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Exploration of Antimicrobial Potential of Soil Actinomycetes from Dal Lake of North-Western Himalayas, India

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ABSTRACT

Humankind explored and exploited all natural resources for his necessities. Many plants and microbes especially Actinomycetes are always targeted for antimicrobial agents against human pathogens. The occurrence of drug resistance is now at an alarming rate among pathogenic bacteria, especially multidrug-resistant bacteria and requires the need to glance at unique ecological niches for the search of new antibiotics which have not explored so far. In this study, 52 Actinomycetes were isolated from the soil sediment samples collected from Dal Lake of Kashmir and screened for antimicrobial activity against both gram-positive and gram-negative pathogenic bacteria by perpendicular streak and agar well diffusion method. During primary and secondary screening 14 Actinomycetes isolates and 8 crude extracts showed a wide range of inhibition zone against MTCC cultures respectively. Out of which 1 isolates (B2) was found to be promising for its broad range of antimicrobial activity. In Phylogenetic studies isolate B2 was identified as Streptomyces indiaensis (SRT1). The influences of various culture/fermentation conditions for bioactive compound were investigated to enhance metabolite production and improved antimicrobial activity. Tryptone soya broth (TSB) was confirmed to be the best culture medium for maximum antimicrobial production on the 18th day at pH 7 and 280C. B2 showed maximum antimicrobial production with starch and casein as the carbon and nitrogen sources respectively, the isolate B2 was found to be halotolerant showed maximum metabolite production at 2% of NaCl. In this study, we were able to determine the optimal growth conditions and optimized fermentation conditions to accumulate antimicrobial metabolite. The further detailed investigation could provide us highly efficient antimicrobial agents against the clinical important human pathogens.

INTRODUCTION

Antimicrobial compounds are produced by various living organisms such as bacteria, fungi, and plants. Among the various groups of organisms that have the capacity to produce antimicrobial agents, the *Actinomycetes* are the most capable candidate (Gebreselema et al., 2013). *Actinomycetes* are slow growing, gram-positive bacteria, having high G+C content from 55-75 % (Ningthoujam et al., 2009). They resemble fungi because of their filamentous appearance and spore production property (Waksman, 1940) and bacteria because of the presence of peptidoglycan in their cell wall and possession of flagella (Mythili and Das, 2011).

Actinomycetes are inexhaustible producers of antimicrobial agents (Atta et al., 2010). Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by *Actinomycetes*, representing 45% of all bioactive microbial metabolites discovered (Berdy, 2005). The secondary metabolites obtained from the class Actinobacteria are of special interest because of their diverse biological activities such as antibacterial, antifungal, antioxidant, antitumor and antiviral. Among *Actinomycetes*, *Streptomyces* Species produce around 7,600 compounds. Many of these secondary metabolites are potent antibiotics, which has made *Streptomycetes* the primary antibiotic-producing organisms exploited by pharmaceutical industry and responsible for the formation of more than 60 % of known antibiotics. Further 15 % are made from a number of related *Actinomycetes, Micromonospora, Actinomadura, Streptoverticillium, and ThermoActinomycetes* (Ramesh et al., 2009; Jensen et al., 2007).

The emergence of resistance in microorganisms to the clinically available antibiotics already in the market and multidrug-resistant pathogenic bacteria are issues of extreme concern in present time for the whole human community. Due to these issues, there is rapid spread of infectious diseases leading to morbidity and mortality especially among the elderly and immuno-compromised patients (Hong et al., 2009; Alanis et al., 2005). To overcome this situation the discovery of novel drugs with lesser side effects is need of present time.

The terrestrial sources and marine ecosystem were traditionally researched for microorganisms especially *Actinomycetes* as antimicrobial agents (Valli S et al., 2012). The rise of resistant

pathogens requires the search for novel *Actinomycetes* and new antibiotics from freshwater sources, which have been less explored and crucial for the discovery of novel *Actinomycetes* as compared to soil and marine water sources, which have been reported extensively.

Recently, many scientists are searching new antibiotics from different untouched habitats. In India, no studies have been conducted so far to isolate and evaluate *Actinomycetes* from different freshwater habitats located in the northwestern Himalayas that could produce useful antibiotics. Therefore, the present study aimed to isolate, screen and characterize antibiotic-producing *Actinomycetes* from the sediment samples of Dal Lake located in Jammu and Kashmir, India. Further, the different parameters viz medium, pH, temperature, RPM and incubation time on the growth of bioactive *Actinomycetes* and production of the bioactive compound were also studied.

