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*Full Length Research Paper*

# Preliminary screening of antimycobacterial effect of psychrophillic Actinomycetes isolated from Manali ice point: Himachal predesh

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**Totally, 128 psychrophilic actinomycetes were isolated and 14 were found to be facultative psychrophilic which were selected to determine the antimycobacterial activity against *Mycobacterium tuberculosis*. The results showed that, four strains were active against the test organism *M. tuberculosis*. The active isolates screened in the present study were found to be highly effective and comes under *Streptomycetes* species (RH7 and RH8), *Micromonospora* species (RH9) and *Micropolyspora* species (RH12). All the isolates capable of producing metabolites and their presence were confirmed by TLC. The 'active culture filtrate' showed 'only one band' and its functional Group Is low molecular weight neutral compounds and amines were determined based on their solubility and pH.**

**Key words:** Psychrophilic actinomycetes, glaciers, drug resistance, *Mycobacterium tuberculosis*, primary amines.

## INTRODUCTION

*Mycobacterium tuberculosis* , the etiological agent of tuberculosis, kills more than 2 million people every year worldwide in concurrence with HIV-related infections. Moreover, appearance of multi-drug resistant (MDR) strains of *M. tuberculosis* to many, if not all, of the existing drugs has been noted. This has necessitated the development of novel anti-tubercular agents (Ducati et al., 2006; Tomioka and Namba, 2007). Tuberculosis (TB) is the most common cause of death due to a single infectious agent worldwide in adults. In 1993, the World Health Organization (WHO) took an unprecedented step and declared TB to be a global emergency (Grange and Zumla, 2002). According to the recent estimates, one third of the human population (about 1.86 billion people) was infected with *M. tuberculosis* worldwide in 1997(Dye et al., 1999). The organism causing TB was described only a century ago by Robert Koch on 24th March 1882. Until middle of the 20th century, there was no definitive treatment available for TB. With the available streptomycin, isoniazid and paraaminosalicylic acid (PAS), in the mid 1940s, predictable, curative treatment for TB became a reality (Sharma and Mohan, 1995). The introduction of rifampicin, pyrazinamide and ethambutol in the subsequent years ushered in the era of short-course treatment.

Studies of Microbiology in glaciers was initiated in the 1960s. At the initial stage, all work was focused on studies on genera, quantity and distribution of microorganisms. During the last two decades more information has been obtained about the diversity and distribution and extensive study has also been done on the physiological and genetic traits and the mechanism of cold adaptation of microorganisms in glaciers (Zlatanov et al., 2001). This progress has led to a declining trend in the discovery of unknown natural products derived from microorganisms (Watve et al., 2001), despite identification of only an estimated 1 to 3% of the existing compounds produced by *Streptomyces* species (Balts, 2006). Natural products have played a major role in antibiotic discovery, since 1941 when penicillin was introduced to the market (Clardy et al., 2006). In 1943, an American named Selman Waksman, together with his

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co-workers, discovered that a fungus called *Streptomyces griseus* produced an antibiotic substance which they named "streptomycin". Streptomycin was the first antibiotic used against *M. tuberculosis* (Zetterstrom, 2007).

The global resurgence of TB and the rapid emergence of MDR-TB have motivated the research of novel anti-tubercular agents and a lot of top researchers in various fields are doing their best to investigate novel compounds with anti-tubercular activity (Coleman et al., 2001; Lourenco et al., 2008; Biava et al., 2008). Studies of actinomycetes to date have been mainly based on terrestrial strains. Actinomycetes are the main sources of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry, making three quarters of all known products; the Streptomyces are especially prolific, producing around 80% of total antibiotic products. *Micromospora* sp. is the runner up with less than one-tenth as many as Streptomyces (Lam, 2006).

The genus Streptomyces was proposed by Waksman and Henrici for aerobic and spore-forming Actinomycetes (Williams et al., 1983). The taxon currently includes Gram positive bacteria, that have a DNA with a high guanine-plus-cytosine content (69 to 73%) and that form extensive branching substrates and aerial mycelia. Indeed, different *Sterptomyces* species produce about 75% of commercially and medically useful antibiotics (Baltz, 2006). They have provided more than half of the naturally occurring antibiotics discovered to date and continue to be screened for useful compounds (Miyadoh, 1993). In the course of screening for new antibiotics, several studies are oriented towards isolation of Streptomycetes from different habitats.

