of antibiotics are produced and implemented in therapeutic world.

1.2 Biosynthetic gene clusters

Biosynthetic gene clusters (BGCs) are organized groups of genes involved in the production of specialized metabolites. Typically, one BGC is responsible for the production of one or several similar compounds with bioactivities that usually only vary in terms of strength and/or specificity (Martinet *et al*., 2019). In this changing research environment, there is an increasing need to access all the experimental and contextual data on characterized BGCs for the lake of comparative analysis, for function prediction and for collecting building blocks to design of novel biosynthetic pathways (Medema *et al*., 2015). This is to mention that Antibiotic biosynthetic genes in Streptomyces usually form a condensed cluster on the chromosome. However, several linear plasmids encoding antibiotic gene clusters have been isolated (Arakawa, 2010).

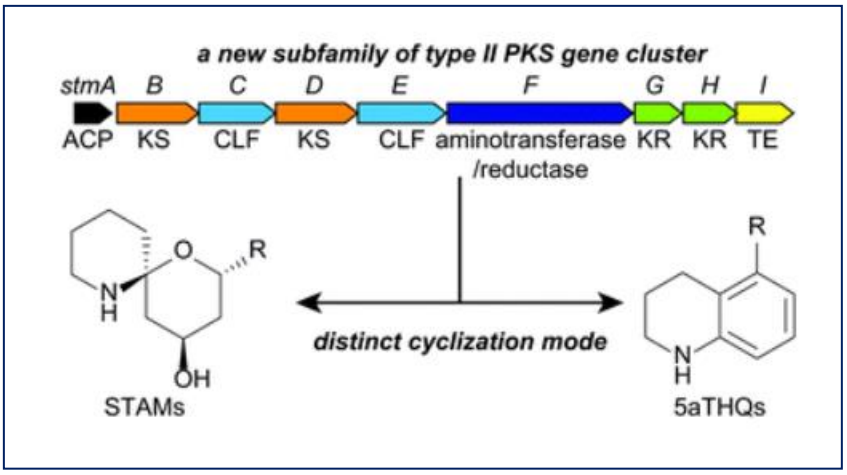

Figure 2: Is showing the subfamily of type II PKS gene cluster comprising *stmA, KS, CLF, KS, CLF,* aminotransferase /reductase, KR and TE genes, which are part of the said family. (Figure is redrawn from Ojaki *et al*., 2019)

Traditional approaches for identification of biosynthetic pathway mainly leverage bioactivity screening to first extract the bioactive compounds with desired properties and subsequently locate the responsible genes by biochemical techniques (Luo *et al*., 2014). Moreover, a large number of BGCs are not or weakly expressed in native hosts under laboratory conditions, known as 'silent' or 'cryptic' gene clusters. Thus, besides the traditional screening and characterization methods, such as phenotype screening, insertional mutagenesis, co-culture and elicitor screening (Cacho *et al*., 2015; Tomm *et al*., 2019; Zhang X. *et al*., 2019), cloning and refactoring the putative BGCs in well-defined hosts become attractive approaches for natural product discovery, achieving functional expression of uncharacterized potentially-valuable natural product biosynthetic pathways (Lin *et al*., 2020). It was not long until scientists noticed that secondary metabolites are usually encoded by genes that cluster together in a genetic package, which was later referred to as a biosynthetic gene cluster (Tran *et al*., 2019).

Thousands of candidate BGCs from microbial genomes have been identified and stored in public databases. Interpreting the function and novelty of these predicted BGCs requires comparison with a well-documented set of BGCs of known function. The MIBiG (Minimum Information about a Biosynthetic Gene Cluster) Data Standard and Repository was established in 2015 to enable curation and storage of known BGCs (Kaustar *et al*., 2020). However, The existing BGC prediction tools often assume that each BGC is encoded within a single contig in the genome assembly, a condition that is violated for most sequenced microbial genomes where BGCs are often scattered through several contigs, making it difficult to reconstruct them (Meleshko *et al*., 2019). Genome sequencing coupled with genome mining allows the identification of a natural product biosynthetic gene cluster using different computer programs (Greule *et al*., 2017).

