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ASSESSMENT OF PROFILE DEPTH, SITE OF SAMPLING, TYPE OF MEDIA AND METHODS USED FOR THE ISOLATION OF ACTINOMYCETES

ALGAFARI R.N.*

Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq.
*Corresponding Author: Email- rebahalgafari@gmail.com

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Abstract- In this work, we investigated the critical conditions affecting isolation of actinomycetes family members. Sites thought to be rich with these bacteria were river banks, farming soils, animal farms, animal manure, bird dung, slaughter houses, and diesel contaminated soils. These were site on which sampling, profile depth, isolation procedure, and cultivation medium were tested. All these sites gave a considerable number of isolates with different criteria. The profile depth of sampling was a critical factor for the isolation. The depth of 10 cm and surface scratches were the best for such purpose. Method of isolation was found to affect isolation procedure dramatically. For the isolation of bio-active actinomycetes, the use of procedures with selective agent like phenol is recommended, whereas, using $CaCo_3$ for this purpose in some procedures was effective in isolating distinct and separated colonies of actinomycetes. Media such as ISP4 and ISP2 are highly recommended for cultivation of newly isolated colonies, since such media were able to enhance specific criteria such pigment formation and helped these colonies to reach maturation at considerable time of incubation. The use of antifungal agents during isolation procedure was very important to obtain uncontaminated colonies, but instead of adding these agents with culture media, we found adding these agents directly on the plates before adding diluted sample from soil was very effective in eliminating fungal growth.

Keywords- actinomycetes family member, streptomycetes, isolation form soil, actinomycetes identification, bio-active actinomycetes, soil microorganisms

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Introduction

The family of actinomycetaceae was created by Buchanan [1] in 1918 and was originally used to accommodate many diverse organisms such as members of the genera *Actinobacillus*, *Leptotrichia*, and *Nocardia, Bofidobacterium, Archnia, Rothia* and genera *Streptomces*, and *Actinomyces* which are widely studied. After several revisions, membership of the family was restricted to bacterial species that appeared to be linked taxonomically by the following phenotypic characteristics: ability to produce Gram positive, branching and, later on, fragmenting filaments without aerial hyphae and spores; comparatively exacting nutritional requirements; facultatively anaerobic (capnophilic) to anaerobic growth; and fermentative carbohydrate metabolism [2].

The term aerobic actinomycetes is an informal designation for bacteria that belong to the order Actinomycetales. Originally, microorganisms of this order were classified with the fungi because they possessed true aerial hyphae, which were considered to be a fungal characteristic. However, on the basis of their cell wall components, in particular, their cell envelope lipid and peptidoglycan compositions, these microorganisms are now recognized as true bacteria that are aerobic. The major groups of the order Actinomycetales, actinoplanetes, maduromycetes, nocardioform actinomycetes, and streptomycetes, probably represent distinct groups, although additional strains need to be examined by molecular methods to confirm relationships based on chemical and morphologic indicators and to clarify apparent anomalies [3]. Actinomycetes are involved in the turnover of organic matter and xenobiotic compounds and are a prolific source of antibiotics and other useful secondary metabolites [4]. Streptomycetes are still a rich source of useful compounds, notably antibiotics, enzymes, enzyme inhibitors and pharmacologically active agents [5]. Out of 22500 biologically active compounds obtained from microbes, 45% are from actinomycetes, 38% from fungi and 17% from other bacteria. Species of *Streptomyces*, account for more than 70% of the total antibiotic production [6].

The isolation of actinomycetes from the mixed microflora present in nature is complicated by their characteristic slow growth relative to that of other soil bacteria. This has resulted in the development of selective isolation procedures based primarily on one or both of the following approaches: (i) nutritional selection, in which media are formulated with nutrients which are preferentially utilized by actinomycetes, and (ii) selective inhibition, in which compounds such as antibiotics are incorporated into media to selectively inhibit non actinomycete bacteria [7].

In this work, the profile depth, type of media, site of sampling, and the type of method used to isolate the members of actinomycetaceae was demonstrated to achieve quick, accurate and efficient

isolation and identification of this type of bacteria.

Materials and Methods

Sampling Sites

Soil samples were collected from farms, river banks, diesel contaminated areas in Baghdad University, animal farms soils, birds dung, animal manure from Al-Tarmiya Destrict, and slaughter houses in Baghdad in in average of 20 sample per site.

