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Development of microphos technology by using alkaliphilic actinomycetes from the soil of Lonar lake

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Abstract
Alkaliphilic actinomycetes were sequestered from a desert soil sample of Lonar, District- Buldhana. The isolate was detected to produce; white, grey, milky white (cotton) color colonies. These colonies were obtained from desert soil sample. These strain produced aerial and substrate mycelium comprising of chain or smooth spore. The colonical growth of strain varied from yellow to grey. All the isolate were later purified and imperiled to a few phosphatic enzymatic screening. Results indicate that number of isolates showed the ability to solubilize phosphate (Microphos – Phosphate Solubilisation).

Keywords: alkaliphilic actinomycetes, Soil, Isolation, Identification, purification, microphos etc.

Introduction
Actinomycetes are gram-positive bacteria viewing a filamentous development like fungi. They are aerobic and extensively extent in nature. Actinomycetes are physiologically miscellaneous group, as evident by their production of frequent extra cellular enzymes and by thousands of metabolic yields they produce. Actinomycetes are one of the major microbial dominant groups and are well known for their saprophytic behaviour as well as for production of diverse bioactive secondary metabolites. Members of alkali-halophilic actinomycetes are not much explored and were poorly understood. Only few reports available pertaining to actinomycetes from saline and alkaline habitats. Recent findings from culturable and electronic supplementary material unculturable diversity have demonstrated that there are tremendous diversities and novelties among the halo tolerant and alkaliphilic actinomycetes present in saline and alkaline environments (Chen *et al.* 2009, Luo *et al.* 2009, Hozzein *et al.* 2004) [2, 3]. *Nocardiopsis alkaliphila* Sp. Nov., a new alkaliphilic actinomycete isolated from desert soil in Egypt (Hozzein *et al.* 2004) [3]. *Streptomyces fukangensis* Sp. Nov., a novel alkaliphilic actinomycete isolated from a saline alkaline soil (Zhang *et al.* 2013) [12]. They are also recognized for their capacity to survive in extreme habitats (Bredholdt *et al.* 2007) [1]. Actinomycetes DNA are rich in G+C content with GC% of 57-75% mainly in dry alkaline soil. Actinomycetes have been well known for the making of secondary metabolites. Many antibiotics are currently and used such as streptomycin, gentamycin and erythromycin are the product of actinomycetes. The actinomycetes are important not just to the pharmacological industries but also the agriculture. Identification of actinomycetes using microscopic techniques alone was not enough to confirm inevitability. Biological methods would be best method to indentify actinomycetes to their type. After isolating actinomycetes, it is primarily acknowledged on the basis of morphological characters so has to have preliminary determination of genus. Microbial natural products have been one of the major incomes for detection of novel drugs. Among the potential sources of natural products, bacteria have been proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds discovered. Morphological and chemotaxonomic characterization are useful aids in the identification of actinomycetes however, they may fail to correctly identify species of several genera that exhibit similar morphological and chemotaxonomic properties (Wang *et al.* 1996, Zhang *et al.* 1998). Polyphasic approaches including the 16S rRNA gene sequence has been widely used to determine taxonomic positions of many organisms in virtually all taxonomic ranks (Wang *et al.* 1999) [10]. Unlike bacteria, actinomycetes are unique in their morphology with widespread diverging substrate and aerial mycelium bearing chain of arthrospores. The substrate mycelium and spores can be pigmented, which makes them most colorful and attractive among microbes. On agar plates they form lichenoid, leathery or powdery colonies. Selective isolation of rare actinomycetes producing novel antimicrobial compounds (Khanna *et al.* 2011) [5].

Materials and Methods

Method for collection of soil sample

Soil sample were composed about 15 cm below superficial of the soil. All soil samples were collected casually from Agriculture Research Center, Lonar. Each sample was occupied from 5-15 cm penetration of the soil by using serried degraded metal tube (30 cm length). Soil samples were assorted and sieved to remove stones, leaf, stem and roots. Then, samples were crammed in cleaned and sterile plastic bags, established and stored at 4 ºC until analysis.

Microbe isolation and enumeration from soil sample media

Soil samples were air dehydrated for 1 week previous isolation. This helps in decreasing the populace of gram negative bacteria. Soil suspension method described by Jeffrey, (2008) [4] was used, where 19 of the soil. Samples were occupied and mix with 100 ml of sterile distilled water.
The soil suspension was stunned dynamically under room temperature on an orbital shaker at 200 rpm for 1 hr. 200 u of the soil suspension was pipette and lawn on to Agar.

