

Full Length Research Paper

Preliminary screening of antimycobacterial effect of psychrophilic Actinomycetes isolated from Manali ice point: Himachal Pradesh

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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 *Streptomyces* 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. *Micromonospora* 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 *Streptomyces* 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 *Streptomyces* from different habitats.

MATERIALS AND METHODS

Sample collection

The soil sample was collected from the Ice Point of Rothang Hill, Himachal Pradesh 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 chemotaxonomical 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 5th and 10th 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 voges 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

Strain coCode	Type of mycelium	Morphology of spore and hyphae	Possible genera
RH1	AM/SM	Rarely branched, septate hyphae, monospore	<i>Micromonospora</i> sp.
RH2	SM	Long vegetative cells	<i>Dactylsporantium</i> sp.
RH3	AM/SM	Oval intergallery vesicle, zygosporium	Intrasporangium
RH4	AM/SM	Irregular branched, non fragmented, rarely septate	Planomonospora
RH5	AM/SM	Spiral chain of spores	<i>Streptomyces</i> sp.
RH6	AM/SM	Medium length chain of spores	<i>Streptomyces</i> sp.
RH7	AM/SM	long chain spore	<i>Streptomyces</i> sp.
RH8	AM/SM	Rarely branched spiral spore	<i>Streptomyces</i> sp.
RH9	AM/SM	Septate hyphae with Monospore	<i>Micromonospora</i> sp.
RH10	AM/SM	Septate hyphae with monospore	<i>Micromonospora</i> sp.
RH11	AM/SM	Long chain spore	<i>Streptomyces</i> sp.
RH12	AM/SM	Rarely branched, Fragmented hyphae, chains of spore.	<i>Micropolyspora</i> sp.
RH13	AM/SM	Monospore, Septate hyphae	<i>Micromonospora</i> sp.
RH14	AM/SM	Spiral chain of spores	<i>Streptomyces</i> sp.

Eppendorf 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 front 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 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 desiccator. 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. The 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 NaHCO₃, 1.5 M HCl and concentrated H₂SO₄.

RESULTS AND DISCUSSION

Based on the spore morphology and mycelium, the isolated strains were identified as *Planomonospora* sp., *Streptomyces* sp., *Micromonospora* sp., *Micropolyspora* sp., *Dactylsporantium* 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 *Dactylsporantium*, *Intrasporangium*, *Planomonospora* 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), *Micromonospora* sp. (RH9) and *Micropolyspora* sp. (RH12) showed antimycobacterial activity 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 *Streptomyces* 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 ₂ SO 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 *Streptomyces*. Purification of desired active compound to the target may mount the way for new antimicrobial metabolites.

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