Profile Depth

Samples collected from different depths, they were collected from the surface of the soil, 10 cm depth, and 50 cm. Each sample was labeled with site of collection and depth. Soil samples with weigh of 10-20 gm were kept in sterile plastic container and dried in oven at temperature of 40°C for 5 days before processing.

Isolation Procedures

Procedure 1: One gram of each soil sample was 10-fold serially diluted in saline solution, 0.1 ml of each solution, at dilutions of 10^{-4}-10^{-6}, then spread on selective media of Pridham and water proline medium, then incubated at 28ºC for 7-14 days. Both screening media were supplemented with 25 µg/ml nalidixic and 50 µg/ml cyclohexamide to prevent growth of other bacteria and fungi, respectively [8].

Procedure 2: The soil was pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37°C for 4 days. It was then suspended in sterile Ringer solution (1/4 strength). Test tubes containing a 10-2 dilution of the samples were placed in a water bath at 45°C for 16 h so that the spores would separate from the vegetative cells and the dilutions were inoculated on the surface of the Actinomycete Isolation Agar (HiMedia) plates. The plates were incubated at 28°C for 7-14 days [9].

Procedure 3: The soils were incubated at 60°C for 40 min and re-suspended in 5 ml of saline. The re-suspended mixture was diluted with 45 ml of saline containing 1.5% (v/v) phenol and shaken for 30 min at 28°C. The media were supplemented with carbendizim (400mg/l) after sterilization and 50µl of samples were inoculated on cultivation media [10,11].

Isolation and Cultivation Media

Pridham medium per 1 liter: 10g glucose, 10g starch, 2g $(NH_4)_2$, SO4, 2g $CaCO_3$, 1g K_2HPO_4, 1g $MgSO_4$, 1g NaCl, and 12g agar, pH 7.0 and water proline medium per 1 liter: 10g L-proline, 12g agar, pH 7.0.

Actinomycete Isolation Agar per liter Sodium Caseinate 2.0g, Asparagine 0.1g, Sodium Propionate 4.0g, Dipotassium Phosphate 0.5g Magnesium Sulfate 0.1g, Ferrous Sulfate 1.0 mg, Agar 15.0 g.

Sodium Propionate agar per liter, Casein enzymic hydrolysate 10.0, Yeast extract 1.0 Potassium dihydrogen phosphate 3.0 Dipotassium hydrogen phosphate 4.8 Ammonium sulphate 3.0 Magnesium sulphate heptahydrate 0.2 L-Cysteine hydrochloride, monohydrate 0.5 Sodium propionate 15.0 Galactooligosaccharide 10.0 Agar 15g/l 15.0 Final pH 7.

ISP2 per liter Yeast Extract 4.0g, Malt Extract 10.0g, Dextrose 4.0g, Agar 20.0 g.

ISP4 per liter, Soluble Starch 10.0g, Dipotassium Phosphate 1.0g, Magnesium Sulfate 1.0g, Sodium Chloride 1.0g, Ammonium Sulfate 2.0g, Calcium Carbonate 2.0g, Ferrous Sulfate 1.0 mg, Manganous Chloride 1.0 mg, Zinc Sulfate 1.0 mg, Agar 20.0 g.

Results

Most of actinomycetes are spore forming bacteria. Such criterion helps greatly in spreading their members in nature. Therefore, all sampling sites gave a considerable amount of isolates with different colors, colony shapes, colony size, and diffusible pigments as shown in [Fig-1].

However, profile depth showed to be critical in providing different species of actinomycetes within isolated cultures. Sheep and cow manure gave the most different types of colonies (132 different types in average) followed by surface samples (113 different type in average) and 10 cm depth (45 different types in average) whereas 50 cm depth yield less isolated colonies. In addition slaughter houses, and finally bacteria isolated from bird dung were rich sources of this family in 30 and 27 average of different colony type respectively [Fig-2].

Assessment of Isolation Procedure

Three recommended procedures of Euanorasetr, et al [8] Ceylan, et al [9], Hayakawa, et al [10], and Ramakrishnan, et al [11] were used for the isolation of members of actinomycetes family members. Each uses different approach which aimed to isolate the most abundant and different types of these bacteria with specific criteria sometimes. Comparing these different procedures sowed that those used by, Ceylan, et al [9], Hayakawa, et al [10], and Ramakrishnan, et al [11] were highly efficient for such purpose since they relied on using selective agents specific physical treatment for actinomycetes over that used by Euanorasetr, et al [8]. The method of Hayakawa, et al [10], and Ramakrishnan, et al [11] gave bacterial isolates with different diffusible pigment which was a distinct feature for that bacterium [Fig-3].

