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Isolation, Screening and Characterization of Actinomycetes from Marine Sediments for their Potential to Produce Antifungal Agents

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ABSTRACT

The marine environmental conditions are exceptionally different from terrestrial ones, it is assumed that marine actinomycetes might produce novel bioactive compounds. Hence marine sediments, collected from the South East coast of Bay of Bengal, were screened 16 isolates were obtained on starchcasein agar media. Preliminary screening was done using crossstreak method against fungal pathogens. The most potent strains were used to extract the antifungal substances. The antifungal activities were performed using agar diffusion method. All of the 16 isolates were active against at least to one of the test organisms. Of these, 5 actinomycetes were active against *Pencillium chrysogenum*, 3 against *Candida albicans*, 4 against *Aspergillus niger* and 3 against *Aspergillus flavus*, 2 isolates were active against *Saccharomyces cerevisiae*, 3 isolates against *Aspergillus fumigatus*, 4 isolates against *Geotrichum candidum* and finally all isolates showed activity against the fungal phytopathogens.Of all the 16 isolates, five best antagonistic actinomycetes isolates were selected for further studies. All the above isolates were characterized and identified by microscopical and macroscopical observations. Identification of the isolates publicized that all isolates belong to the genus *Streptomyces*. The results of this investigation revealed that the marine actinomycetes of Bay of Bengal sediments were potent source of novel antibiotics and bioactive compounds. The marine isolate *Streptomyces sp.* VSBT-501 was found to be more efficient in the production of secondary metabolites. Also further consideration has been paid to study their antifungal properties and their other biologically useful properties.

Keywords: Antifungal activity, Marine Actinomycetes, Streptomyces sp., Cross streak method.

INTRODUCTION

More than 70% of our planet's surface is covered by oceans and life on earth originated from sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than in the tropical rainforests (Edward *et al.*, 2006). Although the diversity of life in the terrestrial environment is extraordinary, the greatest biodiversity is in the oceans (Dubey *et al.*, 2005). As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts, and therefore, might produce different types of bioactive compounds (Fenical *et al.*, 1999; Gesheva *et al.*, 2005). These bacteria are an important group of microorganisms due to their ability to produce a wide array of such as antibiotics, anti-parasitic agents antitumor agents, enzymes cosmetics, secondary metabolites, nutritional materials immunosuppressive agents, vitamins, pesticides and herbicides (Valli et al., 2012; Imada et al., 2005; Atta et al., 2009). It has been reported that marine actinomycetes not only have several new species, but also have plenty novel structures with potent bioactivities (Takizawa et al., 1993). Many researchers have isolated novel antibiotics from the marine environment (Sujatha *et al*., 2005; Biabani *et al*., 1997; Maskey *et al*., 2003; Charan *et al*., 2004; and Li *et al*., 2005).

Actinomycetes are a diverse group of Gram positive, free living, saprophytic, filamentous bacteria and are a major source for the production of antibiotics (Valli et al., 2012). They belong to the order Actinomycetales (Super kingdom: Bacteria, Phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteridae). They are found in soil, fresh water and marine water environments (Gebreselema et al., 2013). They have high G+C (>55%) content in their DNA. They are the best common source of antibiotics and provide approximately two-third of naturally occurring antibiotics, including many of medical importance (Okami & Hotta 1988). Actinobacteria were originally considered as an intermediate group between bacteria and fungi, but latter it has attained a distinct position (Pandey *et al*., 2004). Actinomycetes are the most economically and biotechnologically valuable prokaryotes and are responsible for the production of about half of the discovered secondary metabolites. In the recent times, they have been exploited successfully for their biologically potential secondary metabolites. They produce diverse group of antimicrobial metabolites notably glycopeptides, beta-lactams, aminoglycosides, polyenes, polyketides, macrolides, actinomycins and tetracyclins.