MATERIALS AND METHODS

Sampling area

The samples of sediments were collected in the month of October from Dal Lake situated in heart of Kashmir valley India, popularly known as Jewel in the crown of Kashmir. It is Warm monomictic Lake at an altitude of 1600 m above sea level and lies between 34^0 5’ and 24^0 10’ N latitude and 74^0 8’ and 79^0 9’ longitude. The lake covers an area of 18 square kilometers and the average elevation of the lake is 1,583 meters. The depth of water varies from 6 meters at its deepest to 2.5 meters, the shallowest. The depth ratio between the maximum and minimum depths varies with the season between 0.29 and 0.25metres (Sarina Singh, 2005).

Collection of soil Sediment samples

Soil sediments samples were aseptically collected from Dal Lake of Kashmir at the depth of 10-12 cm by using the sterile spatula and transferred to sterile plastic bags. The samples taken were labeled accordingly and transported to Biotechnology Laboratory, Department of Biotechnology and Microbiology, Arni University (Himachal Pradesh). The samples were stored at 4^0 C until further studies.

Pre-treatment of Samples

The sediment samples were firstly air dried and passed through a 2mm sieve to remove any gravel and debris The air dried and sieved sediment samples were subjected to dry heat treatment at 50^0 C for 1 hr to reduce the number of other bacteria and for preferential isolation of *Actinomycetes*. Media such as starch casein agar, *Actinomycetes* Isolation agar, Tryptone soya agar, Water yeast extract and Glycerol yeast extract agar were used for isolation. Antifungal antibiotics (amphotericin B) at 25 µg/mL were used for *Actinomycetes* growth promotion and for prevention of fungal contamination (Cuesta G et al., 2010; Zhang L et al., 2005).

Isolation of *Actinomycetes*

Isolation and enumeration of *Actinomycetes* were done by applying standard serial dilution plate technique (Elliah P et al., 2004). Different aqueous dilutions (10^{-1} to 10^{-6}) of sediment samples were prepared and spread plated on starch casein agar (SCA), Tryptone soya agar (TSA), and Water yeast extract (WYE), Glycerol yeast extract (GYE) agar and *Actinomycetes* isolation agar (AIA) plates aseptically. Plates were incubated for 7 to 20 days at 30°C. The plates were observed at regular intervals for the growth of *Actinomycetes*. Typical *Actinomycetes* colonies were selected on the morphological basis and maintained in *Actinomycetes* isolation agar slants at 4°C for subsequent studies.

Bacterial Strains

The pathogenic gram-positive bacterial strains such *Staphylococcus aureus* (MTCC 3160), *Streptococcus mutans* (MTCC 890), *Bacillus subtilis* (MTCC 441) and Penicillin resistant *S.aureus* and gram-negative such as *Pseudomonas fluorescens* (MTCC 103), *Pseudomonas aeruginosa* (MTCC 4673), *Escherichia coli* (MTCC 443) were obtained from the MTCC (Microbial type culture collection) Chandigarh, India. Clinical isolates of *S. aureus* collected from different community health centers of Kangra region of Himachal Pradesh India were also included in the study.

Inoculum Preparation and Inoculation

With the help of a sterile wire loop, the pure colony of test bacteria was taken and transferred into test tubes having a sterile nutrient broth. The test tubes were incubated at 37^0 C for 24 hrs until the noticeable turbidity and density are equal to that of 0.5 McFarland standards. After adjusting turbidity, a sterile cotton swab was dipped into suspension and streaked over the whole surface of the plate to make sure the consistent growth of bacterial culture (Gebreselema et al., 2013).

Primary screening of isolates

During the primary screening, isolates were screened against selected bacterial strains by using the perpendicular method. Antimicrobial activity of isolates was first and foremost screened by using perpendicular streak method against Gram-negative and Gram-positive bacteria MTCC bacterial pathogens (Hayakawa M et al., 2004). In perpendicular streak method, nutrient agar media was used and each plate was streaked with individual isolates at the center of the plate and incubated at 30 ^{0}C for 7 days.

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Production of crude extracts

The isolates showing antibacterial activity from the primary screening were subjected to solid state fermentation method to produce the crude extract.

Solid state fermentation and crude extraction preparation

The mixture of rice grain (substrate) weight 100gm and 100 mL distilled water was taken into the conical flask (500 mL); the mixture was boiled until the rice grains become softened. Mineral salt solutions such as K_2HPO_4 (2.00 g/L), NaCl (1.00 g/L), $MgSO_4.7H_2O$ (0.10 g/L) and $CaCl_2$ (0.05 g/L) were supplemented for optimization and autoclaved at 121^0 C for 15 minutes. The solution was allowed to cool and inoculated with 2 mL of culture suspension from 7 days old *Actinomycetes* culture grown on Tryptone soya agar. After inoculation, flasks were incubated at 28 ^{0}C for two weeks. The completely fermented rice grain was grinded with mortar and pestle and then allowed to dry at room temperature for 24 hrs. Later, 100 mL of ethyl acetate and 100

mL of methanol was added to the extract and placed on rotary shaker at 120 rpm for 12 hrs. Finally, by using Whatman No.1 filter paper, the extract was filtered and the solvent phase was removed by using rotary vacuum evaporator set at water bath temperature of 60 ^{0}C (Robinson T et al., 2001).