Moreover, they recommended such procedure for biologically active actinomycetes that are able to produce antibiotics and other enzymes. Our preliminary investigation showed that actinomycetes isolated with such method were active in cellulobios degradation and antimicrobial agent production (unpublished data) since they gave about 18-30 mm clear zone on cellulobios medium as shown in [Fig-4].

The method used by Ceylan, et al [9] was very efficient in isolation of distinct and separated colonies even when low dilutions of soil sample was used. The observation we noted using this method that colonies appeared after incubation were small in diameter mostly similar in shape and color and differentiated to be different after 10 of incubation [Fig-5].

Method used by Euanorasetr, et al [8] gave a high yield of actinomycetes. The only drawback we faced is that it is less efficient in isolation of variety of these bacteria, since most of them were almost the same in shape and criteria, only small different types was observed regarding previously mentioned methods.

Assessment of Cultivation Media

Cultivation media showed a significant effect on growth of newly isolated acinomycetes. This effect was observed on shape of colony and size, pigmentation, time of differentiation, manifestation of specific traits such as pock formation phenomena, presence of mucous drops on the top of the colony, and convention of the center of the colony. [Fig-6] shows pock formation in *Streptomyces* isolate.

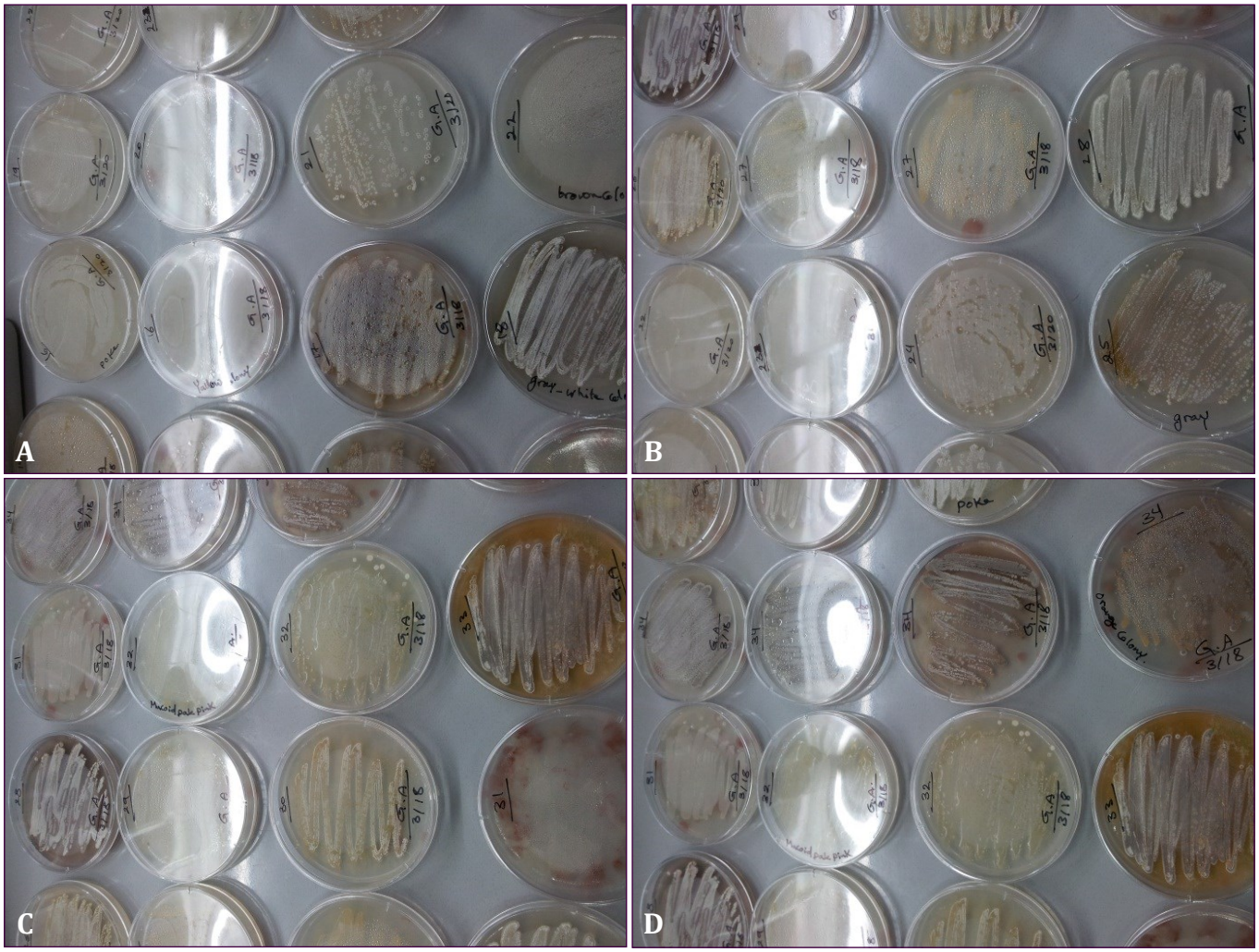

Fig. 1- Isolated actinomycetes samples with different colors, colony shapes, and size from **(A)** animal manure; **(B)** river banks soil; **(C)** farm soil; **(D)** animal farming soils.

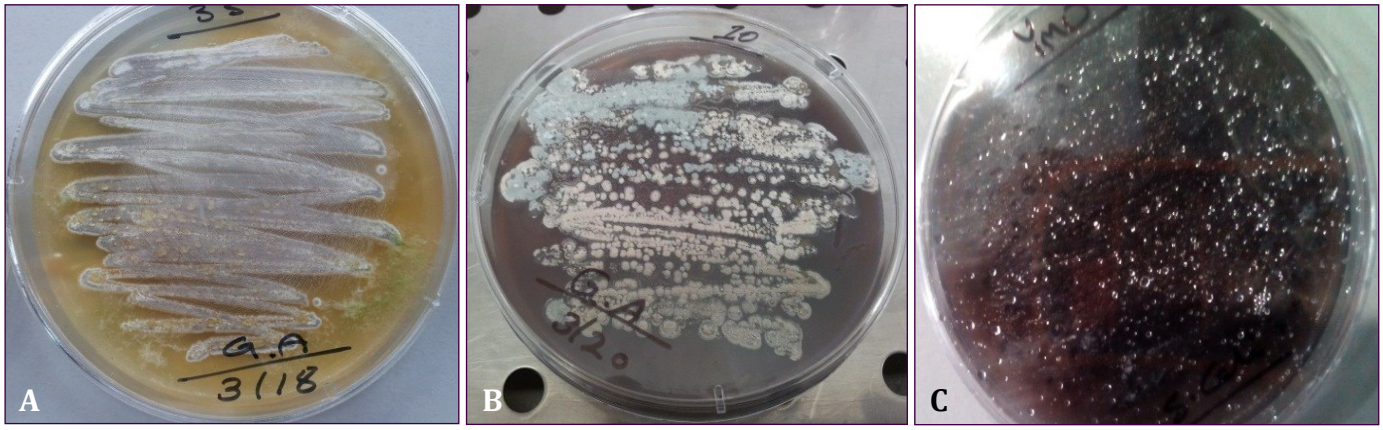

Fig. 2- Samples of isolated actinomycetes each from different site and depth, (A) Soil surface; (B) 10 cm depth; (C) 50 cm depth from left to right respectively as seen.

After completing isolation procedure, samples were first cultivated on ISP 2 medium. This medium helped significantly in growing new colonies by providing required nutrients growth. However, even this medium gave a good boost for actinomycetes growth; it did not affect their differentiation, since some of them did not reach the sporulation stage after 20 days of cultivation as shown in [Fig-7].

The actinomycetes isolation agar was very efficient in cultivating actinomycetes family members. The observation noticed using this medium is late growth of the bacteria, and those colonies grow were small in diameter. [Fig-8] shows the small diameter low count colonies of actinomycetes.

