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Soil Actinomycetes Molecular Characterization for Secondary Metabolites Production

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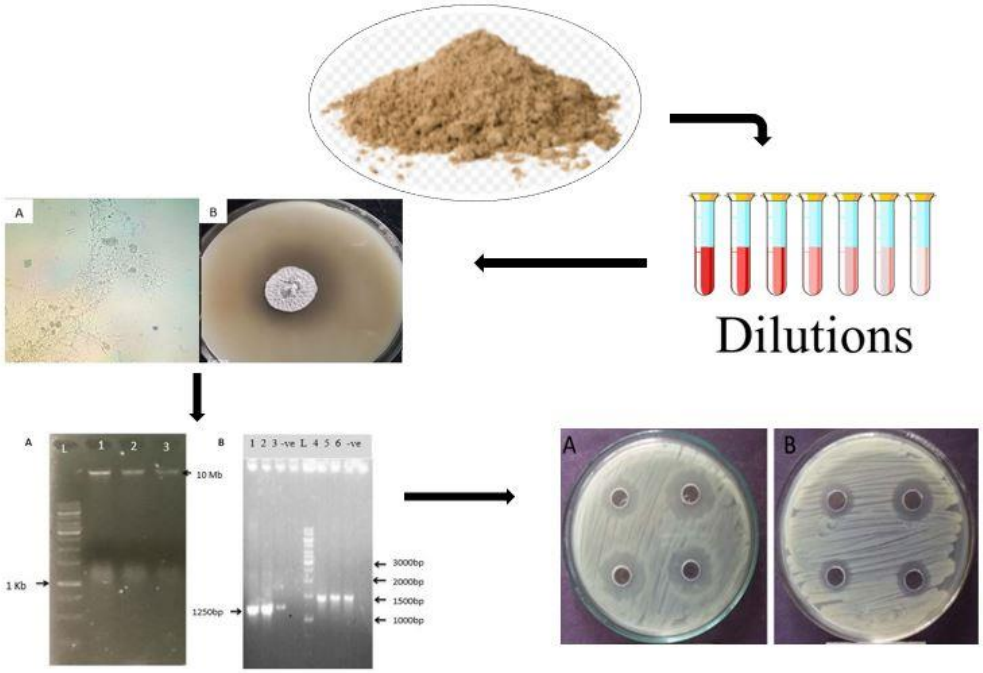
Abstract

Antibiotic resistance issue is really unavoidable, increased due to decrease in production of novel secondary metabolites production. Actinomycetes have the potential to produce biological active compounds used as an antibacterial, antiviral, antifungal, antiprotozoal, antihelminthic, anticholesterol, anticancer, immunosuppressant, pesticides and herbicides. Current research study was design to assess actinomycetes for production secondary metabolites. For this purpose different soil samples were collected and with help of dilution method, actinomycetes were grown on enrichment. Actinomycetes were morphologically analysed under microscope and molecularly through PCR and 16S-rRNA gene-amplification. After confirmation, actinomycetes were grown on LB and GMC media have potential to produce bioactive secondary metabolites. Current research study results that actinomycetes have potential to produce bioactive secondary metabolites. LB-media is the best suitable-medium for growth of actinomycetes and bioactive secondary-metabolites production. It is suggested that actinomycetes strains from various environment should be assessed for novel bioactive secondary metabolites.

Keywords
Actinomycetes, antibiotics, 16S rRNA gene, PCR, *Streptomyces*



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1. Introduction

Microbial natural products are the frontier in the discovery of bio-active compounds of pharmaceutical importance [1]. Different actinomycetes produce more than 80 % naturally occurring antibiotics available in the market. Actinomycetes are Gram positive, filamentous, spore forming, aerobic bacteria, widely distributed in natural ecosystem [2]. Their DNA contain high ratio of GC contents (>55 mol %), have unique spore forming abilities and mycelia structure. Actinomycetes produce aerial hyphae that differentiate into chains of spores [3]. Soil is an important natural source for many microorganisms including actinomycetes which produce many bio-active natural resources including clinically important drugs [4]. Actinomycetes have more genes which encodes for various enzymes that promote the production of bio-active secondary metabolites [5]. According to literature, strain growth conditions effects their secondary metabolites production that's why researchers apply various modification at nutritional and physiochemical level during fermentation process and through genome sequencing, find out genes that involve in production of secondary metabolites [6]. Most of the bioactive chemicals are obtained through metabolic pathways, which is encoded through adjacent chromosomal genes (biosynthetic gene cluster) [7].