Secondary screening

Agar well diffusion method

Tested bacterial suspensions were prepared by suspending 24 hrs grown culture in sterile normal saline. The turbidity of bacterial suspensions was adjusted to a 0.5 McFarland standard ($\cong 1.5 \times 10^8$ CFU/ml). 50 µl of this suspension was swapped in MHA plates. Using sterile cork borer (6 mm in diameter), well were prepared on the plate. A volume of 100 µl of 10mg/mL of crude extract was carefully dispensed into each well and allowed to diffuse for 2 hrs followed by incubation at 37^{0}C for 24 hrs. Standard antibiotic Levofloxacin (10 units per Disc) were taken as positive control for each test organism. Zone of inhibition around each well was checked after 24 hrs of incubation and recorded. Each experiment was repeated three times (Pandey B et al., 2008). Secondary screening of potent *Actinomycetes* confirmed the results of the primary screening.

Screening of *Actinomycetes* against penicillin-resistant *S. aureus* and clinical isolates of *S.aureus*.

The *Actinomycetes* culture showed activity during secondary screening would be further screened (as described above) against the culture of penicillin resistant mutant of *S.aureus* and clinical isolates of *S.aureus* collected from different community health centers of Kangra region of Himachal Pradesh India. Penicillin-resistant mutant of *S.aureus* was constructed in the lab by regular passaging and this mutant will be used for further screening of anti-infective agent / bioactive from *Actinomycetes* cultures.

Morphological characterization of *Actinomycetes*

Morphological characters of the selected isolates were studied by inoculating them into sterile media like starch casein agar, Tryptone soya agar, Water yeast extract and *Actinomycetes* isolation agar (Yang KQ et al., 1995). The media was poured into sterile Petri dishes after sterilization. After solidification, selected isolates were streaked aseptically and incubated at 30 ^{0}C for 7 days. Morphological characters such as colony characteristics, pigment production, absence or presence of aerial and substrate mycelium were observed.

Genomic DNA isolation for identification

DNA was isolated by using CTAB Method (Liu D et al., 2000). The freshly cultured cells were pelleted by centrifuging for 10 min at 10,000 rpm. The cells were resuspended thoroughly in 250µl Lysozyme solution in T.E (2mg/ml) and incubated at 37^{0} C for 1 hour. After incubation, 30 µl of solution B (10% SDS + Proteinase K 10mg/ ml) was added and again incubated at 65^{0} C for 15 min. 25 µl of 5M NaCl and 20 µl of CTAB were added and then the mixture was vortexed carefully and kept at 65 ˚C for 20 min. RNase was added and incubated at 42˚C for 45 min. After incubation, the solution mixture was centrifuged at 12,000 rpm for 20 min. The supernatant was washed with the equal volume of Chloroform: Isoamyl alcohol (24:1). Again centrifugation was done at 10,000 rpm for 10 min. The supernatant was transferred and 3M sodium acetate was added along with 70 % Isopropanol to the aqueous layer and kept at -20^{0} C for overnight. Again centrifugation was done at 10,000 rpm for 10 min. DNA pellet was rinsed with 70% ethanol, air dried and dissolved in T.E. DNA concentrations were measured by running aliquots on 1% Agarose gel. The DNA samples were stored at -20^{0} C until further use.

Amplification of genomic DNA with 16S primers

The primer 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') were used to amplify the 16S ribosomal sequence from genomic DNA in the thermal cycler (ep gradient Eppendorf). The cyclic conditions were as follows: Initial denaturation at 94 ^{0}C for 3 min, annealing at 94^{0} C for 30 sec, 50^{0} for 60 sec, and

72°C for 60 sec and final Extension at 72^0 C for 10 min. The PCR products were confirmed by 1% Agarose gel electrophoresis (Farris MH et al., 2007).

Purification of PCR Production

Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean-up kit (Millipore).The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

DNA Sequence determination

Automated sequencing was carried out according to the dideoxy chain-termination method using Applied Biosystems automated sequencer by Triyat Scientific services (India).

Phylogenetic analysis

Nucleotide Sequence was examined for sequence homology with the archived 16S rDNA sequences in the GenBank database using blastn (Altschul SF et al., 1997). Multiple sequences were aligned with CLUSTAL W (Thompson JD et al. 1994). Phylogenetic analyses were performed according to the neighbor-joining (NJ) method using MEGA version 7.0 (Kumar S et al. 2016).