## MATERIALS AND METHODS

### Sample collection

The soil sample was collected from the Ice Point of Rothang Hill, Himachalpredesh the gate way of Himalayas Situated at an altitude of 3979 m above from the sea level on October 2008. The samples were collected in a sterile container and brought up to the laboratory in ice bag.

### Isolation of actinomycetes from glacier soil sample

One gram of soil sample was serially diluted up to $10^{-8}$ dilution. The dilution such as $10^{-3}$, $10^{-4}$, and $10^{-5}$ were taken and plated on Actinomycetes agar medium. The plate was then incubated at 15°C for 21 days until the colonies were developed. Based on the nature of the colony characters, the actinomycetes was selected and purified by sub cultured on starch casein nitrate agar supplemented with cyclohexamide (0.01 mg/ml).

### Selection of facultative psychrophilic actinomycetes

To determine the nature and kind of psychrophilic actinomycetes, all the isolates were incubated at three different temperatures. Three sets of balanced salt medium (BSM) were prepared and the isolates were streaked on the plates and incubated at three different temperatures respectively; at 0, 15 and at 35°C for 7 to 15 days. Facultative psychrophile can grow at 0°C up through 40°C. The isolates grown at 15 and 35°C were selected for further studies.

### Phenotypic characteristics

Isolated strains were identified through morphological, biochemical and chemotaxanomical studies as follows.

#### *Morphological study*

Isolates grown were facultatively selected and their colony morphology was studied by allowing them to grow on starch casein Nitrate agar and Actinomycetes agar. All the plates were prepared at one week time intervals for proper screening. The plates are streaked with the isolates and kept at 20°C for 10 days. The growth was recorded on the $5^{th}$ and $10^{th}$ day interval. Nature of colony, color of colony, pigmentation and spore mass were recorded and tabulated.

#### *Slide culture method*

This technique is used to find out the spore morphology and type of mycelium produced by the isolated strains. Actinomycetes agar plates supplemented with cyclohexamide were prepared; 4x4 mm of agar was sliced and placed over the surface of sterile glass slide on moist chamber with 60% ethanol. A clean cover glass sterilized by 90% ethanol was kept on the each agar blocks. Isolates were inoculated on the four corners and incubated at 35°C for 2 to 3 days. At the end of the third day, the plates were taken out from incubator and the cover glasses were stained by Sudan black IV. The stained cover glass was air dried and mounted on a new clean glass slide and was examined under Nikon photomicroscope. Aerial mycelium was observed as stained and the unstained are substrate mycelium.

### Biochemical studies

Various Biochemical tests were performed for the identification of potent isolates are as follows; indole, methylred and vogues proskaur, citrate utilization and hydrolysis of starch.

### Utilization of sugar and acid production

This was performed according to the method of Lechevalier with minor modifications. The sugar utilization medium were prepared and sterilized. Six different sugar compounds (arabinose, inositol, lactose, mannitol, mannose, xylose) were taken and 1% concentration of sugar was taken and added to their labelled medium respectively. After the sugar compounds were added to the medium. The isolated Actinomycetes were inoculated in their respective medium. After inoculation the tubes were incubated at 15°C for 4 to 5 days. After incubation, the tubes were observed and the results were tabulated.

## CHEMOTAXONOMIC STUDY

### Cell wall analysis for sugar and amino acid

Five milligram of the dried cell was mixed with 200 µl Tris in an

**Table 1.** Study of spore and morphology of isolated psychrophilic actinomycetes

| StsStrain coCode | Type of mycelium | Mophology of spore and hyphae | Possible genera |
|---|---|---|---|
| RH1 | AM/SM | Rarely branched, septate hyphae, monospore | *Micromonospora* sp. |
| RH2 | SM | Long vegetative cells | *Dactylsporangium* sp. |
| RH3 | AM/SM | Oval intergallery vesicle, zygospore | Intrasporangium |
| RH4 | AM/SM | Irregular branched, non fragmented, rarely septate | Planpmonospora |
| RH5 | AM/SM | Spiral chain of spores | *Streptomyces* sp. |
| RH6 | AM/SM | Medium length chain of spores | *Streptomyces* sp. |
| RH7 | AM/SM | long chain spore | *Streptomyces* sp. |
| RH8 | AM/SM | Rarely branched spiral spore | *Streptomyces* sp. |
| RH9 | AM/SM | Septate hyphae with Monospore | *Micromonospora* sp. |
| RH10 | AM/SM | Septate hyphae with monospore | *Micromonospora* sp. |
| RH11 | AM/SM | Long chain spore | *Streptomyces* sp. |
| RH12 | AM/SM | Rarely branched, Fragmented hyphae, chains of spore. | *Micropolyspora* sp. |
| RH13 | AM/SM | Monospore,Septate hyphae | *Micromonospora* sp. |
| RH14 | AM/SM | Spiral chain of spores | *Streptomyces* sp. |