From this precise introduction about Actinobacteria (AB) and or Actinomycetes (AM), and their biopotency to synthesize antibiotics, it was found

that AB/AM is the treasure-tove of antibiotics. The strains synthesize all forms of antibiotics including Aminoglycosides, macrolides, Tetracyclines and many more related compounds. Although there is trend to opt for synthetic compounds, the emergence of drug resistance, impose to search for newer antibiotics from the AB/AM strains. This is important to note that modifying the natural compounds with pertinent functional groups can be considered as the best Pharmaceutical product with fulfilment of Pharmacological parameters. In this context, haunting for novel antibiotics or conventional antibiotics from novel strains of AB/AM is mostly wanted scenario in clinical research. Therefore, this present pursuit aims at searching for antibiotics from untapped area enriched with potential AB/AM strains. This is further to add that, AB/AM express their putative biosynthetic genes at the time of their need. It is reported that the genes are expressed in stress induced condition (Meij *et al.*, 2017). The various stress induced factors may be chemical compounds, pH, Temperature and more over presence of another organism in the same habitat. When an organism is present in a same niche, there is a competition between the AB/AM strains with the other organism for nutrition and shelter. As a result of which, both the competing partners express their chemical weaponries. AB/AM also expresses the chemical compounds for their own defence. And this is the result of expression of biosynthetic genes, producing antibiotics. Hence, study of gene expression is an important aspect of Actinobacteria research, which will lead a definite route to get the putative antibiotic product.

1.3 Biology of Actinomycetes and antibiotic production

Streptomyces, being well studied can be taken as model organism for Actinomycetes biology. The organism is basically a soil dwelling organism, with profuse aerial branching system and enormous spore producing ability, can undergo autolysis, for reuse of fuel in the process of reproduction. These Gram positive bacteria, slowly break down humates and humic acids in soils, and prefer non-acidic soils with pH ≤5. It is also reported that (Chater *et al.*, 2010), the antibiotics produced by this group of organisms are activated by extracellular signaling pathways (ECSP) with production of diffusible signaling molecules. The genes associated with ECSP are involved in speciation also. The morphogenesis is also greatly influenced by extracellular enzymes and proteins. Dyson *et al.*, (2016) had described about the cell division on Streptomyces is brought about by *FtsZ* and its associated proteins like min C and min D. It was reported that there are two types of cell division in Streptomyces. The aerial mycelia under cell division without cross wall formation resulting coenocytes, where as the other type of cell division resulted into unicytic and unigenomic spores, which eventually detaches from the parental strand. This is important to mention that the initiation of sporulation is linked with the bioactive secondary metabolite production (Meij *et al.*, 2017). Further the antibiotic production is also linked with nutritional status. This biological behavior could be due to advent nutritional stress, thus expression of anti metabolites to inhibit the neighborhood microbes not to be part of the available nutrition.

2. MATERIALS AND METHODS

2.1 Collection of Pond soil

The soil samples were collected from edges fresh water Ponds in the campus of Sambalpur University, Jyoti Vihar, Burla. The ponds are closed from its source Hirakud dam water. The marshy soil samples were collected from the shore of the ponds at two inches deep from the ground level in aluminum foils. The soil samples were collected by sterilized spatula and transferred to wide mouth sterilized bottle. All samples were labeled and transported to Laboratory of Medical Microbiology, Department of Biotechnology and Bioinformatics, Sambalpur University.

2.2 Pretreatment of samples

The soil samples were air dried and exposed 70^{o} C temperature in Hot Air Oven aseptically, for elimination bacteria Kumar *et al.*, (2016).

