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Isolation and Screening of Psychrophilic *Actinomycetes* from Rothang Hill Soil against Dental Carries Causative *Streptococcus* sp

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The objective of this work is to isolate and screen 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*. Six Actinomycetes were isolated from the soil sample through crowded plate technique 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 isolated Actinomycetes exhibits different in the way of utilization of Indole, MR, VP, citrate, nitrate reduction, starch hydrolysis, oxidase, catalase and utilization of sugar.The identification of test pathogenic bacteria is confirmed by haemolytic 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 *S.purpurens*. The *Dactylsporangium* sp produce pertinacious substance which is responsible for antimicrobial activity. Other strains are producing non pertinacious substance. the work is extended to find out the structure of the isolated compounds.

Key word: Psychrophils, *Actinomycetes*, Tooth decay, ZOI, Muller hinton agar, MBS broth.

S.mutans has been implicated as a primary causative agent of Dental carries and periodontal disease. The association of *S. mutans* with dental carries first reported By Clarke (1924). The viridans streptococci have been traditionally regarded as minor opportunist pathogens, being primarily associated with dental caries (Bochud *et al.* 1994). However, over the past decade these bacteria have emerged as significant pathogens of immunocompromised patients and those with haematological malignancies (Awada *et al.* 1992; Bochud *et al.*1994; Classen *et al.* 1990; Cohen *et al.* 1983). Actinomycetes are prokaryotes with extremely various metabolic possibilities. They produce

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numerous substances essential for health such as antibiotics enzymes (Bachmann *et al*. 1991) immunomodulators (Chipeva *et al.* 1996)

MATERIALS AND METHODS

Isolation of pathogenic bacteria from the decayed tooth sample

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

Isolation of Psychrophillic Actinomycetes

The specimens (actinomycetes) used in this study were isolated from the soils of Rothang hill, HP, India.The soil sample was collected from the ice point of manali during October 2008 at a distance of 4061 kilometers 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 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 determined the effect of isolate by agar well diffusion method on Muller Hinton agar. Secondary screening was performed with purified protein extract. The test organisms used were; *Streptococcus mutans* and *Streptococcus oralis.*

Characterization of actinomycetes

The potent actinomycetes selected from secondary screening were characterized by morphological and biochemical method described by WAKSMAN (1961). 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 *et al*. 1959]. The observed structure was compared with Bergey's manual of Determinative Bacteriology, Ninth edition (2000) and the organism was identified. The following biochemical tests are performed for the identification of the potent isolates : Casein hydrolysis, Starch hydrolysis, Urea hydrolysis, Acid production from sugar, utilization of sugar and cell wall analysis (Becker *et al.* 1964) for DAP.

Fermentation process

Fermentation was carried out in a 1L Erlenmeyer flask containing 500 ml of BSM medium. The process carried out for 7 days at 15^0 C with 75 rpm Agitation.

Protein purification

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

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 hour 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 80°-90°C and the residue obtained was weighed. Thus obtained compound was used to determine antimicrobial activity, minimum inhibitory concentration and to perform bioautography.

Determination of the antimicrobial activity

The antimicrobial activity was determined by agar well method (Zamanian 2004).The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1 ml 0.2M phosphate buffer (pH 7.0). Then 100 µl of sample was loaded into well bored and test organism (0.5 McFarland turbidity standards) swabbed Muller Hinton agar plates. The plates were incubated at 37°C for 18-24 hrs and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter.

RESULTS

The isolated Actinomycetes are A: *Intrasporangium* sp, B: *Dactyl sporangium* sp, C: *Micromonospora* sp, D: *Streptoverticilium* sp,D1: *Streptomyces purpures,* and D2: *Streptomyces microflavus*.theire morphological

Table 1. Morphological characteristics of Actinomycete isolates

S. No	Mycelium Type	Colour of Mycelium	Type of Spore	Pigmentation	Gram stain	AFB	DAP	Cell wall Sugar
A	Fragmented branched No aerial mycelium	Whitish grey	Oval shaped intercalary vesicle	Pale brown	+	-	l DAP	Arabinose galactose
B	Smooth, leathery	Pale orange	Sporangiophore, 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

characters are listed on table 1. The test organism used in this study where isolated from fifty of decayed tooth sample is α-haemolytic, indole positive *S.mutans* and a β-haemolytic and indole negative *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. Nearly six different Actinomycetes were isolated from the soil at 10^{-5} dilution. The isolated strains are identified as *Intrasporangium calvum, Dactylsporangium roseum, Micromonospora sp, Streptroverticilliu sp, Streptomyces purpures and Streptomyces microflavus*. This identification is based on their mycellial, spores, starch and nitrate utilization probability described by Bergey's manual of determinative bacteriology 4th edition the morphology and biochemical properties (Table 2). Four of isolates having L-DAP in their cell wall and two are m DAP nature. The isolated actinomycetes having ability to utilize sucrose, mannitol, dextrose, and xylose. But they differ in their utilization of lactose, arabinose, inositol and maltose (Table 3).

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 actinomycetes *Dactylsporangium roseum shows* its maximum activity against *S.mutans* (33mm) and the molecular weight of protein is 66 kDa. The isolate *S.purpurens* more active againsts.oralisthan 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.*

The test organism isolated from decayed tooth is identified as Gram positive cocci in chain (Billroth *et al.* 1874). The isolated two strains are differing in their haemolytic activity (Schottmuller 1903). The *S. mutans* are

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 actinomycetes produced against pathogenic bacteria (in mm)

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

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

β- haemolytic and *S.oralis* is â-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* (33mm).*S.purpureus* also exhibited effectiveness towards *S.mutans* followed by Dactylsporangium. *Micromonospora sp* and *Streptoverticillium* sp are moderatively active against *S.mutans*, they produce 24mm and 26 mm zone of inhibition the least activity 18 mm zone of inhibition is expressed by *S.microflavus* (Isao Kubo *et al*. 1993). . 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 36mm. *Streptoverticillium* is moderatively active against *S.oralis* (24mm). *Intrasporangium, Micromonospora sp and S.microflavus* is less effective than moderate strain.

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