Isolation of Alkaliphilic Actinomycetes strain from soil sample

Media: The alkaliphilic actinomycetes strains were sequestered using alkaliphilic actinomycetes medium and nutrient agar medium. Alkaliphilic actinomycetes medium was confined, 0.2g sodium casinate, 0.010g L-aspergine, 0.4g sodium propionate, 0.050g dipotassium phosphate, 0.1g magnesium sulphate, 0.001g ferrous sulphate, 1.5g agar-agar, 0.5 glycerol and pH was adjusted to 9.0

Isolation method

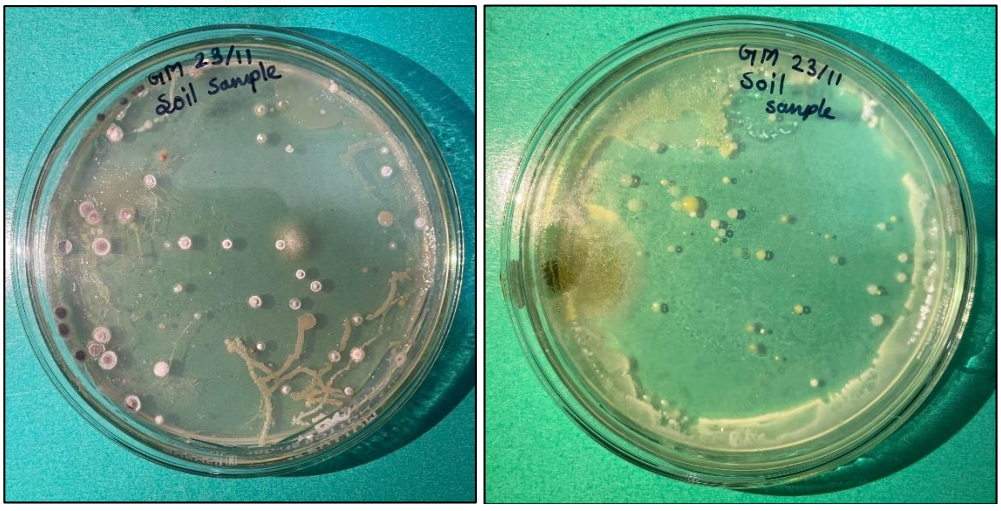
Direct method: For each composed sample, 0.2g of soil samples were extent over the surface of media. Then plates were incubated at 30-35 ºC for one week. The isolated colonies were checked and respreads on designated media.
Soil dilution technique: Actinomycetes were isolated from the soil samples by regular dilution plate technique (10^{-3} to10^{-6}). One milliliter aliquot was taken from each dilution and spread evenly over the surface of the discriminatory isolation media and incubated. Plates encompassing pure cultures were stored until further examinations. Isolates were used in the following experiments.

Characterization of Actionomycetes

Morphological and cultural characterization: The actinomycetes include abundant order of bacteria, which exhibit wide morphological and physiological variety. Morphological, physiological and biochemical belongings of the strains were studied as per International *Streptomyces* Project (Shirling and Gottlieb, 1966) [7] and Bergey's manual of systematic bacteriology (Williams *et al*. 1989).
The isolates were streaked on to 1) starch casein agar (starch 10.0 g; casein 1.0 g; K_2HPO_4 0.7 g; KH_2PO_4 0.3 g; $MgSO_4.7H_2O$ 0.5 g; $FeSO_4.7H_2O$) Glycerol asparagine agar (ISP 5) (L-asparagine 1.0 g; glycerol 10.0 g; K_2HPO_4 1 g; seawater 1 L; agar 20.0 g; pH 9.2

Cover slip culture technique

Cover slip culture is an important tool for learning the micro morphology of filamentous actinomycetes under uninterrupted circumstances. Spore chain morphology, disintegration of substrate mycelium, aerial mycelium, shape and amount of spores in spore chain etc. can be clearly studied by this method. The isolates were inoculated in to Marine actinomycete broth and incubated at 28°C for 1-2 days. Plates containing Casein starch peptone yeast malt extract agar medium (Casein 3.0 g; maize starch 10.0 g; peptone 1.0 g; yeast extract 1.0 g; malt extract 10.0 g; K_2HPO_4 0.5 g; sea water 1 L; pH 7.4; agar 20 g) were organized. Sterile cover slips 3-4 were implanted at an angle of 45°C into the agar medium. A loopful of spore suspension of actinomycetes was dispensed at the intersection of the medium and cover slip. The plates were incubated at 28 °C for 4-8 days. The cover slips were removed at intervals of 2-4 days and were observed under high power and oil immersion objectives. Morphology of aerial mycelium, substrate mycelium, organization of sporogenous hyphae, their morphology (straight, flexuous, spiral shaped) were recorded according to ISP (Shirling and Gottlieb, 1966) [7]. The selected actinomycetes were considered by morphological and biochemical tests. Morphological tests comprises of macroscopic and microscopic approaches. The mycelium structure, color arrangements of spores on the mycelium and colors of colonies were observed and compared with Bergey's manual of determinative bacteriology (Williams *et al*. 1989).