Most of the antibiotics in clinical use are direct natural products or semi synthetic derivatives from actinomycetes or fungi. Resistance of bacteria to the effects of antibiotics has been a major problem in the treatment of diseases. Recently, there has been a fast emergence of newer infections along with several organisms developing resistance, which render already existing antibiotics less effective. One of the important approaches helpful in discovering new microbial species or unknown inactive substances include isolation and characterization of microorganisms from the most extreme habitations (Lee & Hwang, et al., 2002) and relatively unknown or unstudied areas (Moncheva, *et al*. 2002).

METHODS AND MATERIALS

Collection of marine sediments:

The 10 marine sediments were collected by using core sampler from the South East coast of the Bay of Bengal at various depths. The sediment samples were brown to black in colour and of sandy texture. They were maintained at

ambient temperature with sea water and brought to the laboratory in sterile polypropylene bags for isolation of marine actinomycetes.

Isolation of Actinomycetes:

Actinomycetes were isolated by spread plate technique following the serial dilution of soil samples on different media (Williams & Davies 1965) plates containing rifampicin and nystatin.

Identification of Marine Actinomycetes:

Light microscopical studies were carried out by cover slip culture technique and the characters such as aerial mycelia, spores arrangement are observed under microscope. The cover slip culture technique was done by inserting the sterile coverslip at an angle of 45° in to solidified SCA petriplates. A loopful of inoculum of each marine isolate was streaked along the line, where the coverslip meets the agar and the plates were incubated at room temperature. The isolated marine actinomycetes were also cultured on Starch casein agar and their morphological characters were also observed.

Screening of Actinomycetes for antifungal activity:

The screening method consists of two steps, Primary screening and secondary screening. Primary screening of Actinomycete isolates was done by perpendicular streak method (Egorov 1985) on Potato dextrose agar (PDA). The test fungi were *Pencillium chrysogenum, Candida albicans, Aspergillus niger, Aspergillus flavus, Saccharomyces cerevisiae, Aspergillus fumigatus and Geotrichum candidum.* Secondary screening of Actinomycete isolates was done by agar cup assay method on Potato dextrose agar (PDA).

Fermentation:

The isolates possessing broad-spectrum antifungalactivity in both primary and secondary screening were selected for fermentation. Fermentation was carried out by the submerged state culture in Erlenmeyer flask (1 lit.).

Characterization of Actinomycetes:

The potent Actinomycete isolates selected from screening methods were characterized by morphological, biochemical and physiological methods. The morphological method consists of macroscopic and microscopic characterization. Macroscopically the Actinomycetes isolates were differentiated by their colony characters, e.g. size, shape, color, consistency etc. For the microscopy, the isolates were grown by cover slip culture method (Kawato & Sinobu 1979). They were then observed for their mycelial structure, conidiospore and arthrospore arrangements on the mycelia under microscope (1000X). The observed morphology of the isolates was compared with the Actinomycetes morphology provided in Bergey's Manual for the presumptive identification of the isolates. Various biochemical tests performed were catalase, oxidase, citrate utilization, nitrate reduction, starch hydrolysis, starch hydrolysis, gelatin hydrolysis, NaCl resistance and temperature tolerance.

RESULTS

A total of 16 marine actinomycetes were isolated from the 10 sediment samples collected from Bay of Bengal. In the isolation plates, only the *Streptomycetes sp*. colonies were picked up and sub-cultured. All 16 marine actinomycetes were sub-cultured in pure form and maintained as slant culture for further use. Among the different media used only the Starch casein agar proved to be best for the isolation of actinomycetes from marine sediments. Grey colored mycelial cultures were the predominant among the isolates obtained.

In the isolation of actinomycetes from the marine sediments, a total of 16 isolates were obtained from 10 sediments using the method of isolation. The total count of actinomycetes isolated using different media (Fig 1, 2). Out of the five media used for isolation, starch casein agar media and glycerol asparagine agar have shown more number of actinomycetes while humic acid agar and glucose yeast extract malt extract agar resulted in less favorable for actinomycetes isolates. These results are in agreement with the findings of isolates (Fig 3, 4), who used five different selective media for isolation of actinomycetes and observed that starch casein agar and glycerol asparagine agar yielded good growth and more number of actinomycetes than the other types of media.