Eppandorf tube. 300 µl of CTAB solution was added to the test tube and kept at 65°C for 30 min. After cooling, 20 µl of lysozyme (20 µg/ml) was added and incubated for 30 min. The tubes were centrifuged at 5000 rpm for 30 min and the supernatant was decanted. The cell extract pellet was evaporated and redissolved with 0.4 ml of distilled water. Known standard sugar and amino acids 1 mg/ml and sample were prepared and spotted on TLC plate and air dried. The plates were kept in the solvent system until the solvent font reaches ¾ of the plate. Then the plate was air dried and kept at 65°C for 10 min. The plates were sprayed with 10% of acid alcohol for sugar and kept at 100°C for 15 min and 0.2% ninhydrin for amino acid detection.

**Fermentation, antibiotic extraction and purification**

About 1 L of ISP4 medium was prepared and sterilized. The isolated psychrophilic Actinomycetes were inoculated on ISP4 broth and then incubated at 35°C for 7 days under 150 rpm.

**Screening of antibacterial activity of isolated Actinomycetes (well diffusion method)**

The Dubos agar plates were prepared and sterilized aseptically. The test bacterial sample *M. tuberculosis* were swabbed over the agar surface by a sterile cotton swab and kept undisturbed for 15 to 20 min. Then the wells were cut in the agar plates by well cutter. About 200 µl of Culture filtrate of ISP4 broth purified by nitrocellulose filter were loaded on the respective well and allowed to diffuse and then incubated under an anaerobic jar for 2 weeks.

**Solvent extraction**

The active strain grown fermentation medium was centrifuged at 10000 rpm for 10 min and the cell free supernatant was collected and filtered with nitrocellulose acetate filter paper, then it was mixed with equal an amount of ethyl acetate (1:1). The sample was shaken vigorously and the solvent phase were collected and evaporated at 40°C and then kept under desicator. The completely dried residues were re dissolved in distilled water and used for further studies.

**Separation of active compound by TLC**

The concentrated compound of active strain were separated on silica G 60 grade absorbent by mixture of solvent chloroform and methanol (4:1). The TLC plates were UV radiated and then exposed to iodine vapour and the Rf values were calculated. he functional groups of active compound were detected by destructive and nondestructive method.

**Bio-assay of active compound**

Active compound bands were separated by preparative TLC and the bands were collected aseptically from the silica plate and used for bio-assay against the test organism. About 100 µl of sample placed in a sterile disc and kept on test organism swabbed plates.

**Solubility test**

The active compounds were subjected to solubility test, to determine the functional group of compound. The pH of active compound was first determined with the help of pH paper and then mixed with water for their solubility. The water insoluble compound is subjected to react with 2.5 M NaOH, 0.6 M $NaHCO_3$, 1.5 M HCl and concentrated $H_2SO_4$.

## RESULTS AND DISCUSSION

Based on the spore morphology and mycelium, the isolated strains were identified as *Planpmonospora* sp., *Streptomyces* sp., *Micromonospora* sp., *Micropolyspora* sp., *Dactylsporangium* sp. and *Intrasporangium* sp. Among these 14 Actinomycetes, 6 comes under the genus Streptomyces, 4 comes under the genus Micromonospora, and the remaining isolates are each one under Dactylsporangium, Intrasporangium, Planamonospora and Micropolyspora (Table 1). All of the 14 isolates were morphologically different and showed luxuriant growth on starch casein nitrate agar than actinomycetes agar (Table 2). The presence of xylose,