Currently, multi drug resistance is a major issue, increasing due to decrease in production of new antibiotics. The possible way to access new antibiotics is to find new bacteria and fungi isolating approaches and to search for new sites or environment for selection of microbes [8]. However, in past two decades, there has been decrease in discovery of novel antibiotic compounds [9, 10]. Hence, research is now focused in search for next generation pharmaceutical molecules with previously unexplored habitats of actinomycetes. Some important antibiotics produced by actinomycetes are actinomycetin, mycetin and micromonosporin lysozyme, actinomycin, streptothricin, proactinomycin and streptomycin. These antibiotics differ greatly in their structure, antimicrobial and toxicity properties [11].

Streptomyces has largest genus most commonly found in terrestrial habitat, at least 90% actinomycetes isolated from soil have been reported to be *Streptomyces* spp. such as *streptomyces kanamyceticus, streptomyces fradiae, streptomyces griseus, streptomyces antibioticus, streptomyces ambofaciens, streptomyces venezuelae, streptomyces lincolnensis, streptomyces roseosporus* and *actinoplanin teichomyceticus* that produce important antibiotic[12]. *Streptomyces* are characterized by production of various extra-cellular enzymes and different kind of bio-active secondary metabolites that have great structural and functional diversity used as an antibacterial, antiprotozoal, antifungal, antiviral, antihelminthic, anticholesterol, anticancer, immunosuppressant, pesticides and herbicides [13]. Due to their useful biological activities, bio-active secondary metabolites produced by *Streptomyces* have received considerable attention especially in effects of human health [14]. A continuous screening of potential bacterial taxa for production of secondary metabolites is crucial for discovery of novel compounds [15].

Current research study was designed to assess actinomycetes for secondary metabolites production and also to find most suitable medium for growth of actinomycetes

2. Material and methods

Soil samples were collected from Quetta, Pakistan, the coordinates of sampling sites were 31°23'18.2768" N; 28°87'22.4548" E; 32°19' 16.876" N; 58°16' 0.4578" E; 29°20' 28.7658" N; 62° 60' 10.8670" E. All the samples were collected from landscape (6-12 cm depth) with help of spatula in sterilized polyethylene zipper bags and stored at 5ºC [16].

2.1 Actinomycetes isolation

Soil samples were dissolved in distilled water and dilutions were prepared. 1% Sodium dodecyl sulfate (SDS) was added for disruption of mycelia. For actinomycetes growth, enrichment media was used in which nistatin was added as antifungal agent. From each dilution, 150 µl was spread on enrichment media plates and incubated for 7 to 13 days at 31ºC [16].

2.2 Morphological identification of actinomycetes

Morphological identification of actinomycetes strains were done through classical microscopy procedures using different magnification lenses ranging from 10 xs to 100 xs. The morphology of these actinomycetes were then compared with already known strains of actinomycetes.

2.3 Molecular characterization of actinomycetes

Polymerase chain reaction (PCR) and 16S rRNA gene amplification procedures were used for the molecular identification of actinomycetes. Genomic deoxyribonucleic acid (DNA) isolation was performed according to the protocol of [16].

a. **a. Gel electrophoresis and nano drop**

The concentration of agarose Gel in gel electrophoresis for DNA used were 1%. The Isolated DNA, (5ul) was mixed with loading dye (2µl) and loaded into gel. For DNA measurement, DNA ladder (1kb) was used as marker. Nano-drop spectrophotometer was used to measure the concentration of isolated DNA where de-ionized water (1µl) was used as blank at 260/280 nm wavelength for optimization of nano-drop spectrophotometer.