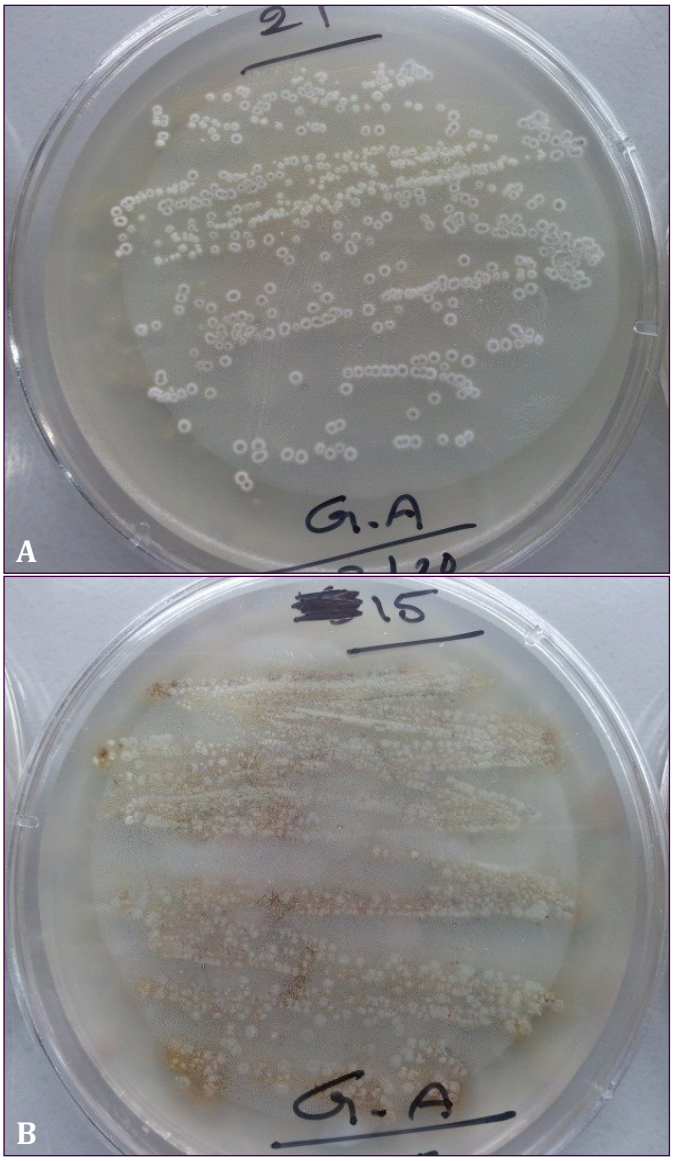

Fig. 3- Samples of actinomycetes isolated using different methods which shows difference in colony size, shape, and pigmentation, (A) Method recommended by Ceylan, et al [9]; (B) Method recommended by Hayakawa, et al [10].

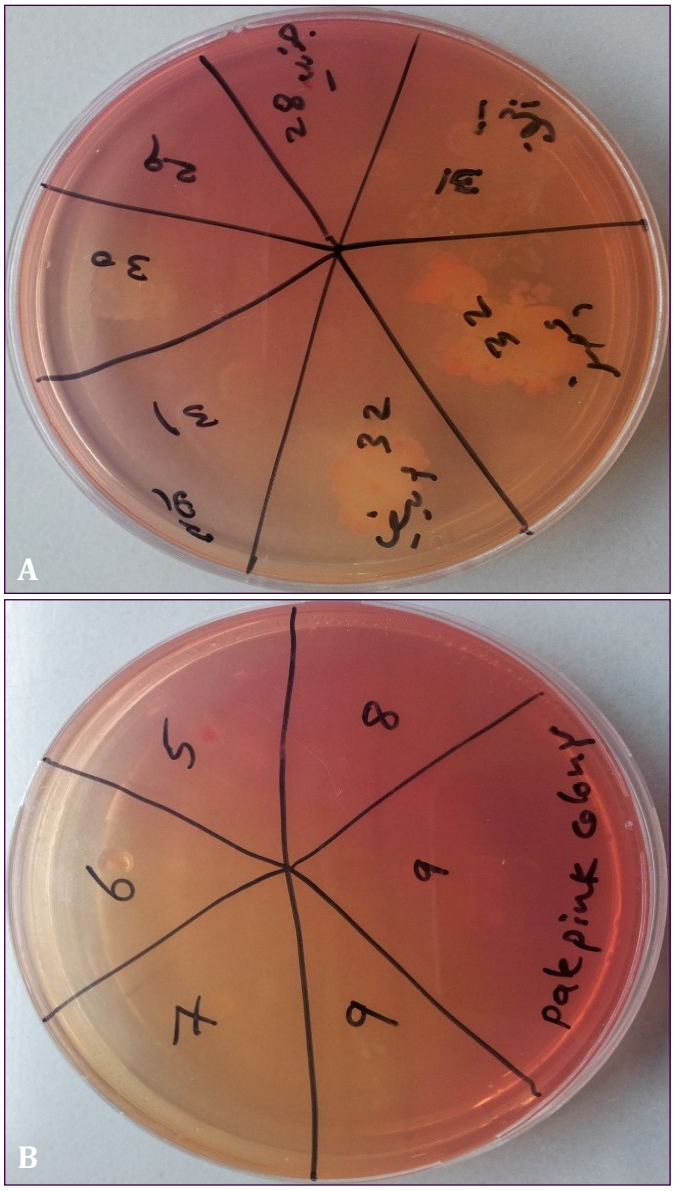

Fig. 4- Preliminary test of cellulose degrading bacteria. The figure shows the transparent zone surrounding some colonies with this activity.

