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Isolation and Screening of Antibiotic Producing Psychrophilic Actinomycetes and its Nature from Rothang Hill Soil Against Viridans *Streptococcus* sp.

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Abstract: The objective of this study is to isolate and screen new antibiotic producing psychrophilic Actinomycetes and to find out their antimicrobial activity against *Streptococcus* by agar well diffusion method. The antibacterial activities of isolated actinomycetes were found against *S. mutans* and *S. oralis*. A total Six Actinomycetes were isolated from the soil sample through crowded plate technique were subjected to primary screening and identified as *Intrasporangium* sp., *Dactyl sporangium* sp., *Micromonospora* sp., *Streptoverticilium* sp. and two *Streptomyces* sp. The bacterial species were isolated from fifty of tooth samples which are collected from the dental hospital and identified as *S. mutans* and *S.oralis*. The identification of test pathogenic bacteria is confirmed by hemolytic activity on blood agar plates and biochemical tests. The test organism *S. mutans* is highly sensitive to *Dactylsporangium* sp. and the *S. oralis* sensitive to *streptomyces*. The *Dactylsporangium* sp. produce pertinacious substance which is responsible for antimicrobial activity. Other strains are producing non pertinacious substance. This is the first time we report that the species other than *Streptomyces* showed antibacterial activity against streptococcus associated with dental disease.

Key words: Psychrophiles, dental carries, zone of inhibition, mbs broth, antibiotic

INTRODUCTION

New antibiotics that are active against resistant bacteria are required. Bacteria have lived on the Earth for several billion years. During this time, they encountered in nature a wide range of naturally occurring antibiotics. To survive, bacteria developed antibiotic resistance mechanisms. Therefore, it is not surprising that they have become resistant to most of the natural antimicrobial agents that have been developed over the past 50 years (Hancock, 2007). The emergence of antibiotic resistance is an evolutionary process that is based on selection for organisms that have enhanced ability to survive doses of antibiotics that would have previously been lethal (Cowen, 2008). Antibiotics like penicillin and erythromycin, which used to be one-time miracle cures are now less effective because bacteria have become more resistant (Pearson and Carol, 2008).

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Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005), notably antibiotics (Strohl, 2004). Actinomycetes are prokaryotes with extremely various metabolic possibilities. They produce numerous substances essential for health such as antibiotics enzymes (Bachmann and McCarthy, 1991) immunomodulators. If we include secondary metabolites with biological activities other than antimicrobial, Actinomycetes are still out in front, with over 60% *Streptomyces* sp. accounting for 80% of these (Hopwood *et al*., 2000). Psychrophiles or Cryophiles (adj. cryophilic) are extremophilic organisms that are capable of growth and reproduction in cold temperatures. They can be contrasted with thermophiles, which thrive at unusually hot temperatures. The environments they inhabit are ubiquitous on earth, as a large fraction of our planetary surface experiences temperatures lower than 15°C. *Streptococcus mutans* has been implicated as a primary causative agent of Dental carries and periodontal disease which compromise most oral disease (Law *et al*., 2007). The mouth contains a wide variety of oral bacteria, but only a few specific species of bacteria are believed to cause dental caries: *Streptococcus mutans* and *Lactobacilli* among them. *Lactobacillus acidophilus*, *Actinomyces viscosus*, *Nocardia* sp. and *Streptococcus mutans* are most closely associated with caries, particularly root caries. Bacteria collect around the teeth and gums in a sticky, creamy-colored mass called plaque, which serves as a biofilm. Some sites collect plaque more commonly than others. The grooves on the biting surfaces of molar and premolar teeth provide microscopic retention, as does the point of contact between teeth. Plaque may also collect along the gingiva (Rogers, 2008). Antibacterial activity of actinomycetes strains was confirmed in batch culture. They were active against clinical isolates from the species *Staphylococcus aureus* and *Streptococcus pneumoniae*.. The antibacterial compounds produced by these strains probably possessed non-polar structure and consisted of several active components (Moncheva *et al*., 2002).

MATERIALS AND METHODS

The study was carried out at Department of Microbiology, Jmal Mohamed College during January 2009 to may 2009.

Isolation of Pathogenic Bacteria from the Decayed Tooth Sample

About fifty decayed tooth samples were collected from dental clinic, used for the isolation of test pathogen by routine microbiological laboratory procedure and identified by routine clinical tests.

Isolation of Psychrophillic Actinomycetes

The specimens (actinomycetes) used in this study were isolated from the soil of Rothang hill, Himachal Prethesh, India. The soil sample was collected from the ice point of Manali during October 2008 at a distance of 4061 km from the sea level were brought to the laboratory in aseptic condition. Actinomycetes from the soil had been isolated by pour plate technique on Starch-casein agar and Glycerol-arginine agar and incubated at 15°C for 15 days.