Standardization of fermentation conditions for growth and metabolite production:

Effect of Media on growth and metabolite production

The isolate was preliminary tested for its antimicrobial potentiality and was further studied for the optimal production of antimicrobial agent in liquid medium at shake flask level. The outcome of culture media on the production of antimicrobial agent was examined by taking different liquid media such as Starch casein broth, *Actinomycetes* isolation broth, Tryptone soya broth, Water yeast extract broth, Glycerol yeast extract. Microbial metabolite production was conceded out using 50 ml broth medium in the 500ml flask and inoculated with 1ml of active isolate incubated at 28^0 C±1 in an orbital shaking incubator maintained at 150 rpm for fifteen days.

After incubation, each culture broths were checked for antimicrobial activity by standard agar well diffusion method (Thenmozhi M et al., 2010) against MTCC strains.

Effects of pH on growth and metabolite production

The initial pH levels of the Tryptone soya broth (TSB) media were adjusted from 4 to 10 and the isolates were grown for 15 days at 150 rpm and 28^0 C. Agar well diffusion method was used for estimation of metabolite production. The optimum pH achieved by this step was fixed for subsequent experiments (Oskay M et al., 2011).

Effect of temperature on growth and metabolite production

The isolates were inoculated into the TSB medium and incubated at different temperatures (25, 28, 31, 34 and 37^0 C) at pH 7 for 20 days in a rotary shaker at 150 rpm. The antimicrobial activity was evaluated against MTCC strains by agar well diffusion method. Optimum temperature was kept at the standard temperature for further experiments (Saadoun et al., 2008).

Effect of Incubation Period on growth and metabolite production

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The isolates were inoculated into the TSB medium and incubated up to 20 days in a rotary shaker at 150 rpm at 28^0 C. For the determination of antibiotic production, 1 ml aliquots were withdrawn after every 24 hrs to test the antibiotic production rate. Inhibition zone was tested by agar well diffusion method (Ready et al., 2011). The optimum incubation period was preset for succeeding experiments.

Effect of Carbon Source on growth and metabolite production

The b2 isolate was supplemented with different carbon sources such as glycerol, sucrose, maltose, glucose, and starch individually to a final concentration of 1% (w/v) in the Tryptone soya broth to test the effect of different carbon source on antibiotic production. Influence of various level of best carbon source on the bioactive metabolite synthesis was examined. After incubation in an optimal condition, the antibacterial activity was tested by agar well diffusion method (Borodina et al., 2008).

Effect of Nitrogen source on growth and metabolite production

Different nitrogen source such as yeast extract, oatmeal, Casein, Peptone and corn meal was supplemented separately to a final concentration of 1% (w/v) in the Tryptone soya broth. Suitability of nitrogen source following antibiotic production was examined. After incubation in optimal conditions, the antibacterial activity was tested by agar well diffusion method (Saadoun et al., 2008).

Effect of salt on growth and metabolite production

Different concentration of Sodium Chloride as 0.5, 1.0, 1.5, 2.0 and 2.5 % have been prepared in the production medium to determine the best concentration of NaCl for antibacterial production. These NaCl concentrations were prepared in the 50 ml broth that has been seeded with the B2 isolate and then incubated in an orbital shaker. Antibacterial activity was tested by agar well diffusion method.

Statistical Analysis

The collected and recorded data were subjected to One-way ANOVA ranked with Duncan's multiple range tests with descriptive analysis type on different isolates against different MTCC Cultures using SPSS 16 Version software. The zone of inhibition was measured in triplicate. The results with $P<0.05$ were considered to be statistically significant.

RESULTS

Isolation of *Actinomycetes*

After 7-day incubation powdery, pigmented and smooth colony was observed in the dilution plate. Fifty-two *Actinomycetes* were isolated and inoculated on AIA medium for purification. The pure active colonies were designated as B1-B52. These isolates were maintained on AIA medium.

Primary screening for antimicrobial activity

All *Actinomycetes* isolates isolated from soil sediment of Dal Lake were screened against MTCC bacterial strains by perpendicular streak method. After 24 hrs incubation, 14 were active against MTCC bacterial strains. Among them, only eight isolates (B2, B4, B14, B15, B20, and B 21, B39, B45) were taken for further studies based on their antibacterial activity. (Table 1) These eight isolates widely inhibited the growth of the MTCC bacterial cultures. Among them, B2 showed the maximum zone of inhibition against all MTCC cultures taken in the study.

Table 1: Zone of inhibition (mm) of the isolates against MTCC pathogenic bacterial strains using perpendicular streak method.