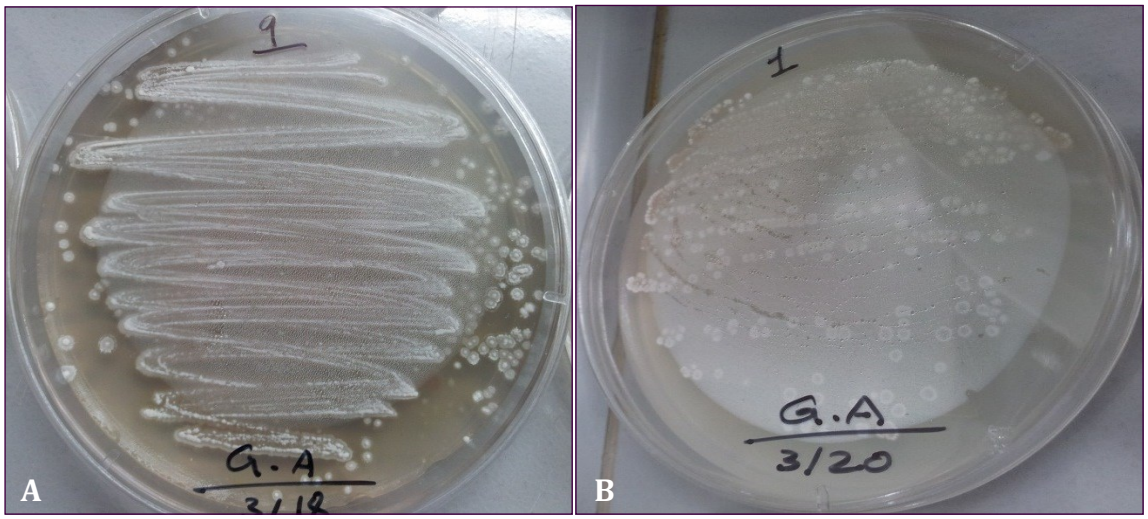

Fig. 5- Bacterial culture of actinomycetes isolated using different methods according to, **(A)** Hayakawa, et al [10]; **(B)** Ceylan, et al [9]

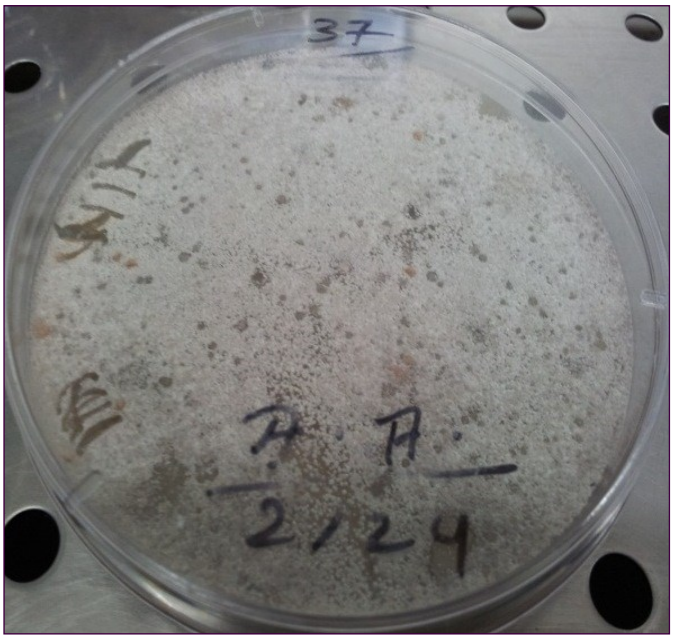

Fig. 6- Pock formation around some colonies those shows inhibition and lysis of affected bacteria. This is the result of fertility plasmid transfer from donor to recipient.