Screening of Psychrophilic Actinomycetes for Antimicrobial Activity

The screening method consists of two steps; Primary screening and secondary screening. In primary screening the antimicrobial activity of crude culture filtrate were used

to determine the effect of isolate by agar well diffusion method on Muller Hinton agar per National Committee for Clinical Laboratory Standards (NCCLS, 1999). The medium is slightly modified with the addition of anti coagulated blood for enhance the growth of Haemolytic bacteria. Secondary screening was performed with purified protein extract by ammonium sulphate precipitation and dialysis. The test organisms used were *Streptococcus mutans* and *Streptococcus oralis.*

Characterization of Psychrophilic Actinomycetes

The potent actinomycetes selected from secondary screening were characterized by morphological and biochemical method described by Nakazawa *et al*. (2006). Morphological methods consist of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method. The mycelium structure, color and arrangement of conidiospore and arthrospore on the mycelium was observed through the oil immersion (Kawato and Shinobu, 1959). The biochemical tests used in this study for the identification of the potent isolates are casein hydrolysis, starch hydrolysis, urea hydrolysis, acid production from sugar, utilization of sugar and cell wall analysis for Diaminopimelic acid (DAP) (Staneck and Roberts, 1974).

Fermentation Process

Fermentation was carried out in a 1L Erlenmeyer flask containing 500 mL of Balanced Salt Medium (BSM). The process carried out for 7 days at 15°C with 75 rpm agitation.

Protein Purification

The crude extract was mixed with saturated ammonium sulphate and kept over night at 4^0C then centrifuged. The precipitate dialyzed in phosphate buffer overnight to purify the protein. The sample is characterized by SDS Page electrophoresis.

Isolation of Antibacterial Metabolites

Antibacterial compound was recovered from the filtrate by solvent extraction method following the process. Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 h for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 8-90°C and the residue obtained was weighed. Thus, obtained compound was used to determine antimicrobial activity.

Determination of the Antimicrobial Activity

The antimicrobial activity was determined by agar well method (Zamanian *et al.*, 2005).The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1 mL 0.2 M phosphate buffer (pH 7.0). Then 100 µL of sample was loaded into well bored against test organism prepared as per 0.5 McFarland turbidity standards as follows:

McFarland standard No.	0.500	1.000	2.000	3.000	4.000
1.0% barium chloride (mL)	0.050	0.100	0.200	0.300	0.400
1.0% sulfuric acid (mL)	9.950	9.900	9.800	9.700	9.600
Approx. cell density (1X10^8 CFU/mL)	1.500	3.000	6.000	9.000	12.000
% transmittance at 600 nm	74.300	55.600	35.600	26.400	21.500
Absorbance at 600 nm	0.132	0.257	0.451	0.582	0.669

The test plates were incubated at 37°C for 18-24 h and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter with the help of zone scale.

RESULT

Nearly six different Actinomycetes were isolated from the soil. The isolated Actinomycetes are identified as A: *Intrasporangium* sp., B: *Dactylsporangium* sp., C: *Micromonospora* sp., D: *Streptoverticilium* sp., D1: *Streptomyces* sp. and D2: *Streptomyces* sp. Their morphological characters are listed on Table 1. This identification is based on their mycelia, spores, starch and nitrate utilization probability described by Shirling and Göttlieb (1966). The morphology and biochemical properties are listed on Table 2. Four of isolates having L-DAP in their cell wall and two are meso- DAP. The isolated actinomycetes having ability to utilize sucrose, mannitol, dextrose and xylose. However, they differ in their utilization of lactose, arabinose, meso-inositol and maltose (Table 3).

The test organism used in this study where isolated from fifty of decayed tooth sample is *S. mutans* and *S. oralis*. Their biochemical characteristics are listed on the (Table 1). The results shows these two organisms are predominant and primary bacteria's responsible for dental carries.

The antibiotic production of isolated Actinomycetes and its activity against the test organism such as *S. mutans* and *S. oralis* was listed in the Table 4. Among the six

Table 1: Morphological characteristics of actinomycete isolates

S. No	Mycelium and nature of colony	Colour of mycelium	Type of spore	Pigmentation	Gram stain	AFB	DAP	Cell wall sugar
A	Fragmented branched aerial mycelium nil	Whitish grey	Oval shaped	Pale brown intercalary vesicle	+	-	L DAP	Arabinose galactose
B	Smooth, leathery both mycelium	Pale orange	Sporangiop hore, motile spores	brown	+	-	m DAP	Arabinose xylose
C	Septate, branched, coloured aerial mycelium	White to grey	mono Sporophore	yellow	+	-	m DAP	Galactose xylose
D	Aerial and subtrate mycelium, regular branched	White cottony	Spiny spore surface	violet	+	-	L DAP	Arabinose
D1	Extensively branched, floccose, aerial and substrate mycelium	Ash	Short chain of spores	brown	+	-	L DAP	Galactose
D2	Smooth, granular aerial and substrate mycelium	White pink	Long chain spore	Wine red	+	-	L DAP	Arabinose