Isolates	Gram-negatives			Gram-positive				
	E.coli (MTCC443)	*P.aeruginosa* (MTCC4673)	*P.fluorescens* (MTCC 103)	*B.subtilis* (MTCC441)	*S.mutans* (MTCC890)	*S.aureus* (MTCC 994)	Clinical Isolates of *S.aureus*	Penicillin resistant *S.aureus*
B2	+++	+++	+++	+++	++	++	+++	+
B4	++	++	++	+++	+	+	–	–
B14	+++	+	++	+++	+	–	–	–
B15	+	++	++	+	–	+	–	–
B20	+++	+++	++	+++	+	+	+++	–
B21	+	++	++	++	+	–	–	–
B39	++	++	+	+++	+	–	+	–
B45	+	+++	++	++	+	–	++	–

+++: Good activity; ++: Moderate activity; +: Weak activity; – : No activity

Secondary screening of crude extracts

The crude extracts prepared from 8 potential isolates by using solid state fermentation methods were subjected to secondary screening by agar well diffusion method. Table 2 showed the results of the secondary screening. In agar, well diffusion method isolate B2, B4, B14, B15, B20, B21, B39 and B45 was active results against *E.coli*. Isolates B2, B14, B15, B20, B21, B39 showed promising results against *P.fluorescens*. B2, B4, B15, B20, B21, B45 showed inhibition against *P.aeruginosa*. Isolates B2, B4, B14, B15, B20, B39, and B45 showed positive results against *B.subtilis*. Isolates B15, B21, and B39 showed the zone of inhibition against *S.mutans*. Isolates B2, B15, B20, and B45 showed promising results against *M.smegmatis.* Isolates B2, B20 and B39 showed promising results against Penicillin resistant *S.aureus*. The inhibition zone of B2 was maximum of 21.6mm and 21.2mm against *E.coli* and *P.fluorescens* respectively and it was comparatively greater than positive control (19.4mm and 19mm) taken in the experiment. Results of secondary screening shown in Figure 1. All the results were statistically significant Table 2.

Table 2: Zone of inhibition (mm) of the isolates against MTCC pathogenic bacterial strains using agar well diffusion method.

Isolates	Gram-negatives			Gram positive						
	E.coli (MTCC443)	*P.fluorescens* (MTCC 103)	*P.aeruginosa* (MTCC4673)	*B.subtilis* (MTCC441)	*S.mutans* (MTCC890)	*S.aureus* (MTCC 3160)	Clinical Isolates of *S.aureus*	Penicillin resistant *S.aureus*	LSD(p<0.05)	F-ratio
B2	21.1±0.76[a]	21.5±1.36[a]	21.1±1.15[a]	19.2±0.68[b]	10.8±0.15[d]	9.2 ±1.11[e]	15.5±0.51[c]	19.23±0.75[b]	0.37	89.65
B4	9.3±1.00[b]	_	13.73±0.64[a]	7.9±0.36[c]	_	_	_	_	0.18	484.63
B14	14.4±0.66[a]	12.4±0.66[b]	_	13.0±1.00[b]	_	_	_	_	0.20	603.27
B15	11.5±0.50[b]	8.6±0.45[d]	10.7±0.68[c]	12.3±0.41[a]	6.6±0.56[e]	_	_	_	0.17	501.78
B20	17.7±1.25[b]	17.5±0.50[b]	19.83±1.25[a]	19.9±1.00[a]	_	7.8±0.30[d]	3.9±0.37[c]	__	0.32	366.10
B21	_	12.1±0.95[b]	13.43±0.58[a]	_	8.96±1.15[c]	_	_	_	0.23	343.94
B39	14.2±0.87[b]	17.8±0.56[a]	_	17.0±0.50[a]	7.9±0.36[c]	5.9±0.65[d]	_	_	0.20	746.22
B45	16.1±0.76[c]	_	20.03±1.00[a]	18.7±0.64[b]	_	_	13.9±0.36[d]	_	0.25	989.27
Positive Control	19.4±0.26[c]	19±0.50[c]	21.73±2.05[ab]	20.7±0.64[bc]	23.1±1.10[a]	22..5±0.64[a]	20.5±0.40[bc]	_	0.39	192.56

Values are mean ± SD of three replications; denotes inactive; Positive control: 25 µg/mL of standard Levofloxacin.

