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Pretreatment Techniques for Isolation and Screening of Actinomycetes: A Complete Approach

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ABSTRACT: Actinomycetes have been providing many industrially important bioactive compounds possessing great economic value and always being curious organisms for secondary metabolite production. They are recognized as prolific producers of enzymes, antibiotics, anti cancerous agents etc. and play significant role in recycling of organic matter. Numerous methods have been advocated for the isolation of actinomycetes to facilitate the discovery of natural compounds specially antibiotics. They are widely distributed in the natural habitats, hence various methods like pretreatments, combinations of antibiotics, specific isolation media and some novel methods has been adapted for their isolation. In addition, rare actinomycetes are also isolated by providing pretreatment combinations and high throughput screening methods. Strategy for a range of pretreatment techniques and isolation methods have been mentioned in this review for isolating actinomycetes.

KEYWORDS: Actinomycetes, pretreatment techniques, selective isolation

I. INTRODUCTION

Actinomycetes serve as promising source of bioactive secondary metabolites with great importance in pharmacology and commercial purposes. They play crucial role in the production of different enzymes as well as antibiotics like aureomycin, streptomycin, erythromycin etc. They also play significant roles in degradation or decomposition of organic substances such as cellulose, starch, chitin, organic acids, proteins and fats. Besides contributing to plant growth promotion (PGP), they also serve as biocontrol agents with their antagonistic effect against phytopathogenic microbes.

Actinomycetes are filamentous Gram-positive bacteria, mostly aerobic and belong to phylum Actinobacteria and order Actinomycetales. The G+C content in their DNA is very high about 70%. They are mostly mesophilic in nature and temperature range between 25 and 30°C is optimal for their growth. Most actinomycetes show maximum growth around neutrality but grow best in pH range between 6 and 9. The colonies of actinomycetes are compact, tough and often leathery with a dry surface on culture media and frequently covered with aerial mycelium and spores¹.

The taxonomy of actinomycetes has evolved significantly over time. It includes 6 classes, 22 orders, 54 families, 250 genera and 3000 species. Some rare actinomycete genera are Actinomadura, Actinoplanes, Amycolatopsis, Actinokineospora, Acrocarpospora, Actinosynnema, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kibdelosporangium, Kineospora, Kutzneria, Microbiospora, Microtetraspora, Nocardia, Nocardiosis, Nonomurea, Planomonospora, Planobispora, Pseudonocardia, Saccharomonospora, Saccharopolyspora, Saccharothrix, Streptomyces, Streptosporangium, Spirilliplanes, Thermomonospora, Thermobifida and Virgosporangium².

Discovery of novel metabolites from actinomycetes can be enhanced by isolating diverse group of actinomycetes. Hayakawa and Nonomura developed various methods for isolating desirable rare actinomycete genera which include a variety of pretreatment techniques in combination with enrichment techniques that appropriately supplement agar media with selective antibacterial agents³. An attempt is made in this review to explore existing and improved methodologies for isolation of actinomycetes.

Soil factors affecting the actinomycetes population:

The distribution and abundance of actinomycete taxa in soil is significantly influenced by environmental and physical factors including geographical location, pH, temperature, organic matter content, moisture content and nutrient availability. Soil salinity is one of the major environmental factors that could be the prime target for discovering novel microbial biodiversity producing rare secondary metabolites⁵.

It is worth emphasizing extreme environments that have extreme alkalinity, acidity, salt, hot springs, radioactivity, cold (polar-regions) harbour many unique actinomycetes. Diverse groups of actinomycetes such as Actinokineospora, Aerococcus, Arthrobacter, Brevibacterium, Glycomyces, Kribbella, Kocuria, Micromonospora, Nocardioidea, Nocardioopsis, Pseudonocardia, Promicromonospora and Rhodococcus were isolated from calcium sulphate rich soils of rock gypsum-salt forest (Gaolin) of Yuanjiang, China⁶.

Temperature plays key role for growth and survival of actinomycetes. Angel et al. (2012) investigated actinomycete biogeographic studies in hot springs of Zambia, China, New Zealand and Kenya⁷. The temperature and pH values of the sampling locations ranged between 44.5 and 86.5°C and 5-10 respectively.

Pretreatment of soil samples:

Lamari et al. (2002) reported a variety of selective methods to isolate novel antibiotic producing actinomycetes¹⁰. The pretreatment of samples with the addition of toxic chemicals for isolation becomes a selective approach¹¹.

Moist and dry heat treatment:

Pretreatment of samples by drying and heating stimulated the isolation of rare actinomycetes. The rare genus Streptosporangium is difficult to isolate by traditional methods as its sporangiospores could withstand and resist physical or chemical pretreatments. Hayakawa et al. (1991) found that dry heat treatment (120°C for 1 h) of natural samples greatly induces growth of Streptosporangium spp¹². Qin et al. (2009) subjected different samples to continuous drying at 100°C for 15 min, directly plating on different selective media enabled the isolation of 280 strains belonging to the genera Pseudonocardia, Nocardioopsis, Micromonospora and Streptosporangium¹³.