Among the 16 isolates of marine actinomycetes, about 9 marine actinomycetes showed antibacterial activity and the remaining 7 had no activity. The activity against fungal phytopathogens showed that a total of 9 marine actinomycetes have antifungal activity out of 16 marine actinomycetes isolated. Of these, 5 actinomycetes were active against *Pencillium chrysogenum*, 3 against *Candida albicans*, 4 against *Aspergillus niger*and 3 against *Aspergillus flavus*, 2 isolates were active against *Saccharomyces cerevisiae*, 3 isolates against *Aspergillus fumigatus*, 4 isolates against *Geotrichum candidum* and finally all isolates showed activity against the fungal phytopathogens. In the secondary screening, all the 5 actinomycete isolates shows activity in well diffusion assay (Fig 5, 6). From the primary screening (Table 1) and secondary screening (Table 2), about 5 actinomycetes were selected based on their efficiency.

Table 1: Primary screening of antifungal activity of the actinomycetes isolated.

Isolate No.	Name of the Test Organism (Inhibition zone diameter in mm)						
	P.chrysogenum	*C.albicans*	*A.niger*	*A.flavus*	*S.cerevisiae*	*A.fumigatus*	*G.candidum*
VSBT-103	+	+	+	+	--	+	+
VSBT-201	+	+	--	+	--	--	--
VSBT-302	+	--	+	--	--	+	+
VSBT-501	+	+	+	+	+	+	+
VSBT-503	+	--	+	--	+	--	+

Table 2: Secondary screening of antifungal activity of the actinomycetes isolated.

Isolate No.	Name of the Test Organism (Inhibition zone diameter in mm)						
	P.chrysogenum	*C.albicans*	*A.niger*	*A.flavus*	*S.cerevisiae*	*A.fumigatus*	*G.candidum*
VSBT-103	08	10	08	12	--	08	10
VSBT-201	12	16	--	10	--	--	--
VSBT-302	10	--	10	--	--	08	08
VSBT-501	22	20	16	18	18	12	16
VSBT-503	14	--	12	--	12	--	10

Of all the 9 isolates, five best actinomycetes isolates were selected for further studies. The potential isolates are VSBT-103, VSBT-201, VSBT-302, VSBT-501, and VSBT-503. According to 5 marine actinomycete isolates often show more active antifungal activity against different fungal pathogens. Similarly, the present study also indicated that the marine actinomycete isolates exhibited more antifungal activity against pathogenic fungi. All the 5 marine actinomycetes were identified up to genus level. The light microscopical studies and the growth characteristics on starch casein agar medium revealed that all the isolates possess characters which are similar to the genus *Streptomyces* and illustrated in Table 3.

Table 3: Morphological and Cultural characteristics of selective actinomycete isolates

Morphological and Cultural characteristics of the active isolates						
Isolate No.	**Morphological characteristics**			**Cultural characteristics**		
	Spore bearing hyphae	**Spore mass colour**	**Growth**	**Vegetative mycelia colour**	**Aerial mycelia colour**	**Soluble pigment**
VSBT-103	Retinaculum apertum	Brown	Abundant	Light brown	Brown	Reddish Brown
VSBT-201	Flexous	Light yellow	Good	Yellow	Brown	Brown
VSBT-302	Retinaculum apertum	Dark brown	Abundant	Dark yellow	Brown	Reddish brown
VSBT-501	Monoverticillus	Black	Abundant	Light brown	Brown	Nil
VSBT-503	Retinaculum apertum	Green	Abundant	White	Green	green

The morphological and cultural characteristics of the most active isolate VSBT-103, VSBT-201, VSBT-302, VSBT-501 and VSBT-503 were studied on International Streptomyces Project (ISP) media. The growth characteristics, presence of mycelium and soluble pigments were observed. The morphological characters of the active isolates were also studied microscopically under oil-immersion (100x) after gram-staining. The observations revealed that all the isolates are gram positive. Biochemical tests were conducted for melanin formation, nitrate reduction, coagulation and peptonization of milk, gelatin hydrolysis, starch hydrolysis, and carbon assimilation. All the isolates namely VSBT-103, VSBT-201, VSBT-302, VSBT-501, VSBT-503 showed positive results in starch hydrolysis (Table 4).