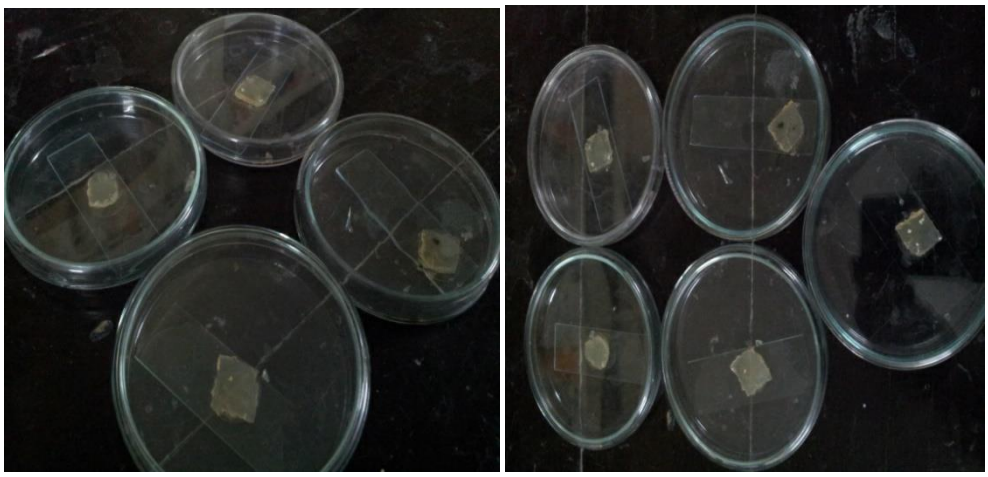


Fig 1: Cover slip technique for micro morphology of filamentous actinomycetes

Morphological characteristics

Morphological characteristics of the strains were considered according to methods of Shirling and Gottlieb (1966) [7]. The morophology of mycelial structures, spore chains and spore surface was observed with a light microscope x100 and various tests (pigmentation of substrate mycelium, diffusible pigments were performed for the characterization of the actinomycetes isolate. Following are some plates showing growth of actinomycetes on actinomycetes isolation medium.