Additionally, along with dry heating of samples treated with chemicals such as 0.01 % benzethonium chloride, 0.03 % chlorhexidine gluconate, 0.05 % sodium dodecylsulfate (SDS), 6 % yeast extract and 1.5 % phenol and supplemented with different selective antibiotics such as leucomycin, nalidixic acid drastically eliminated the unicellular bacteria and other unwanted Actinomycete propagules (Streptomyces spp.) from the isolation plates and increased the selectivity for Streptosporangium spp., Microbispora spp., Actinomadura spp., Micromonospora spp., Nocardia spp. and Nonomuraea spp¹⁴. Niyomvong et al. (2012) showed that pretreatment of samples with moist (50°C/6min) and dry (120°C/1h) heating and 1.5 % phenol reduced the number of undesirable bacteria and enhanced the selective isolation of Actinoplanes, Gordonia, Microbispora, Micromonospora, Nocardia and Nonomuraea¹⁵.

Phenol Treatment:

Phenol is a biocide and lowered the number usual contaminant microorganisms. The samples treated with phenol (1.4-1.5%) have the significant positive effect controlling unwanted microbes and recommended for the selective isolation of actinomycetes¹⁶. Although phenol treatment of soil suspension lowered the number of fungi and other bacteria. This pretreatment may keeping the Micromonosporae and Microbisporae were in alive condition. In another study, the rare genera Micromonospora (49.2%), Actinomadura (13.1%), Microbispora (9.8%) and Polymorphospora (3.3%) were successfully obtained from soil samples using 1.5 % phenol pretreatment¹⁷.

Calcium Carbonate Treatment:

Treatment of natural habitat samples with calcium carbonate increased the populations of rare genera of actinomycetes⁹. El-Nakeeb and Lechevalier (1963) identified the increased number of actinomycetes colonies with treatment of calcium carbonate from soil samples⁸. Similarly, Alferova and Terekhova (1989) studied the soil samples were subjected to calcium carbonate treatment under humid conditions have proved to be efficient in increasing the number of strains and rare genera⁹. Samples mixed with calcium carbonate where the pH is altered in favour of the growth of actinomycete propagules and the calcium ions have the ability to stimulate the formation of aerial mycelia^{18,19}. The calcium carbonate in combination with other selective isolation methods are recommended for the isolation of rare genera of actinomycetes from soil samples²⁰.

Microwave Irradiation:

Many studies have examined the use of microwave energy for sterilization of soil, as well as increasing the culturability of Actinomycetes. Ferriss (1984) reported that microwave irradiation of soil reduced total fungal and total prokaryote counts in soil extracts²¹. Yang et al. (2008) reported that short periods of microwave irradiation increased the number of antagonistic actinomycete isolates²². Recently, Xue et al. (2010) reported that microwave irradiation of a calcareous soil increased the total counts of culturable actinomycetes such as Streptomyces and Micromonospora²³. Furthermore, Wang et al. (2013) isolated biologically active Streptomyces spp., Nocardia spp., Streptosporangium spp. and Lentzea spp. using microwave irradiation of soil samples²⁴. In addition, some researchers used other physical agents such as electromagnetic radiation¹⁵, electric pulses and super high frequency radiation, ultrasonic waves and extremely high-frequency radiation for the selective isolation of actinomycetes in natural samples²⁵.

Centrifugation Process:

Another physical method, centrifugation, eliminates Streptomyces and other non-motile actinomycetes from the liquid phase, thereby facilitating the selective growth of rare motile actinomycetes on isolation plates subsequent to inoculation¹⁶. The combined enzymatic hydrolysis and differential centrifugation method was particularly useful for isolating endophytic rare actinobacteria Pseudonocardia, Nocardiosis, Micromonospora, Amycolatopsis, Nocardia, Nonomuraea, Actinomadura, Gordonia, Promicromonospora and Mycobacterium¹³.

Chemoattractants And Chlorination Methods:

Selective isolation of sporulating actinomycetes known to produce motile spores is done by the use of xylose, chloride, γ -collidine, bromide and vanillin which act as chemoattractants for accumulating spores of Actinoplanes, Dactylosporangium and Catenuloplanes¹⁶. Further, selective isolation of rare genera such as Herbidospora, Microbispora, Microtetraspora and Streptosporangium can be achieved by chloramine treatment, as chlorination is known to suppress growth of contaminant bacteria and promote the growth of rare actinomycetes when plated on humic acid-vitamin-enriched media²⁶.