b. **b.Gene 16S rRNA amplification**

Molecular identification of actinomycetes were also done through amplification of 16S rRNA gene. Specific primers for actinomycetes and *Streptomyces* were used. PCR conditions (**Table 1**) and constituents for the amplification of 16S rRNA gene for actinomycetes were thermo scientific PCR master mix (12.5 µl), forward primer (1.5 µl) reverse primer (1.5 µl) nuclease free water (9 µl) DNA template (1 µl) and for *Streptomyces* were thermo scientific PCR master mix (25 µl), forward primer (1.5 µl) reverse primer (1.5 µl) nuclease free water (1 µl) DNA template (2 µl).

Table 1. Polymerase Chain Reaction (PCR) profiles for actinomycetes and *Streptomyces* 16S rRNA gene amplification

PCR	Temperature	Time	Number of cycles
Bacterial 16S rRNA			
Initial denaturation	95ºC	10 min	
Denaturation	95ºC	1 min	
Amplification	56ºC	0.50 sec	35
Extension	72ºC	0.40 sec and 10 min	
Streptomyces 16S rRNA			
Initial denaturation	95ºC	10 min	
Denaturation	95ºC	1 min	
Amplification	56ºC	0.70 sec	35
Extension	72ºC	0.60 sec and 10 min	
Primers			
Actinomycetes	Forward primers	5′-GGGTCACGGGCGCTGGCCAT-3′5′-	
	Reverse primer	GGATGCGCTGGCGACGC-3′	
Streptomyces	Forward primers	5′-GGCTCACGCGCGCCGGCCTA-3′	
	Reverse primer	5′-GGTAGCGTCGGCGACGCA-3′	

2.5 Actinomycetes secondary metabolites production

For the growth of actinomycetes Luria-Bertani (LB) Broth and Golden Media Composition (GMC) growth media were used. 250ml of each media was prepared in 500ml flask respectively maintained at pH (7.2) and autoclaved at 121 ºC, 15 psi for 20 minutes (**Table 2**). The spores of actinomycetes were inoculated under sterile conditions. For production of secondary metabolites, the cultures were incubated in shaking incubator at 120 rpm, 30°C for 8 days. All these experiments were done in triplicate [18].

Table 2. LB broth and Golden media composition.

Ingredients	Concentration (g/L)
LB broth	
Dextrose	10
Na2SO4	5
NaCl	5
K_2HPO_4	2
Tris Base	2
NaNO3/ Urea	4.5/2
ZnSO4.7H2O	0.0178
MgSO4.7H2O	1
FeSO4.7H2O	0.010
Golden Media	
Dextrose	30
(NH4)2SO4	3
KH2PO4	0.5
NaCl	1
CaCO3	6
K2SO4	5
MgSO4.7H2O	0.3
ZnSO4.7H2O	0.04
FeSO4.7H2O	0.010

2.6 Cell mass calculation

Each media, 10 ml sample was collected into weighed Eppendorf tube after every 12 hours and to collect the cell mass, these samples were then centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded, and cell mass was dried for 24 hours at 50 ºC. Then dry cell mass was calculated [19].

2.7 Antimicrobial Assays

For antimicrobial assay 1.5 ml supernatant was taken into Eppendorf tubes soon after the incubation period from fermented flask and for assessment of antimicrobial activity of actinomycetes, LB and GMC media containing *Escherichia coli*, *Salmonella enterica* and *Bacillus subtilis* colonies were used. When wells were formed in *E. coli* containing media plate then 0.1 ml supernatant was drawn and kept in incubator at 30 °C for 5-8 days. Same procedure was performed for *S. enterica* and *B. subtilis* [19].

3. Results

In the Current research study, selective isolation technique has been used for the evaluation of actinomycetes. Actinomycetes were morphologically identified, and colonies were selected based on actinomycetes colony morphology (**Figure 1**).