For each row, values with the same letter are not significant

(A) (B) (C)

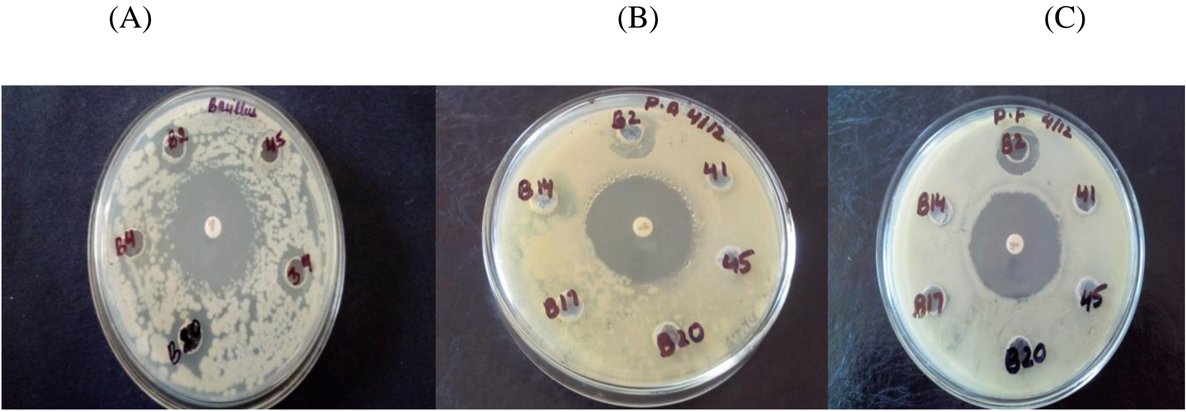
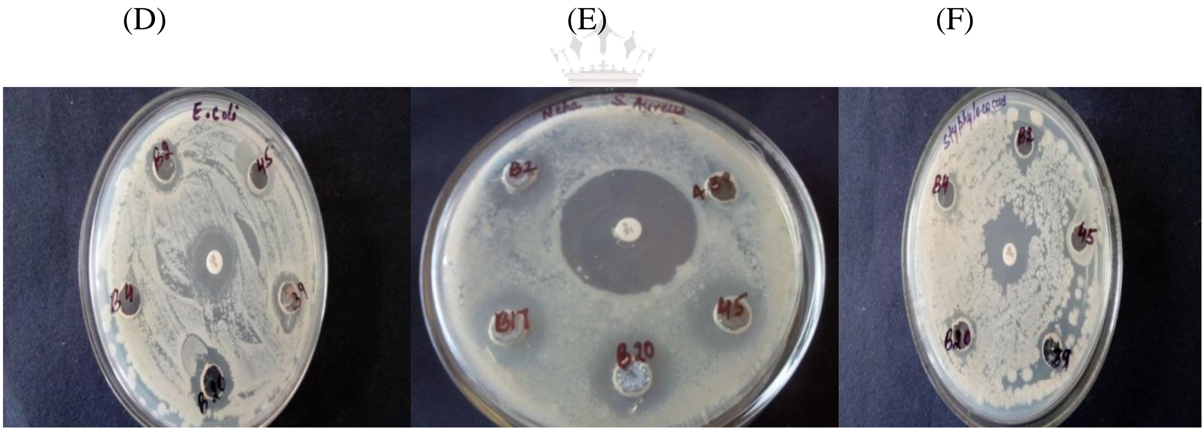


Figure 1: Secondary Screening of isolates by Agar well diffusion method.

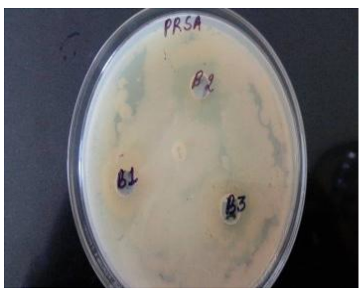
B2, B39, and B20 Shows antimicrobial B2 shows antimicrobial B2 shows antimicrobial against activity against ***B.subtilis*** activity against ***P.aeruginosa*** against ***P.fluorescens***

(D) (E) (F)



B2 and B20 Shows antimicrobial B2, B17, B20 and 45 shows antimicrobial B2 shows antimicrobial activity ***against E. coli*** activity against the clinical isolate of ***S.aureus*** activity against ***S.aureus***

(G)0



B2 shows antimicrobial against ***Penicillin resistance S.aureus***

Morphological characteristics of selected isolates

Morphological characterization of eight active isolates was studied by streaking on *Actinomycetes* isolation agar, Water yeast extract agar, Starch casein agar, Tryptone soya agar, Glycerol yeast extract agar. The Gram staining showed Gram-positive filamentous organism. Results of morphological characteristics of the selected isolates revealed that the growth of the isolates was excellent in AIA, SCA, and TSA. The isolates B2, B4, B15, and B20 showed excellent growth in Tryptone soya agar. The isolate B14 and B45 showed excellent growth in *Actinomycetes* isolation agar. The isolate B21 and B39 showed excellent growth in Starch casein agar. The aerial and substrate mycelium color varied among the isolates such as B2 was observed with Yellow pigment and B20 was observed with light yellowish pigment (Table 3).