Enrichment Methods:

Pinus pollen grain baiting was another simple technique employed to isolate Actinoplanes species from soil¹². The bait bearing the sporangia in the dried soil particles is desiccated (30°C/2h) with the aid of silica gel and the spores are liberated upon immersion in water. A portion of the enriched zoospore sample is plated on HV agar containing nalidixic acid. The desiccation stage almost completely eliminates contamination of bacteria from the colonized bait and resulted in 83% selective isolation of Actinoplanes spp. More recently, Matsukawa et al. (2007) also successfully used desiccation for the selective isolation of Streptomyces species, with anti-phytopathogenic activity from root fragments of healthy herbaceous plants²⁷.

The rehydration and centrifugation (RC) method is a simple enrichment method incorporating differential centrifugation for the isolation of motile actinomycetes¹⁶. RC consists of immersing air-dried natural habitat samples in 10mM phosphate buffer containing 10% soil extract, allowing the preparation to stand at 30°C for 90 min, followed by centrifugation (1:500g/20 min). The RC method consistently achieved 37 to 86% selective isolation of motile actinomycetes from various samples. The most frequently isolated motile actinomycetes were Actinoplanes, Dactylosporangium, Actinokineospora, Actinosynnema, Catenuloplanes, Couchioplanes, Geodermatophilus, Kineosporia and Sporichthya were also recovered. In other studies, Otoguro et al. (2001) found that the combined use of calcium carbonate treatment with the RC method resulted in the selective isolation of Actinokineospora spp. on HV agar supplemented with fradiomycin, kanamycin, nalidixic acid and trimethoprim²⁸. Yamamura et al. (2003) applied sucrose-gradient centrifugation for selective isolation and ecological study of Nocardia spp. in soil and sediment samples²⁹.

The supernatant enriched with actinomycete zoospores is plated on HV agar containing nalidixic acid and trimethoprim. The phosphate buffer-soil extract solution liberates motile zoospores and centrifugation eliminates Streptomyces and other non-motile actinomycetes from the supernatant, thereby facilitating selective growth of motile actinomycetes on the isolation plates subsequent to inoculation.

A simple enrichment technique incorporating differential centrifugation which enabled the isolation of motile rare actinomycetes was reported by Hayakawa et al. (2000). This technique was designated a rehydration and centrifugation method and it consistently achieved 37–86% preferential isolation of zoosporic actinomycete genera from various soil and plant litter samples. The most frequently isolated motile actinomycetes using the RC method were Actinoplanes

and Dactylosporangium. Other strains were also recovered they are Actinokineospora, Actinosynnema, Catenuloplanes, Couchioplanes, Geodermatophilus, Kineosporia and Sporichthya. The combined use of the RC method along with the calcium carbonate treatment resulted in the selective isolation of Actinokineospora spp. on HV agar supplemented with antibiotics such as fradiomycin, kanamycin, nalidixic acid and trimethoprim²⁸. A water suspension of air dried soil was centrifuged through a gradient consisting of 10, 20, 30, 40, and 50 % sucrose at 240×g for 30 min. The 20% sucrose layer, which was enriched with Nocardia spp., was then diluted and plated on HV agar supplemented with antibiotics.

Chemical Treatment:

Selective isolation of actinobacteria can be effective by chemical treatment of samples using calcium carbonate and chitin treatment, calcium chloride, phenol, sodium dodecyl sulfate (SDS), yeast extract, germicide, chemotactic agents, and chloramine-T. Carbon and nitrogen sources such as calcium carbonate and chitin support growth of actinomycetes. The growth of Streptomyces and other genera on nalidixic acid containing HV agar is favored by treatment of soil sample with 0.05% SDS and 5% yeast extracts. Treatment of soil samples with chloramine-T was found to promote the growth of Herbidospora, Microbispora, Microtetrastora, Nonomurea and Streptosporangium on nalidixic acid containing HV agar³. Endophytic actinomycetes were also isolated mostly by chemical treatment for surface sterilization using sterilants including ethanol (70-95%), sodium hypochlorite (1-2%) and hydrogen peroxide (3%). Some surfactants such as Triton X-100, Tween 20 and Tween 80 can also be added followed by serial dilution³⁰.

Selective Media and Selective Antimicrobial Agents:

A soil sample contains mixture genera of microbes, selective media were always preferred for isolation of actinomycetes. The isolation media must be design to reduce the competing contaminants with adversely affecting actinomycetes colonies. Appropriate selective media containing macromolecules and selective antimicrobial agents are widely influenced for promoting growth of actinomycetes simultaneously suppressing the contaminant bacterial, fungal colonies. Addition of antimicrobial and antifungal antibiotics to the selective media enhances the selection of members of the family actinomycetales.