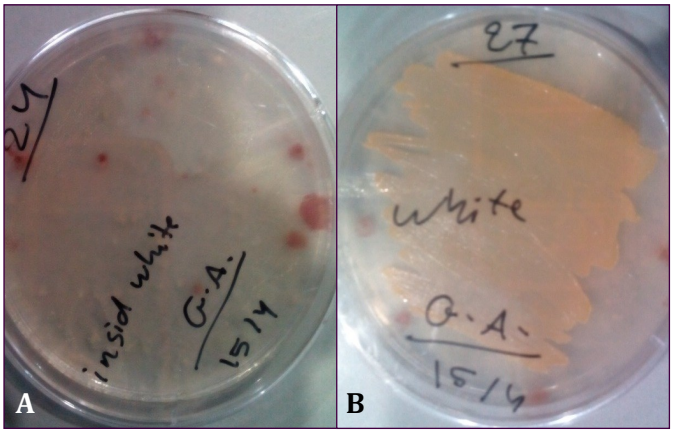

Fig. 7- Bacterial isolates cultivated on ISP2 medium. The figure shows the formation of substrate mycelia only after 10 days of incubation in some isolates without the formation of aerial mycelia.

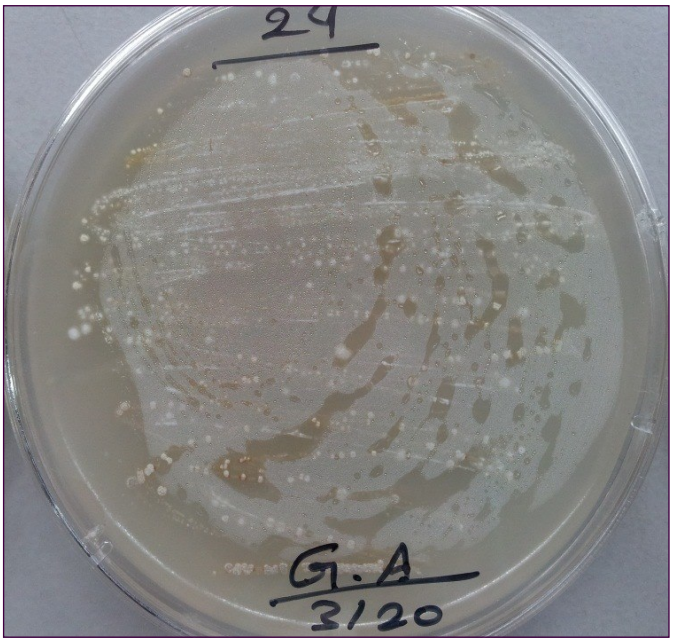

Fig. 8- Bacterial colonies cultivated on actinomycetes isolation agar. This photo was captured after 14 days showing small size of colonies that did not exceed 1.5 mm in diameter.

The same observation was noticed when sodium propionate medium was used. Colonies took 5 days to appear distinctly on the cultivation plate, and about 15 days to differentiate.

During our 5 months of experiments in isolating members of actinomycetes, the ISP4 medium was superior over other types of media in cultivation of these bacteria since all colonies failed to reach complete growth and sporulation, was able to complete this phase when grown on ISP4. Growth on this medium did not affect specific traits that might each bacterium shows, since pigment formation, pock formation, diffusible pigments were observed after growth [Fig-9].

Discussion

Actinomycetes comprise of novel species that are characterized by their shape. The formation of substrate and areal mycelia is the distinct feature of these bacteria which made some researchers to consider them as an evolutionary state toward eukaryotes, especially fungi.