Thus, morphological, cultural and biochemical characterization indicated that the isolates belong to Streptomyces genus of Actinomycetes. The culture supernatants were tested for activity against overnight culture of *Pencillium chrysogenum* by cup plate technique. Zone of inhibition were measured after overnight incubation at 37°C.

Table 4: Physiological and Biochemical characteristics of promising isolates

Isolates					
Reaction	VSBT-103	VSBT-201	VSBT-302	VSBT-501	VSBT-503
Melanin reaction					
a. ISP-1	--	+	+	+	+
b. ISP-6	+	+	+	+	+
c. ISP-7	+	+	+	+	+
H_2S production					
a. ISP-6	+	+	+	+	+
Tyrosine reaction					
a. ISP-7	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Casein hydrolysis	--	--	+	--	--
Gelatin hydrolysis	+	+	+	--	+
Milk coagulation& peptonization	+	+	--	+	--
Nitrate reduction	+	--	+	+	+
Methyl red	--	+	--	--	+
Voges-Proskauer	--	--	--	--	--
Citrate	+	--	+	+	+
Oxidase	--	--	--	--	--
Urease	+	--	+	+	+
Catalase	--	+	--	--	--

Growth temperature					
a. 10˚C	+	+	+	+	+
b. 20˚C	+	+	+	+	+
c. 28˚C	+	+	+	+	+
d. 37˚C	--	+	+	+	+
e. 42 ˚C	--	+	+	+	--
pH tolerance	**6-10**	**5-10**	**5-10**	**5-10**	**5-10**
Cell wall Type	**I**	**I**	**I**	**I**	**I**

DISCUSSION & CONCLUSION

The incidence of multidrug resistant organisms is on the rise now-a-days, with several microorganisms showing resistance to the very drugs once potent against them. As a result, the treatment of a growing number of infectious diseases has now become a challenging task. Hence, there is an urgent need for developing new drugs which are effective against current existing antibiotic resistant pathogens.The marine habitat is proven to be an outstanding and a fascinating resource for new innovative and potent bioactive producing microorganisms. Actinomycetes are a potential source of bioactive compounds and the richest source of secondary metabolites (Suthindhiran et al., 2009). They are gram positive and filamentous in nature (Kokare et al., 2004). Recent investigations indicate the tremendous potential of marine actinomycetes, particularly *Streptomyces* species as a useful and sustainable source of new bioactive natural products (Valli et al., 2012).

The present study was aimed to isolate actinomycetes from the marine environment and screen them for the production of secondary metabolites. The medium was supplemented with nystatin to eliminate fungal contamination (Mohseni et al., 2013). Actinomycetes were identified by the presence of powdered colonies on the surface of the agar plates. A total of 16 marine actinomycetes were isolated from the 3 sediment samples collected from Bay of Bengal. The use of selective media (starch casein media) incorporation with antibiotics like rifampicin (50µg/ml) and nystatin (50µg/ml) were crucial in inhibiting the contaminating microorganisms. Both the primary and secondary screening methods were used to screen Actinomycetes for their antifungal activity. Primary screening was used to select the antifungal isolates and determine the range of microorganisms that were sensitive to the antifungal isolate. The secondary screening method was crucial to select the isolates for further studies. Both the primary and secondary screening methods were used to screen Actinomycetes for antibacterial activity. The first screening was used to select the antibacterial isolates and regulate the range of microorganisms that were sensitive to the antibiotic. The secondary screening method was crucial to select the isolates for further studies.

Out of 16, only 5 (48.15%) active Actinomycetes selected from primary screening showed antifungal activity in the secondary screening. This difference might be due to the difference in the morphology of Actinomycetes when grown in solid and liquid media as filamentous mycelia and fragmenting mycelia respectively (Bushell, 1993), or the chemical modification of the active compounds rendering them inactive in broth culture. From the result of primary and secondary screening, three isolates VSBT-201, VSBT-501 and VSBT-503 were found to be the best strains as they showed broad spectrum antifungal activity with a large zone of inhibition.