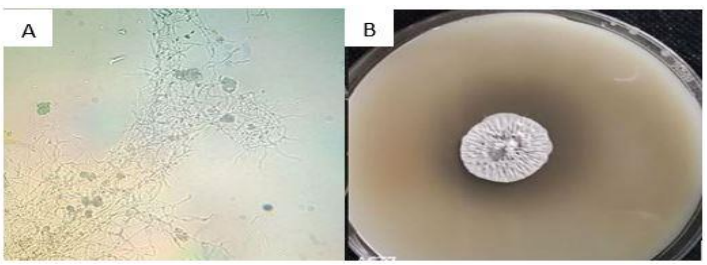

Figure 1. (**A**) Microscopic view of *Streptomyces* , (**B**) Growth of *Streptomyces* on media plate.

Successful identification of actinomycetes and *Streptomyces* were done through 16S rRNA specific polymerase chain reaction. The range of amplified PCR product of actinomycetes and *streptomyces* were in the range of 1250 to 1500 bp and 600 bp respectively (**Figure 2**)

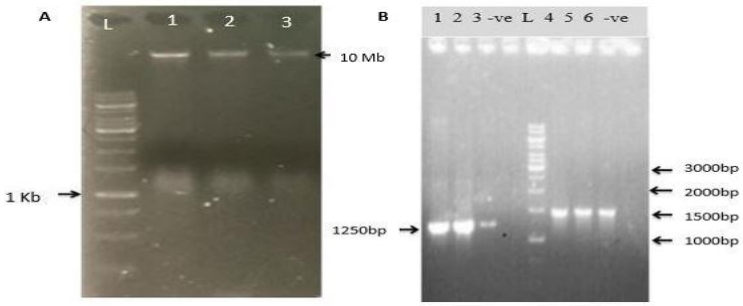

Figure 2. (A) Actinomycetes genomic DNA gel (B) *Streptomyces* specific 16S rRNA gene amplification gel.

Strong and mild antimicrobial activity of actinomycetes secondary metabolites was noted when *E. coli, S. enterica* and *B. subtilis* cultures were spread on media plates then clear zone was observed around the wells (**Figure 3)**. Actinomycetes have strong antimicrobial activity against *E. coli* as compared with *B. subtilis* and *S. enterica* as shown in **figure 4**.

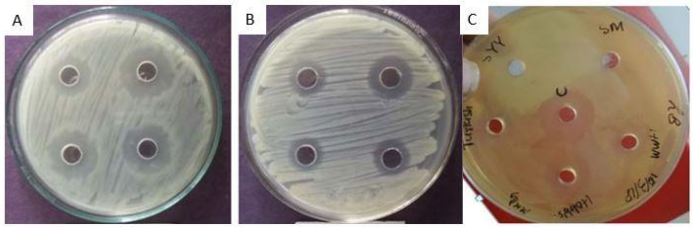

Figure 3. Antimicrobial activity of actinomycetes secondary metabolites (A) *E. coli* (B) *B. subtilis* (C) *S. enterica*

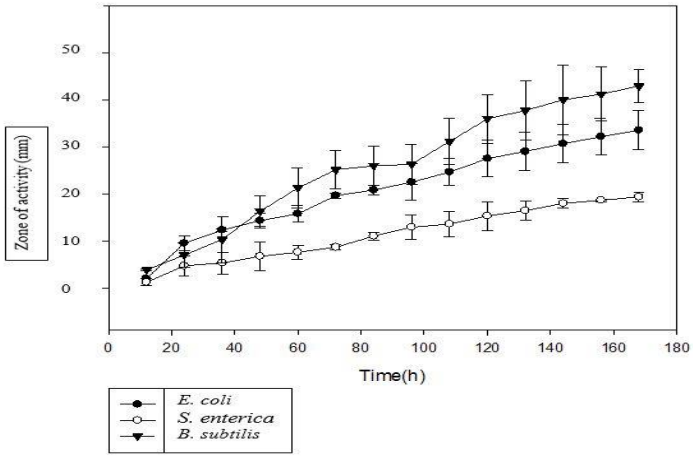

Figure 4. Comparison of antimicrobial activity of actinomycetes secondary metabolites against *E. coli, S. enterica* and *B. subtilis.*

Maximum amount of antimicrobial activity was observed on LB media as compared to GMC media. The results indicate that for production of actinomycetes secondary metabolites, LB growth medium was the best suitable medium as shown in **Table 3.**

Table 3. Antimicrobial activity of actinomycetes in (mm). Poor (-), Good (+), Very Good (++), Excellent (+++).