Table: 3 Morphological Characteristics of Isolates

Isolates	Culture media	Pigments	Aerial mycelium	Substrate mycelium	Growth
B2	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar Glycerol yeast extract agar	None None None None None	White White Light Yellow White White	Yellow Yellow Yellow Yellow Yellow	Very good Good Very good Excellent Good
B4	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar Glycerol yeast extract agar	None None None yellowish None	Light Grey Grey Light Grey Light Yellow Light Yellow	White White White White White	Very good Good Very good Excellent Good
B14	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar Glycerol yeast extract agar	None None None yellowish None	White White Light Grey White Light Yellow	Yellow Yellow White White Yellow	Excellent Good Very good Very good Good
B15	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar	None None None yellowish None	Chocolate Chocolate Light Grey Light Grey Light Grey	Black Black Red Red Red	Excellent Good Very good Excellent

	Glycerol yeast extract agar				Good
B20	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar Glycerol yeast extract agar	None Orange None None None	White White White White White	Yellow Yellow Light Yellow Light Yellow Yellow	Very Good Good Very good Excellent Good
B21	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar Glycerol yeast extract agar	None yellowish None yellowish None	Light Green Green Yellow Yellow Yellow	Green Green Green Yellow Yellow	Very good Good Excellent Very good Good
B39	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar Glycerol yeast extract agar	None None None None Red	White White White Grey White	Yellow Yellow Yellow Yellow Yellow	Very good Good Excellent Good Good
B45	*Actinomycetes* isolation agar Water yeast extract agar Starch casein agar Tryptone soya agar Glycerol yeast extract agar	None None None yellowish None	Light yellow White Yellow Yellow Yellow	Red Red Black Black Red	Excellent Good Very good Very good Good

Phylogenetic studies and species identification

The partial 16S rRNA sequence of isolate B2 was compared with the sequences in the GenBank database using blastn (Altschul SF et al., 1990). The isolate B2 showed 95% homology to *Streptomyces indiaensis* strain SRT116S ribosomal RNA gene. In order to further classify B2, a Phylogenetic tree was constructed using 16S rRNA sequences from other Streptomyces reported in DNA database of NCBI (Figure 2).

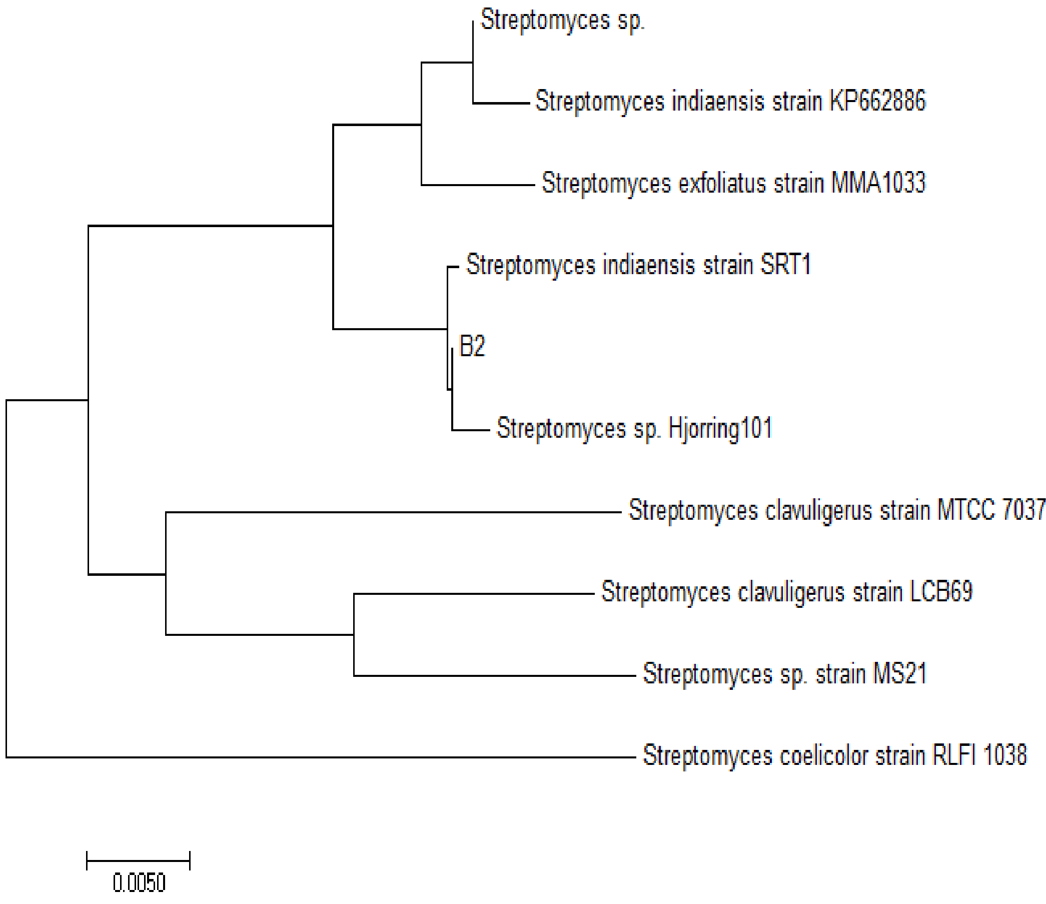


Figure: 2 Phylogenetic tree of 16S rRNA gene sequences of B2 and other Streptomyces at DNA database of NCBI. Sequence alignment was performed by Clustal W 1.86 and tree was constructed using MEGA 7 .1 version.