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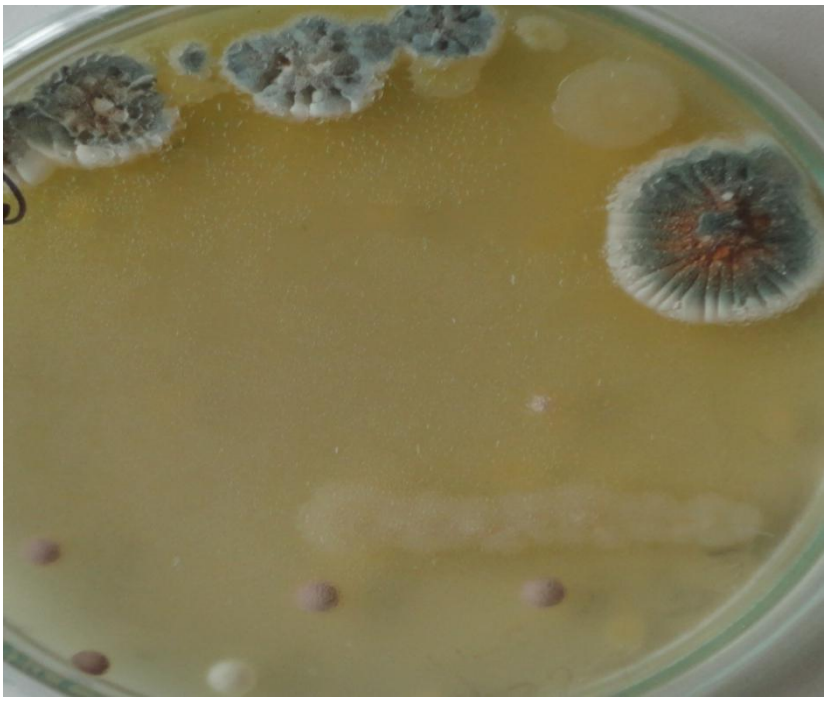

Figure 1: Isolation plate showing colonies in GYM agar media.

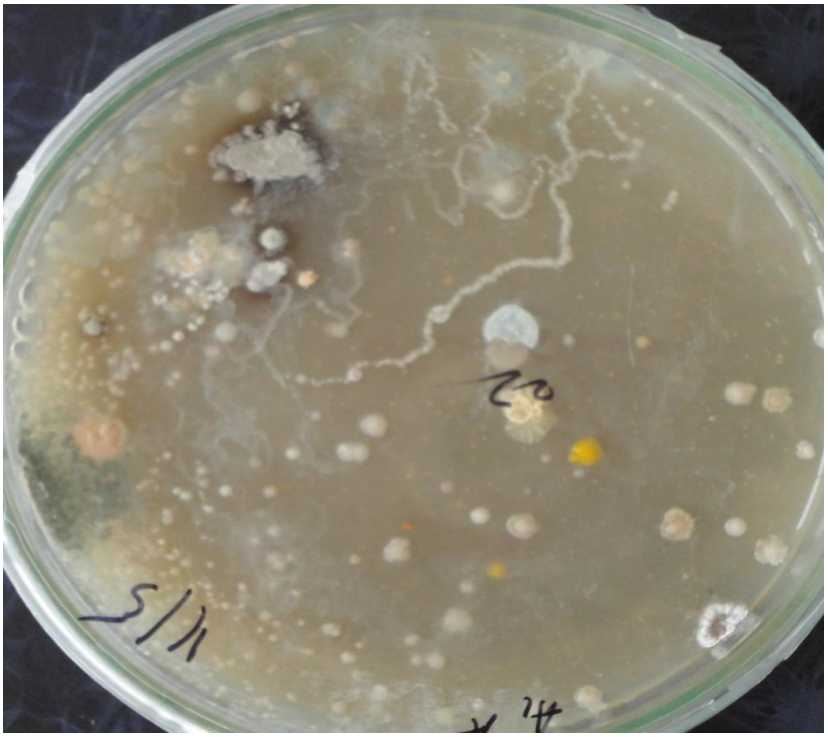

Figure 2: Isolation plate showing colonies in Starch Casein agar media.