Hours	LB	GMC
24	-	-
48	+	-
72	+	+
96	++	+
120	++	+
144	+++	++
168	+++	++

1. **4. Discussions**

Actinomycetes molecular identification was successfully done through amplification of 16S rRNA gene. Amplified region of 16S rRNA helped in identification of *Streptomyces* strain [20]. According to literature, actinomycetes species, 16 S rRNA gene are about 1250 base pairs while *Streptomyces* have 600 base pairs confirmed by actinomycetes specific primers and amplification of 16 S rRNA gene [21]. Analysis of 16S-rRNA gene sequence amplification has been demonstrated to be a potential method for microorganism's phylogeny investigation. It is reported that 16S rRNA gene sequencing provides genus identification in most cases more than 90% [22]. For identification of novel strains, 16S rRNA sequence method is most prevalently used due to its presence in almost all bacteria and the function of 16S rRNA gene over time has not changed [23].

Antibiotics are the most vital bio-active secondary metabolites for the treatment of infectious diseases. Since last two decades, multidrug resistance (MDR) increased due to absence of new antibiotic that is a basic challenge for effective treatment of infectious diseases [16]. Due to increased burden of multidrug resistance, there has been increasing interest of researcher for searching of novel bio-active secondary metabolites to overcome multidrug resistance of pathogens [24]. Actinomycetes have potential to produce novel bio-active secondary metabolites to cure infectious disease. For this purpose different soil samples were taken from different spots for isolation of actinomycetes. Previous research studies showed that novel actinomycetes are most prominently found in soil so soil samples were an important activity for isolation of antibiotic producing soil actinomycetes [18].

According to previous studies, in inoculated plates, clear zones around the wells is an indication of antimicrobial activity of actinomycetes secondary metabolites against test organisms. Gurung at al reported that 0-18 mm inhibition zone of actinomycetes secondary metabolites against test organisms [25]. From present study a range of recorded inhibition zone of actinomycetes secondary metabolites were 0-44 mm which is higher than reported by Gurung et al [25]. Luria Bertani (LB) media supports growth of actinomycetes and it is a best suitable medium for secondary metabolites production. LB growth

medium is one of robust growth medium for bacterial culture as it contain a complete nutrition required for bacterial growth [26]. It is reported that LB medium is effective for the diversification of cultivable bacteria and suitable medium to isolate a novel bacterial strains [27].

Streptomyces genus contains Gram positive bacteria, having guanine cytosine (GC) rich (70%) genetic material [28]. Versatile soil bacteria, having high biotransformation process and active metabolite production act as antivirals, antifungals, anti-tumbrels, immune-suppressants, anti-hypertensives and also various antibiotics are produced [29]. Most antibiotics are produced from specific strains of *streptomycetes* genera [30]. They produce more economical antibiotics and enzymes which have applications in pharmaceuticals and agriculture fields as insecticides, herbicides and anti-parasitic compounds [31, 32].

Actinomycetes have ability to produce antimicrobial activity against many Gram-positive and Gram-negative bacteria such as *E. coli*, *B. subtilis* and *S. enterica*. [33, 34]. Actinomycetes secondary metabolites production started in the stationary growth phase when growth substrates are limited in media [35]. In current research study soil actinomycetes have been assessed for production of antibiotics, their secondary metabolites were checked with help of antimicrobial assays against *E. coli*, *B. subtilis* and *S. enterica* that shows strong antimicrobial activity against *E. coli* and *B. subtilis.*

5. Conclusion

Molecular characterization of actinomycetes for the production of secondary metabolite offers a strong research perspective. The findings of this research study demonstrated that actinomycetes have potential to produce bioactive secondary metabolites. LB media is the best suitable medium for growth of actinomycetes and secondary metabolites production. It is suggested that actinomycetes from different environment should be assessed for novel antimicrobial compounds.

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