2.3 Media Preparation

The Nutrient agar (NA), Nutrient broth (NB), Actinomycetes Isolation agar, Phenol Red Dextrose agar (PRDA), Mac Conkey agar (MAC) and Eosin Methylene Blue (EMB) agar media were prepared following Hi-media instructions, as the media were procured from said company.

2.4 Bacterial strains

Bacterial strains of *Escherichia coli* (BME4) *Klebsiella pneumoniae* (BMKl4), *Salmonella typhi* (BMSa4), *Staphylococcus aureus* (BMS4) were obtained from dept. of Microbiology, Veer Surendra Sai Institute of Medical Sciences and Research (VSSIMSR), BURLA, Odisha, with prior permission and following ethical guidelines.

2.5 Study of Colony characterisation

The actinomycete grown on the isolation agar were observed for its typical charchteristic features, like colour, odour, consistency, optimum time period for full growth (initiation of sporing stage).

2.6 Optical microscopy of actinomycetes isolates

The actinomycetes isolates were viewed under optical microscopy by performing Grams staining, using Crystal violet as primary staining reagent and Safranine as secondary reagent. While absolute alcohol was used as recoloring agent. The microscopic structure was observed using 100 x objective / less.

2.7 Test for Starch degradation activity

The isolated Actinomycetes were biotype considering Starch degradation activity as one of the

parameter, to typify the isolated actinomycetes. The screening was made following Al-Dhabi *et al*., (2020) method, with partial modification. Purposefully, nutrient agar (2.8%) and starch (0.28gm %) were taken and Starch agar plates were prepared. Further, patch inoculum of pure actinomycetes were placed onto Starch agar Plates and were incubated at 37^0C for 24hrs. The post incubated plates were observed for appearance of halos if any, indicating starch degradation activity of test actitinomycetes (Sivanandhini *et al*., 2015).

2.8 Test for Citrate utilization activity

The Citrate utilization test by candidate actinomycetes strains were carried out with Nutrient agar plates incorporated with Citrate and Bromothymol Blue as indicator. The Citrate agar slants were prepared and actinomycetes inoculum was placed in the slants and the tubes were incubated at 37^0C for 24hrs. The Change of Green colour to Blue colour was considered as citrate utilization by the test actinomycetes strains (Kathiravan *et al*., 2016).

2.9 16s r RNA sequencing of Actinomycetes strains

The actinomycetes, executing bacterial growth inhibition activity, were sequenced for 16 s r RNA sequence based identification. The 5 day old cultures were considered for sequencing. For this purpose, the out sourcing facility (Agrigenome Pvt. Ltd., Kochi) was availed.

2.10 BLASTn analysis of 16 s r RNA sequencing

The sequence, received from the outsourcing lab, in FASTA format, were BLASTn (Basic Local Alignment Tool for nucleotides) analysed and similarity index was inferred with the public sequence databases, pasting the sequence into the textbox on one of the BLAST Web pages, at NCBI portal (www.ncbi.nlm.nhm.gov).

2.11 Phylogenetic analysis

The 16 s r RNA sequences were phylogeneticallyanalysedby generating a phylogenetic tree from the 16 s r RNA sequences of LMA4, LMA5 and LMA6 (Rassem *et al*., 2018).

3. RESULTS AND DISCUSSION

3.1 Sample Collection sites

The sites of the ponds from where the samples were collected are shown in Figure 3 (a, b and c). The soil samples were marshy, and were observed to be under category of clay soil.

Figure 3: The edges of ponds (a, b and c) from where the marshy and clay soils were collected

3.2 Post heat treated soil samples

The post heat treated soil samples Figure 4 (a, b and c) were found to be dried and found to be cleaned from bits and pieces.

Figure 4: The three number of soil samples after heat treatment at 72^0C

3.3 The growth of actinomycetes on the Actinomycetes agar from soil in water

The plates showing mixed cultures of all isolated microbes (bacteria, fungi and actinomycetes) are depicted in Figure 5. The colonies with dry powdery appearance were selected as presume actinomycetes and were further subcultures, which shown in Figure 5 (a, b and c) for LMA4, LMA5 and LMA6 respectively.