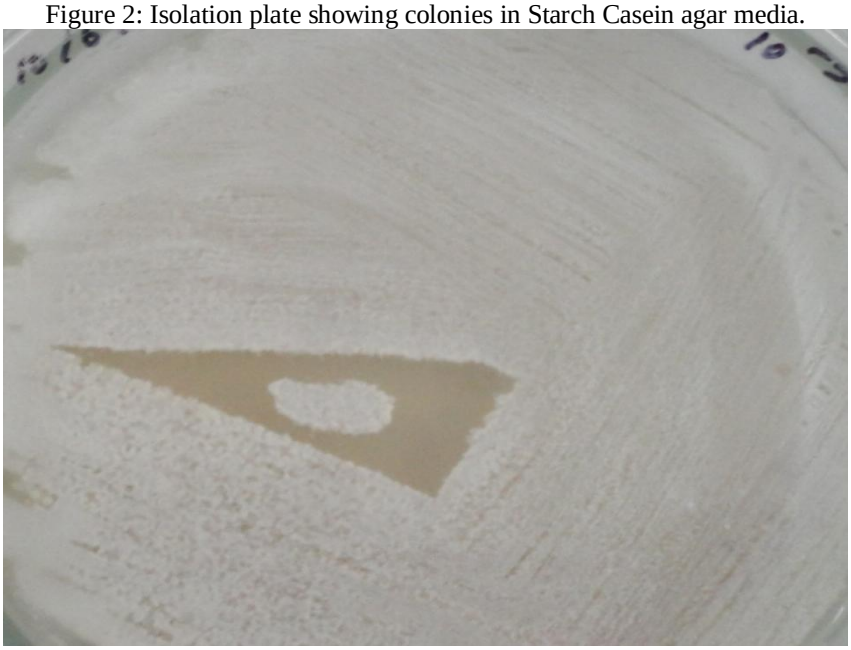

Figure 3: Pure culture plate of the Actinomycetes with white chalky colonies.

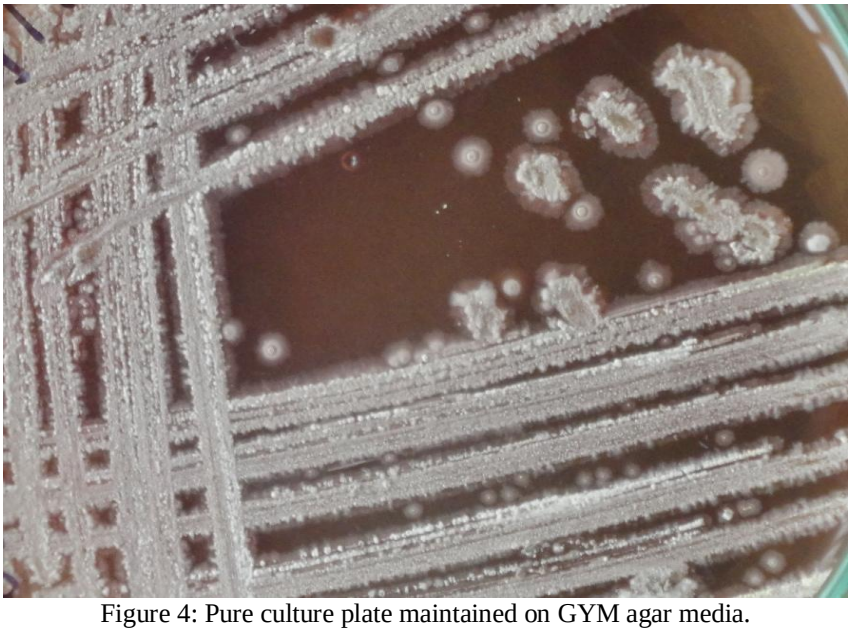

Figure 4: Pure culture plate maintained on GYM agar media.