**Table 2.** Growth of isolated psychrophilic actinomycetes on Actinomycetes agar and starch casein nitrate agar.

| Strain code | Growth on actinomycetes agar | Growth on starch casein agar |
|---|---|---|
| RH1 | Light ash, powdery, slightly rough, pale yellow pigmentation (non diffusable) | Greenish grey, powdery, rough, yellowish green pigmentation (non diffusable) |
| RH2 | Mucoid, white, no pigmentation | Mucoid, white, no pigmentation |
| RH3 | White, powdery, moist, no pigmentation | Ash, powdery, pale yellow pigmentation |
| RH4 | Chalky white, powdery, pale red wine, diffusable | Ash, powdery, diffusable brown pigment |
| RH5 | Light greenish ash to white, rough, no pigmentation | Dark greenish ash, powdery, no pigmentation |
| RH6 | Grey, powdery, slightly rough, no pigmentation | Grey, fine powdery, blackish brown pigmentation |
| RH7 | Sandal white, powdery, no pigmentation | Sandal, powdery, no pigment |
| RH8 | Ash, powdery, no pigmentation | Grey, powdery, brown pigmentation |
| RH9 | Dull white, , non diffusable pale yellow pigment | Grey, powdery, yellowish brown, diffusable |
| RH10 | White fine powdery, pinkish red | Whitish ash,powdery, diffusable red wine pigmentation |
| RH11 | Fine powdery, sandal white, no pigmentation | Dark ash, powdery, no pigmentation |
| RH12 | White powdery, , non diffusable pale yellow pigment | Grey, powdery, diffusible yellowish brown pigmentation |
| RH13 | Whitish ash, pale yellow pigment, non diffusable | Dark grey, powdery, pink pigmentation |
| RH14 | Milky White powdery, | White to Pink powdery |

mannose, levulose, inositol and arginine, lysine, proline and methionine were also detected in all 14 isolates.

Although, various biochemical tests were performed (Tables 3 and 4), it was unable to identify the actinomycetes up to species level, due to the lack of other tests. All the isolated strains are unanimous in starch hydrolysis and slightly differ in their ability to utilize various sugars. For proper identification of genera and species of actinomycetes, besides morphological and physiological properties, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined. Likewise, Micropolyspora is differentiated from Streptomyces "only by the fragmenting nature of the vegetative mycelium" is hardly appropriate, either from a morphological or from a chemical point of view (Thirumalachmar and Sukapurre, 1964). Genera *Streptomyces* sp. (RH7 and RH8), *Micromonspora* sp. *(RH9) and Micropolyspora* sp. (RH12) showed anti-mycobacterial ativity against the test pathogen. Among the four, *Micropolyspora* sp. most effectively inhibited the growth of test organism and the zone of inhibition was 28 mm. The bio-assay also supported this study positively. Based on several studies, the Streptomycetes are especially prolific antibiotic producer (Waksman and Curtis, 1916). The active compound was purified and separated by preparative thin layer chromatography. Single separated band was observed in all four samples in thin layer chromatography. The presence of fluorescence band at 365 nm and dark band at 254 was observed and indicates the presence of organic compounds. In chemical screening, development of pink color spot was observed by methyl yellow, which indicates presence of antimicrobial agents. According to Ph and solubility test (Tables 5 and 6) and pH of concentrated compound (Table 7) indicates the presence of low molecular weight compound on *Streptomycetes* sp. and *Micromonospora* sp. and Amines on *micropolyspora* sp. The most effective compound obtained from RH12 were soluble in NaOH and its pH is 10 and indicates it has amine compound. This is the first time the anti-mycobacterial activity of Micropolyspora was determined. At present, the chemotherapeutic options for TB treatment have been restricted to a handful of compounds introduced 40 to 50 years ago; this must be administered in combination for extended periods. Early stage drug discovery is a key bottleneck to find the novel antimycobacterial drug. Of the 1,556 chemical compounds marketed worldwide