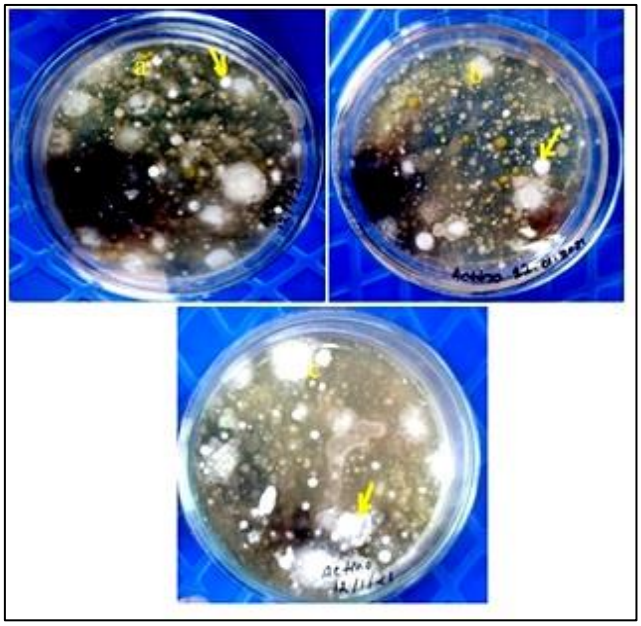

Figure 5: The mixed microbial growth on Actinomycetes Isolation agar. Yellow colour arrows indicate the actinomycetal colonies

3.4 Optimization of growth of isolated Actinomyceyes using different media

The growth pattern of isolated actinomycetes (LMA4, LMA5 and LMA6) had been observed in Actinomycetes Isolation agar, Phenol Red Dextrose agar, Mac Conkey agar and EMB agar (Figure 6) respectively. As the actinomycetal isolates were growing luxuriantly it is suggested that the isolates could have used the Glucose in Phenol Red Agar media but might not be for fermentation purpose, which was evidenced with no colour change of media from Red colour to Yellow colour, due to acid production as end product of fermentation, as there was presence of Phenol Red as pH indicator. This is to mention that Proteose peptone and HM Peptone B which is free from fermentable carbohydrates is added in the medium thereby preventing the production of false positive reactions. However, there was also no gas production by the isolates, which could have indicated by the splitting of agar or by the bubbles formation.