The aminoacid L-arginine was suggested by Benedict et al.,(1955) for isolating actinobacteria³⁴. Gauze's agar medium is selectively used for isolation of specific actinobacteria³⁵. Glycerol-arginine medium with antifungal antibiotics nystatin and pimarin encouraged the development of soil inhabiting actinomycetes⁸. Starch-casein agar medium, Bennett's agar suitable for selective isolation of actinomycetes³⁶. Williams and Davies (1965) employed antibiotics like actidione (50µg/ml), nystatin (50µg/ml), polymyxin B sulphate (5µg/ml) and penicillin (1µg/ml). While novobiocin (100µg/ml), Gentamicin (5µg/ml), azithromycin (50µg/ml), rifampicin (25µg/ml), leucomycin and tobramycin (25µg/ml) were used for the preferential isolation^{37,38}.

For successfully eliminating the fast growing bacterial contaminants, Hayakawa et al.,(1991) supplemented the HV agar with synthetic antimicrobial agents nalidixic acid and trimethoprim¹². International Streptomyces Project ISP agar media³³ and Hayakawa et al.,(1996) formulated the LSV-SE agar medium containing Kraft lignin as the carbon and nitrogen source to support and increased recovery of rare actinobacterial genera³⁹. Specialized growth media such as Oatmeal agar³³, Chitin agar, Raffinase-histidine agar, Humic acid-vitamin agar⁴⁰ by supplemented with antifungal antibiotics either gentamicin, oxytetracycline for facilitating good sporulation and selectively isolation of rare actinomycetes Dactylosporangium, Streptosporangium, Micromonospora, Saccharomonospora and Microtetrastora. You and Park (1996) developed the coal-vitamin agar medium for isolation of rare actinobacteria, pretreatment of soil suspension with peptone (6%), lauryl sulphate (0.05%) at 50°C/10min and also greatly enhance the colony count of actinomycetes and inhibited the growth of soil bacteria⁴¹. Sandoval et al.,(2015) formulated a simple method for isolation of actinomycetes by modifying santon medium supplemented with activated charcoal and selected antimicrobial agents⁴².

Yeast-extract malt-extract dextrose agar was suggested as a most suitable medium for isolation of actinomycetes⁴³. Suzuki. (2001) suggested an alkaline isolation medium (pH=9.0) i.e, Humic acid-MOPS (3-(N-morpholine) propane-sulfonic acid) gellan gum isolation medium (HMG) for actinobacteria selective isolation (Actinobispora, Planobispora) and it incorporated with antibiotics Gentamicin sulfate (100µg/ml), trimethoprim (50µg/ml), ampicillin sodium salt (20µg/ml) and nalidixic acid (10µg/ml) ⁴⁴. Seong et al.,(2001) designed hydrolysate vitamin agar (HHVA) ⁴⁵, Zakharova et al.,(2003) reported Gauze-I agar⁴⁶ and Hamaki et al.,(2005) employed soil extract agar as suitable for the isolation of novel actinomycetes⁴⁷. The selective isolation and enumeration of actinomycetes especially from marine and mangrove soils Actinomycetes isolation agar (AIA), from desert soils Minimal medium (MM), have been

developed^{48,49}. Qin et al.,(2009) suggested the media containing aminoacids as nitrogen sources (arginine, asparagine and proline) and cellulose, sodium propionate, sodium succinate, xylan as carbon sources had prominent isolation effectiveness especially for rare actinomycetes¹³.

Raghavarao et al.(2012) used the starch-casein agar, glucose-yeast extract malt extract agar amended with antimicrobial agents rifampicin (5µg/ml) and fluconazole (25µg/ml) and Kiranmayi et al.,(2014) suggested the starch-casein nitrate agar incorporated with nalidixic acid (20µg/ml), cycloheximide (50µg/ml) for extreme isolation of rare actinomycetes. Munaganti et al.,2015; Mogili and Muvva (2018), employed yeast extract malt extract dextrose agar (ISP-2) supplemented with rifampicin, fluconazole, streptomycin and nystatin (20µg/ml) respectively, for selective isolation of Actinobacteria. Ragalatha et al.(2020) attempted Vegitone agar medium in addition with different selective media amended with cycloheximide and streptomycin (25µg/ml) for retrieval of rare actinomycetes. All of these methods have significantly increased the total number of actinomycetes.

II. CONCLUSION

Actinomycetes are famous for antibiotic production and continued to be explored in hope of getting novel antibiotics. Different generalized and advanced methods such as pretreatment, enrichment, different media and integration of antibiotics for isolation of novel genera of the actinomycetes. The methods can provide significant impetus towards the isolation and screening of novel actinomycetes which will be ultimately significant for discovery of antibiotics and other industrially important bioactive compounds. Different strategies have been used for isolation and screening of antibiotic producing actinomycetes was mentioned in this review.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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