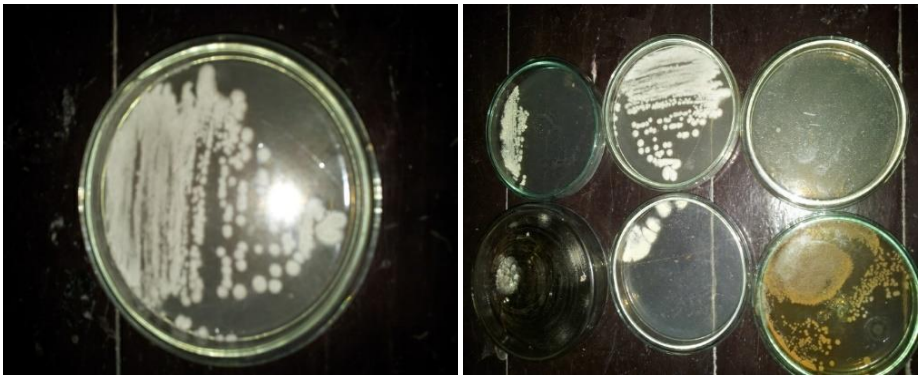


Fig 1: Microscopic appearance of spore chain morphology of actinomycete isolates

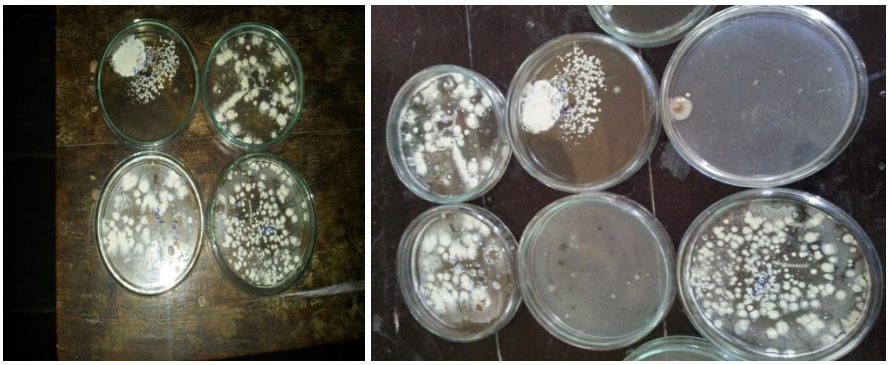


Fig 2: Colony appearance of actinomycetes isolates

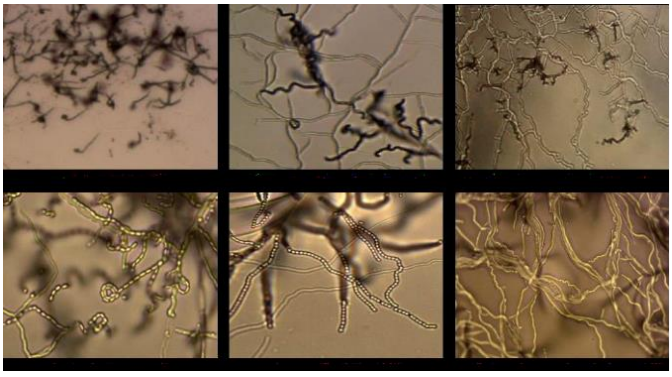


Fig 3: Microscopic appearance of spore chain morphology of actinomycete isolates

Fig 3: Colony appearance of actinomycetes isolates

The spore chain morphology of actinomycetes developed in coverslip observed under high power and oil immersion objectives exposed four types of spore chain morphology. The most prominent spore chain morphology was the spiral one, and 34% of the cultures exhibited spiral spore chain (mostly verticillate type) followed by 28% revealing rectiflexibiles (straight to flexuous) and 13.9% retinaculiaperti (open hooks, loops or spirals with one to two turns) spore chain morphology. (Fig 3-4). Remaining 23.9% of the isolates unveiled long chain of spores with zigzag fragmenting hyphe.

Screening of phosphate solubilizing alkaliphilic actinomycetes

Alkaliphilic actinomycetes were inoculated on starch casein agar medium encompass 2% of tricalcium phosphate as a sole phosphorus source for selective screening of actinomycetes which have capability to release inorganic phosphate from tricalcium phosphate. Inoculated plates were incubated at 30 degree temp for 7-10 days. After incubation period plates perceiving zone of clearance around the colonies which displays the degree of phosphate solubilization (Sahu and Sivakumar 2007) [6].

Results and Discussion

Isolation of alkaliphilic actinomycetes and distinguished their morphological and physiological characteristics. A total of 4 isolates were isolated from the soil samples on the actinomycetes isolation medium and 2 isolates on the starch casein agar. Aerial mycelium colours were observed for the detection of strain varieties in the different soil samples from dissimilar areas of lonar. Colour type was more plentiful in isolates grown actinomycetes isolation medium than in isolates grownup on starch casein agar medium. This medium used for the observation of spores and sporophores development in actinomycetes it was noted as medium for characterizing actinomycetes colony and the colour alteration after the development of sporulation regardless the media differences. This data provided that all the isolates are alkalipilic organisms. Alkaliphilic organism is recognized to be able to grow optimally at pH above 9, usually between 10 and 12. Nonethless, it cannot grow or grow slowly at the near neutral pH value 6.5. Zone of clearance around the colonies which indicates the degree of phosphate solubilization i.e. alkaliphilic actinomycetes display phosphate solubization activity. The appearance of colonies ranged from concentric, wrinkled, umbonate, and chrysanthemum (radial furrows) type (Fig. 1). Reviewed that neutrophiles sterptomyces species are able to cultivate between pH 5.0 and 9.0 with optimum growth close to neutrality. The soil samples composed in this study were sandy and alkaline (range of pH 7.76 and 8.80). Isolation of actinomycetes from sandy soil had been done with various media.

Conclusion

Phosphorous, an essential plant nutrient is a non renewable resource whose availability depends on mined rock phosphate. Even through synthetic phosphetic fertilizer has played some major roles in enhancing crop production, its excessive use has also dark side to it where it has found that to damage the environment and destruct soil fertility, seriously affect the human health. There is an urgent demand by the agriculture to find non hazardous strategy that can overcome the use of agrochemicals.

But research on PSM (Phosphate solublizing microorganism) that is bacteria and fungi are carried out worldwide in highly way but due to pH of soil get changes so their viability in soil get decreases because it cannot tolerate that conditions so we are focusing research on alkaliphilic actinomycetes which are generally tolerate alkaline conditions and phosphate solubilization and from that we are scale up the production and get pot trial and preparing efficient phosphate solubalizer in market. Finally we are concluded that the potential roles of actinomycetes are as phosphate solubilizers enhancing crop production.

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