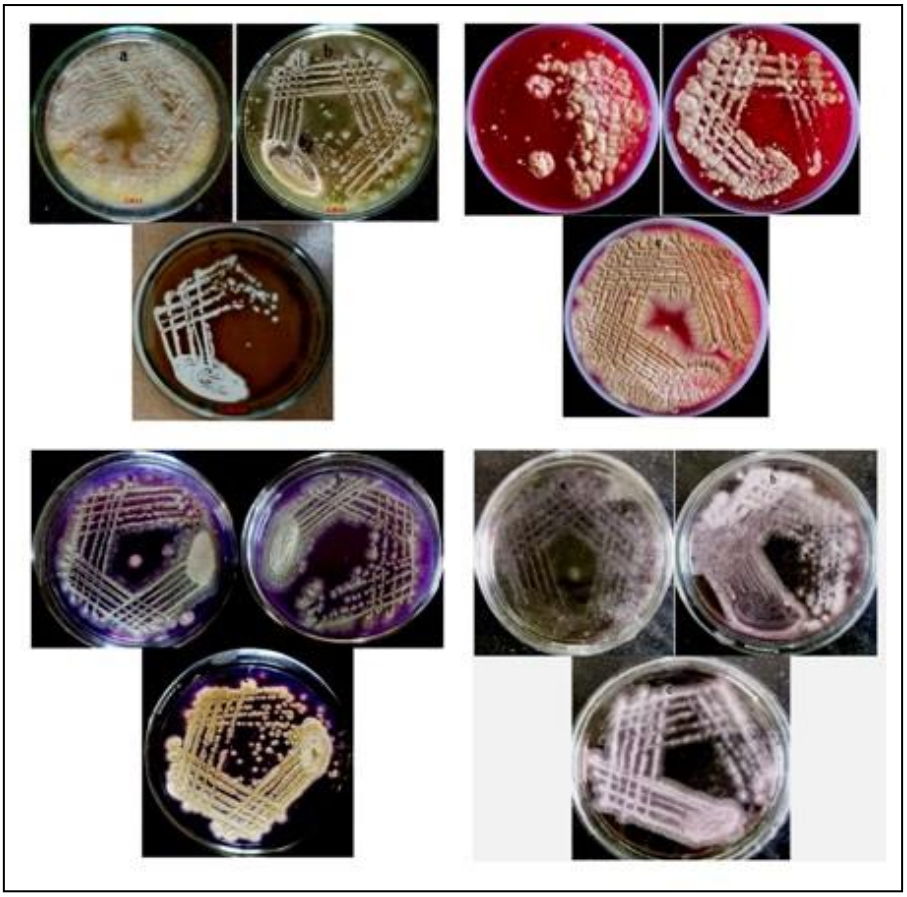

Figure 6: The luxuriant growth of actinomycetes isolates on Actinomycetes Isolation agar, Phenol Red Dextrose agar, Mac Conkey agar and Eosin Methylene Blue (EMB) agar media; a: LMA4, b: LMA5 and c: LMA6 respectively

In addition to growth in Phenol Red agar media, it was observed that there was also growth on Mac Conkey agar plates, which is traditionally used as selective media for differentiation of lactose fermenting and non-lactose fermenting bacteria, with demonstration of changing of colour change. In this test as Bromo cresol Purple was used as indicator of pH. The MAC plates were observed as Purple colour initially as well as after diffuse colonial growth of Actinomycetes. Hence, it may be inferred that the isolates were not fermenting the lactose present in the medium. This was in agreement with Phenol Red Dextrose medium grown actinomycetal colonies.

Further, the growth pattern as observed on the EMB agar plates, it was seen that there were also growth of all the actinomycetes. By studying the grow pattern of EMB agar grown actinomycetes, it was observed that there was also no manifestation of fermentation of Lactose (an ingredient of EMB agar), so also no colour change of media after growth of Actinomycetal strains. This is to mention that Eosin Y and Methylene Blue (1:6 ratio) were the pH indicator dyes had been used in the said EMB media.

From this isolated Actinomycetal growth pattern observation, it was understood that the three actinomycetal isolates were not carbohydrate fermenters, but were users of carbohydrates from the media for the purpose of generation of energy as well as specific enzyme production. This is significant to note that the isolates were using the complex carbohydrate, Glucose and also comparatively simple carbohydrate, Lactose.

3.5 Starch degradation and Citrate utilization activity by isolates

The results observed for Starch degradation activity (Figure 7) and Citrate utilization activity (Figure 8) are illustrated in respective figures from the observation, it was inferred that the test isolates namely LMA4, LMA5 and LMA6 had no starch degradation activity. But the actinomycetes were luxuriantly growing on the said agar plates. Therefore the activity may be defined as starch utilizing activity. For which, it may be suggested that starch could have been source of carbon for the growth and multiplication of test actinomycetes. While, the tests comprising the Citrate utilization activity, it was found that the actinomycetal strains had grown in Citrate agar conferring citrate utilization. It is to mention that Citrate is an important intermediate compound in the primary metabolic path way of Krebs cycle. Bromothymol Blue being a pH indicator had changed from Green colour to Blue colour due to degradation of ammonium salts into Ammonia, which is

responsible for increase in alkaline pH (7.6). It was observed that there was gradual increase of alkalinity in the test tubes, as evidenced from the culture tubes. It is assured that the test isolates were utilizing the citrate, how -ever slowly. The slow grow is the basic growth criteria of actinomycetes.