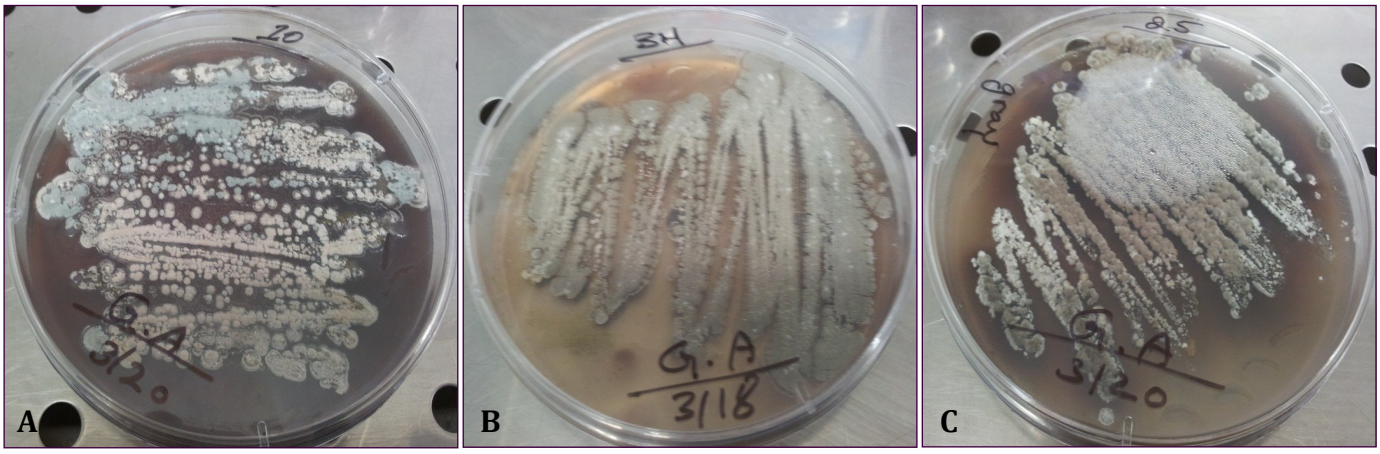

Fig. 9- Actinomycetes grown on ISP4 medium. The figure shows large colonies, complete differentiation of the bacteria, and presence of diffusible pigment.

Optimizing sampling sites, procedures, methods, and cultivation conditions showed high impact on isolation of new colonies with promising criteria.

Depth of sampling at 10 cm and surface is recommended for the isolation purpose, since the availability of nutrients and oxygen may help in propagation of these bacteria. Animal manure and bird dung can be considered as a collective factor to obtain different types of this bacterium due to the movement of these animal to different areas and feeding on what is available their as food which may help in providing different species of this family. Slaughterhouse can provide highly concentrated nutrients from blood and slaughtered animals, and rich niutrients to provide a propitiate conditions to assist their growth. In addition, species of this family were isolated from soils with diesel fuel contaminated soils. Presence of such contamination may be considered as a selective agent for bacteria with high enzymatic activity which a criteria for members of actinomycete family that can degrade complex organic compounds [12].

Our study showed that isolation procedure is a critical point in sample processing. The use of specific agents like $CaCo_3$ and phenol, affected isolation procedure dramatically combined with physical factors like heating and stirring which helped in elimination of other types of bacteria and enforce disassociation of spores from vegetative form resulting in a suspension rich with actinomycetes spores.

Cultivation media was also essential in a successful isolation procedure. The use of ISP 2 medium aided growth of actinomycetes in a significant way and resulted in wide range of colonies with wide diameter. The only thing that this medium failed with is that some bacteria did not reach spore formation state due to lack of some menial salts that they were available in ISP4 medium. All those bacteria that did not reach spore formation state were able to complete this phase on ISP4.

The observation that we recorded during this work that the presence of sodium propionate in Actinomycetes isolation agar, and sodium propionate medium had affected the size of the colony and time required to be seen as a distinct colony. This was regarded to the toxic effect of this organic salt on bacterial metabolism that was overcome by some members of actinomycetes that survive the effect of selective factors during isolation procedure.

It is noteworthy to mention that in all of our cultivation process after sample is processed, cyclohexamide was added from 50 mg/ml stock solution in a volume of 100µl on each petri dish and spread before adding the sample. This is modification was found to be better than adding this antibiotic to medium during formulation since it helped effectively in elimination of all types of fungal contamination.

Conclusions

Depending on this study, we found that sampling sites from farms, animal manure, animal farms, and bird dung are rich sources for biologically active actinomycetes if sampling, in some cases, was performed from surface or 10 cm depth. The isolation procedure is a critical stage in processing samples that require the selection of a procedure aided by selective agents that help elimination of bacteria other than actinomycetes when it is applied. The use of ISP2 medium for the first time inoculation of samples followed by ISP4 medium gave the best results for actinomycetes isolation and cultivation.

The addition of antifungal agents directly on cultivation medium right before adding the sample, not during media preparation, gave totally uncontaminated colonies of actinomycetes.

Recommendations

This study recommends sampling from surface scratches of soil and down to 10 cm depth for the isolation of actinomycetes. The use of selective agents during sample processing such as phenol for bacteria with high biological activity and $CaCO_3$ to obtain separated colonies. The use of enrichment medium such as ISP2 with provoke bacterial growth, whereas the use of ISP4 is highly recommended to deliver complete growth of bacterial colonies.

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