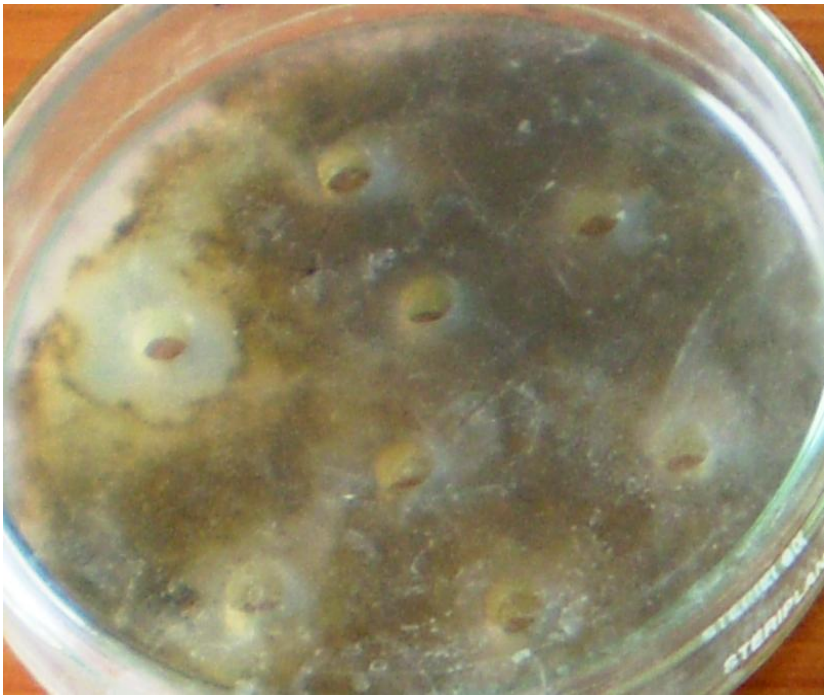

Figure 5: Actinomycetes showing zone of inhibition towards fungi.

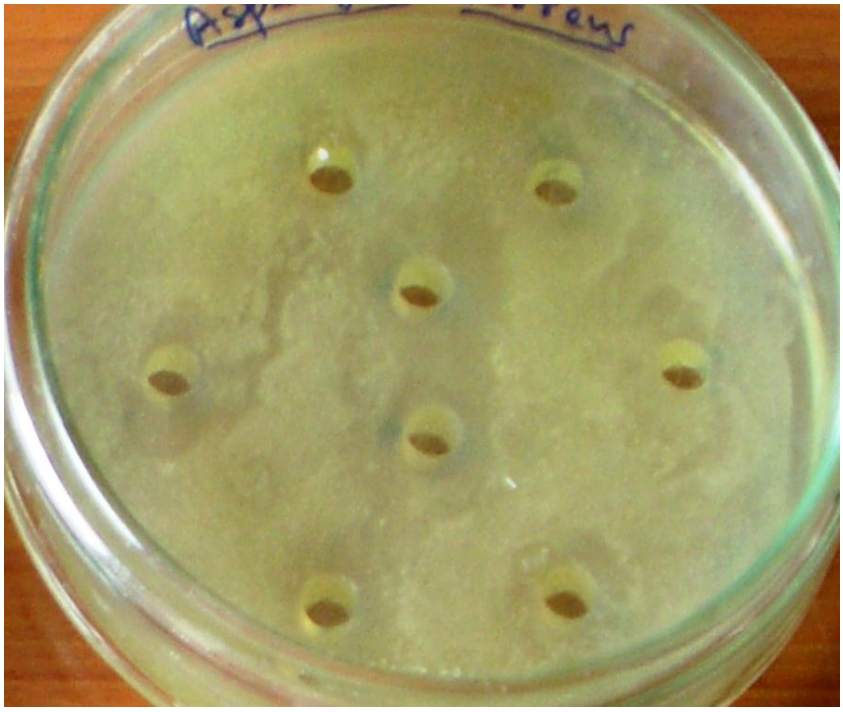

Figure 6: Actinomycetes showing zone of inhibition towards fungi.