Figure 7: The plates showing the growth of LMA4, LMA5 and LMA6 on Starch Agar plates

Figure 8: Is illustrated with Citrate utilization activity of LMA4, LMA5 and LMA6 respectively, at 24 hrs, 48 hrs (upper panel; a and b), 72hrs and 96 hrs (lower panel, c and d) respectively. There was gradual increase of alkaline pH which were evidenced from Blue colour to Green colour change, from 24 hrs to 96 hrs

The details of colony morphology of each of isolated actinomycetes (LMA4, LMA5 and LMA6) are given in Table 1.

Table 1: Showing the details of colony morphology of LMA4, LMA5 and LMA6

Name of isolated Actinomycetes	Colour	Reverse colour	Appearance	Odour	Growth period till onset of sporing	Starch utilization activity	Citrate utilization activity
LMA4	Off white	Straw Yellow	Powdery aerial mycelia	Earthy	5days	Yes	Yes
LMA5	Turquoise	Straw Yellow	Powdery Aerial mycelia	Earthy	5days	Yes	Yes
LMA6	Milky White	Straw Yellow	Powdery aerial mycelia	Earthy	5 days	Yes	Yes

3.6 Test Bacterial strains

The bacterial strains used in this study were availed from dept. of Microbiology, VSSIMSR, Burla and were maintained in the laboratory of Medical Microbiology, Dept. of Biotechnology and Bioinformatics, Sambalpur University using the clinical ethical guidelines. Two numbers of bacterial strains namely *Escherichia coli* (BME4) and *Staphylococcus aureus* (BMS4) were observed to be pure as evidenced from streaked plates (Figure 9). Further, the Gram stained microscopic observation had substantiated the basic features of the said bacterial strains (*E.coli*, Gram negative small hyphen like rods and *S. aureus*, Gram positive clustered spherical balls), was also reported.

Figure 9: The streaked plates depicting isolated and pure colonies of *Escherichia coli* (BME4) and *Staphylococcus aureus* (BMS4) respectively on Nutrient agar plates

Identification and Microscopic visualization of Actinomycetes

The Microscopic features are depicted in Figure 10 (a, b and c)

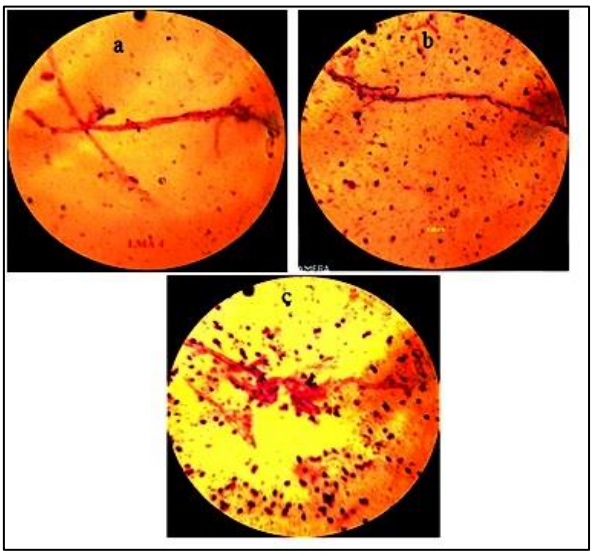

Figure 10 a, b and c: The microscopic observation of LMA4, LMA5 and LMA6 respectively. The mycelia are slender with profuse branching and more over Gram positive

3.7 The 16s r RNA sequencing of Actinomycetal isolates and BLASTn analysis

The results of the 16s r RNA of LMA4 obtained from the Sequencing lab, obtained in FASTA format are given in Figure 11. It was observed that LMA4 16 s r RNA was eluted with Full Length(1483bp) | A(20% 307) | T(26% 374) | G(23% 364) | C(31% 477), LMA5 16s r RNA with Full Length(435bp) | A(21% 293) | T(25% 308) | G(22% 310) | C(32% 441) and LMA6 with (1455bp) | A(20% 295) | T(25% 349) | G(23% 342) | C(32% 466) respectively.