**Table 3.** Biochemical properties of isolated psychrophilic Actinomycetes.

| Strain code | Grams stain | AFB | Catalase | Oxidase | Starch | Urease | Indole | MR | Vp | Citrate |
|---|---|---|---|---|---|---|---|---|---|---|
| RH1 | + | - | + | + | + | + | ++ | + | ++ | - |
| RH2 | + | - | + | + | - | - | ++ | + | + | - |
| RH3 | + | - | - | - | ++ | + | ++ | - | - | - |
| RH4 | + | - | - | - | ++ | - | ++ | + | - | - |
| RH5 | + | - | + | + | + | - | ++ | + | ++ | - |
| RH6 | + | - | - | + | ++ | + | ++ | ++ | ++ | - |
| RH7 | + | - | - | + | ++ | - | ++ | + | - | - |
| RH8 | + | - | + | + | ++ | - | ++ | - | - | - |
| RH9 | + | - | - | - | - | + | ++ | + | + | - |
| RH10 | + | - | + | + | - | + | ++ | - | ++ | - |
| RH11 | + | - | - | + | ++ | + | ++ | + | ++ | - |
| RH12 | + | - | - | + | ++ | - | ++ | ++ | + | - |
| RH13 | + | - | - | + | ++ | + | ++ | ++ | ++ | - |
| RH14 | + | - | + | + | + | - | ++ | + | ++ | - |

**Table 4.** Utilization of various carbohydrates by isolated psychrophilic actinomycetes.

| Strain code | Arabinose | Mannose | Maltose | Lactose | Inositol | Sucrose |
|---|---|---|---|---|---|---|
| RH1 | - | ++ | ++ | ++ | + | ++ |
| RH2 | - | ++ | - | ++ | ++ | ++ |
| RH3 | + | ++ | ++ | ++ | ++ | ++ |
| RH4 | - | + | - | ++ | ++ | ++ |
| RH5 | - | + | - | ++ | ++ | ++ |
| RH6 | - | + | + | ++ | ++ | ++ |
| RH7 | - | + | ++ | ++ | ++ | ++ |
| RH8 | - | ++ | - | ++ | ++ | ++ |
| RH9 | - | ++ | ++ | ++ | ++ | ++ |
| RH10 | + | - | ++ | ++ | ++ | ++ |
| RH11 | + | + | - | ++ | ++ | ++ |
| RH12 | - | + | ++ | ++ | ++ | ++ |
| RH13 | - | + | ++ | ++ | ++ | ++ |
| RH14 | - | + | - | ++ | ++ | ++ |

+: weakly positive, ++: Positive, -: Negative.

**Table 5.** Concentration of ethylacetate extracted compounds isolated from culture filtrate.

| Strain code | Initial weight of watch glass (g) | Final weight of Watch Glass (g) | Concentration of compound (g) |
|---|---|---|---|
| RH1 | 20.214 | 20.228 | 0.014 |
| RH2 | 20.446 | 20.453 | 0.007 |
| RH3 | 21.238 | 21.244 | 0.006 |
| RH4 | 21.581 | 21.586 | 0.005 |
| RH5 | 20.647 | 20.653 | 0.006 |
| RH6 | 20.974 | 20.981 | 0.007 |
| RH7 | 22.009 | 22.039 | 0.03 |
| RH8 | 21.500 | 21.506 | 0.006 |
| RH9 | 20.184 | 20.188 | 0.004 |
| RH10 | 33.089 | 33.110 | 0.021 |
| RH11 | 32.237 | 32.252 | 0.015 |

**Table 5.** Continued.

| | | | |
|---|---|---|---|
| RH12 | 85.931 | 85.939 | 0.008 |
| RH13 | 35.309 | 35.326 | 0.017 |
| RH14 | 38.205 | 38.235 | 0.030 |

**Table 6.** Solubility test.

| Strain code | Water | 2.5 M NaOH | 0.6M $NaHCO_3$ | 1.5 M HCl | Conc $H_2SO_4$ |
|---|---|---|---|---|---|
| RH7 | Insoluble | Insoluble | Insoluble | Soluble | NA |
| RH8 | Soluble | NA | NA | NA | NA |
| RH9 | Soluble | NA | NA | NA | NA |
| RH12 | Insoluble | Soluble | insoluble | NA | NA |

NA- Not Applicable.

**Table 7.** pH of the ethyl acetate fraction.

| S/No. | Strain code | pH | Nature of compound |
|---|---|---|---|
| 1 | RH7 | 8 | Low MW neutral compounds |
| 2 | RH8 | 7 | Low MW neutral compounds |
| 3 | RH9 | 6 | Low MW neutral compounds |
| 4 | RH12 | 10 | Low MW amines |

between 1975-2004, only three were for TB (Casenghi et al., 2007). In the present study, the isolates Micropolyspora and Micromonospora were found to be highly effective and belong to non Streptomycetes. Purification of desired active compound to the target may mount the way for new antimicrobial metabolites.

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