A: Intrasporangium sp., B: *Dactyl sporangium* sp., C: *Micromonospora* sp., D: *Streptoverticilium* sp., D1: *Streptomyces* sp., D2: *Streptomyces* sp, +: Positive, -: Negative

Table 2: Biochemical characters

Organism	Indole	Mr	Vp	Citrate	Catalase	Oxidase	Nitrate	Starch
Act A	-	+	-	+	+	+	+	+
Act B	-	+	-	+	+	+	+	-
Act C	-	+	-	+	+	+	+	Weakly +
Act D	-	+	-	+	+	+	-	+
Act D1	-	+	-	+	+	+	+	-
Act D2	-	+	-	+	+	+	-	+

A: *Intrasporangium* sp., B: *Dactylsporangium* sp., C: *Micromonospora* sp., D: *Streptro verticillium* sp., D1: *Streptomyces purpures,* D2: *Streptomyces microflavus*

Table 3: Utilization of sugar and acid production

Sugar	Act A	Act B	Act C	Act D	Act D1	Act D2
Sucrose	+	+	+	+	+	+
Lactose	+	+	-	-	-	-
Arabinose	+	-	+	-	+	+
Mannitol	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+
Xylose	+	+	+	+	+	+
Inositol	+	-	-	-	+	-
Maltose	-	-	+	+	+	-

A: *Intrasporangium* sp., B: *Dactylsporangium* sp., C: *Micromonospora* sp., D: *Streptro verticillium* sp., D1: *Streptomyces purpures*, D2: *Streptomyces microflavus*

Table 4: Zone of psychrophilic actinomycetes produced against pathogenic bacteria (mm)

Isolate	Sample nature	*S. mutans*	*S. oralis*
A	protein	Nil	Nil
	crude extract	Nil	16 mm
B	protein	33 mm	Nil
	Crude extract	Nil	Nil
C	protein	Nil	Nil
	crude extract	24 mm	17 mm
D	protein	Nil	Nil
	crude extract	26 mm	24 mm
D1	protein	Nil	Nil
	crude extract	30 mm	36 mm
D2	protein	Nil	Nil
	crude extract	18 mm	19 mm

A:*Intrasporangium* sp., B: *Dactylsporangium* sp., C: *Micromonospora* sp., D: *Streptro verticillium* sp., D1: *Streptomyces purpures,* D2: *Streptomyces microflavus*

psychrophilic actinomycetes *Dactylsporangium roseum* shows its maximum activity against *S. mutans* (33 mm) and its molecular weight of is 66 kDa. The isolate *Streptomyces* sp. more active against *S. oralis* than other strains. The inhibition activity is 36 mm of zone. *Intrasporangium* sp. is not effective against *S.mutans* similarly *Dactylsporangium roseum* is ineffective towards *S. oralis* the rest of the strain are produced metabolites which are effective towards *S. mutans* and *S. oralis.*

DISCUSSION

The test organism isolated from decayed tooth is identified as Gram positive cocci in chain. The isolated two strains are differing in their haemolytic activity. The *S mutans* are α-haemolytic and *S. oralis* is β-haemolytic in nature. Both are comes under viridians group of *streptococci*. The inhibitory effect of six isolated Actinomycetes against *S. mutans* shows five are effective except *intrasporangium* among those five *Dactylsporangium* sp. *shows* its maximum activity against *S. mutans* (33 mm). The strain capable to produceTetracyclin derivative active against Gram positive organism (wells *et al*., 1992). *Streptomyces* sp. also effective towards *S. mutans* (Wanbanjob *et al*., 2008) followed by Dactylsporangium. *Micromonospora* sp. and *Streptoverticillium* sp. are moderatively active against *S. mutans*, they produce 24 and 26 mm zone of inhibition the least activity 18 mm zone of inhibition is expressed by *S. microflavus* (Kubo *et al*., 1993). In this study the genus Micromonospora also exhibit antimicrobial activity against test organism (Charan *et al*., 2004). The activity of inhibitory effect against *S. oralis* is not produced by *Dactylsporangium roseum*, more efficient activity is observed on the *S. purpurens* which is 36 mm. It was also found that the crude extract shows maximum inhibitory against *Streptococcus oralis* and *S. mutans.* The only pertinacious substance active against *S. mutans* is reported by *Dactylsporangium*

roseum and no one protein active against *S. oralis.* In this study *Streptoverticillium* is moderatively active against *S. oralis* (24 mm) and *Intrasporangium*, *Micromonospora* sp. and *S. microflavus* are less effective strain.

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