Further, the GC % was calculated as 55.1 for LMA4, 55.4 for LMA5 and 55.4 for LMA6 respectively. From the GC % as calculated for the 16 s r RNA sequences of said actinomycetes strains, it was observed that each of the isolates were with higher % of GC content which reflects a higher side of GC content. This is to mention that Actinomycetes are the organisms, with high percentage of GC content in their genome. Although 16 s r RNA sequences are not destined for transcription, but are the signatory sequences. These sequences are also considered for GC% calculation.

Therefore, it may be suggested that the three isolates are convincingly the strains of Actinomycetes.

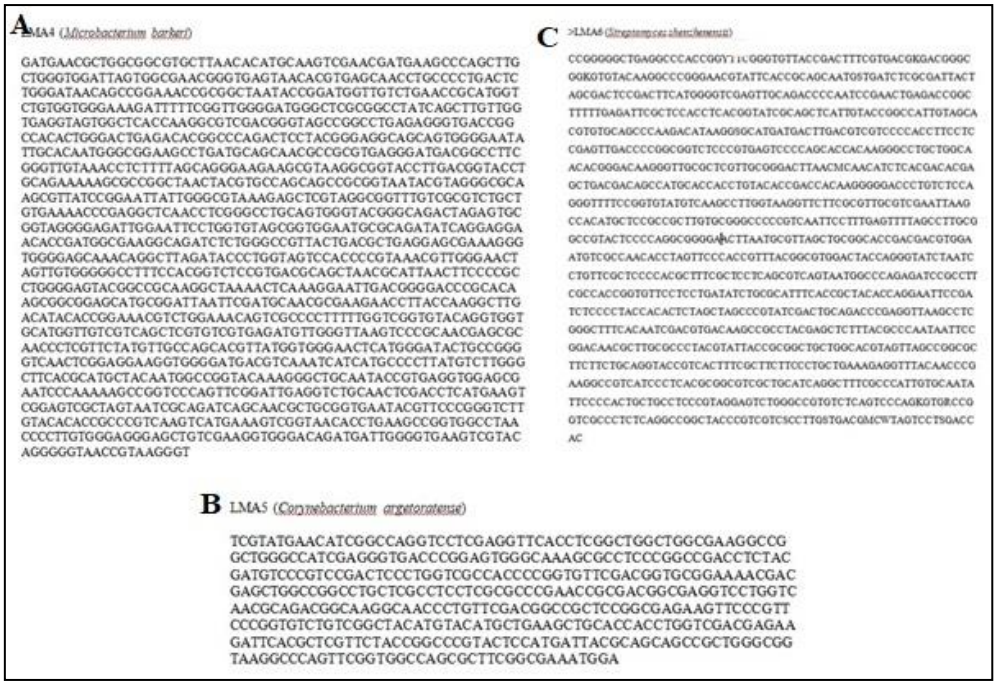

Figure 11: The 16s r RNA sequences of A. LMA4, B. LMA5 and C. LMA6 were eluted with sequences of Length 1483bp, 435bp and 1455bp respectively

When the query sequences of LMA4, LMA5 and LMA6 were analysed in NCBI (National Center for Biotechnology Information) portal using Basic Local Alignment Search Tool (BLASTn) for nucleotides, it was found that LMA4 had sequence similarity 100.00% with *Microbacterium barkeri* 16 s r RNA sequence, LMA5 had sequence similarity 99.93% with *Corynebacterium argetoratense* and LMA6 had 100.00 % with *Streptomyces shenzhenensis* strains.

3.8 Phylogenetic analysis of Actinomycetal isolates

The Phylogenetic tree, generated including the sequences of LMA4, LMA5 and LMA6, is depicted in Figure 12. It is observed that LMA4 and LMA6 have been originated from a common nodal point and diverted as two parallel branches, inferring about parallel evolution. However, the third strain namely, LMA5 was observed to originate from the said nodal point but it has taken its course as a different branch, thus suggesting its linkage is little far away from LMA4 and LMA6.

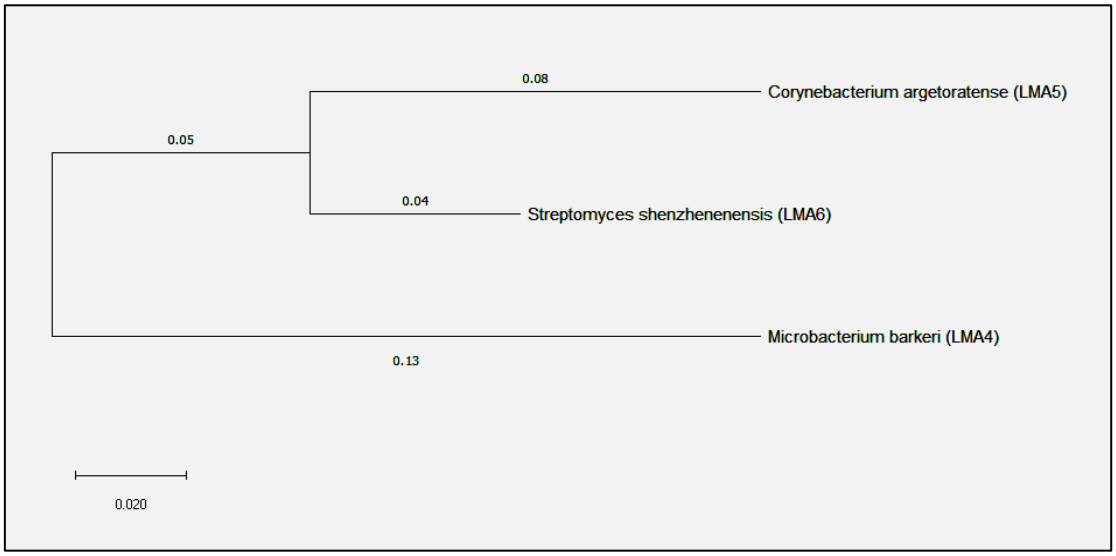

Figure 12: The un-rooted Phylogenetic tree generated using Mega X software online. The LMA5 and LMA6 are two branches (0.08 and 0.04) originated from a common node, LMA5 (0.13) is originated from the same node but have taken its course independently (0.05)

4. CONCLUSION

In conclusion, the soil isolated strains of Actinomycetes were pure cultures, which were luxuriantly growing in Actinomycetal isolation agar, Phenol Red Dextrose Agar, Mac Conkey agar and also EMB agar, inferring about the use of carbohydrates as source of energy for their growth and multiplication. Further, it was observed that the strains being Gram positive were profusely branched mycelial forms with spore production within 74 hrs of incubation at 37^0C. In addition, it was found that the strains were starch degraders as we all as Citrate users, evidenced from Starch degrading activity as well as Citrate tests. In the tests, for screening of bacterial growth inhibiting metabolite production (degree of biopotency), using patch inoculum diffusion method, it was found that LMA5 (*Corynebacterium argetoratense*) had relatively better bacterial growth inhibiting activity than the LMA4 (*Microbacterium barkeri*) and LMA6 (*Streptomyces shenzhenensis*). Further, the 16s rRNA sequencing, GC% analysis and more over Phylogenetic linkage analysis had suggested that LMA5 (*Corynebacterium argetoratense*) is of different linkage. Therefore phylogenetic linkage was well corroborated with bacterial growth inhibitory activity (biopotency) of respective strains.

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