Optimization of fermentation conditions

Effect of medium on growth and metabolite production

The effect of different medium on antibiotic production by the isolates B2 is shown in Figure 3. Several culture mediums such as Starch casein broth, *Actinomycetes* isolation broth, Tryptone soya broth, Water yeast extract broth, Glycerol yeast extract were used for the production of desired bioactive metabolite for B2 isolate. TSB was confirmed to be the effective culture medium for maximum antibiotic production by showing 19.4 mm zone of inhibition.

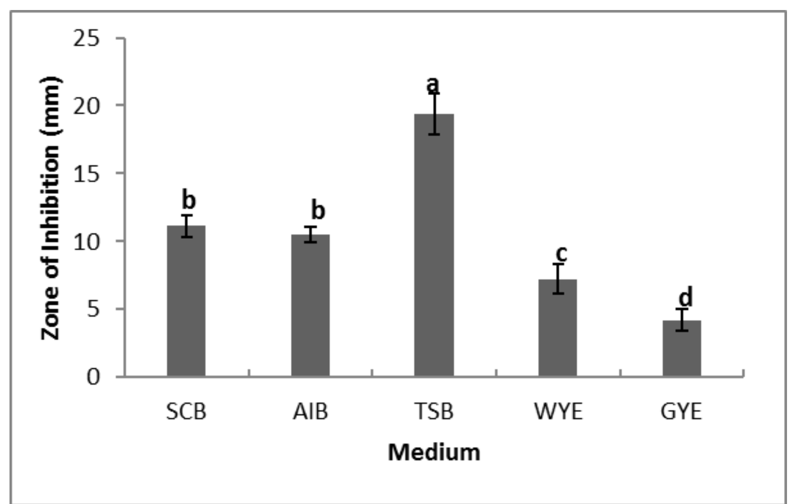


Figure 3: Effect of various medium on the production of antibiotic by B2 Isolate. Data expressed as mean± SD (n=3) P<0.05

Effect of pH on growth and metabolite production

The pH affects the production of antibiotic by B2 Isolate. The initial pH of the TSB (Tryptone soya broth) medium was adjusted to (4, 5, 6, 7, 8, 9, and 10) individually. The antibiotic production was maximum for isolate B2 at pH 7. Figure 4 represents the effect of initial pH on antibiotic production by the Isolate B2.

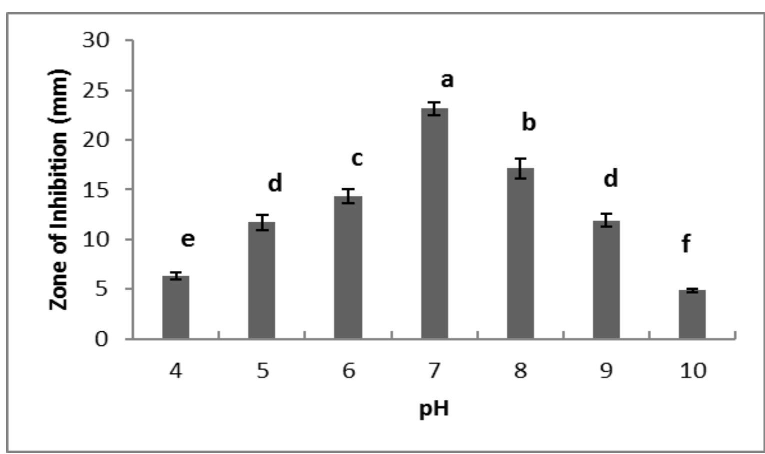


Figure 4: Effect of pH on the production of antibiotic by B2 Isolate. Data expressed as mean± SD (n=3) P<0.05

Effect of Temperature on growth and metabolite production

Temperature played an important role in the metabolite production and activity. The effect of incubation temperature on bioactive metabolite production was studied for B2 isolate, TSB medium with selected B2 isolate was incubated at different temperature (25, 28, 31, 34, 37^0 C) on the rotary shaker. 28^0 C was observed to be the optimum temperature for the antibiotic production. Figure 5 depicts the effect of incubation temperature on antibiotic production by the isolate.

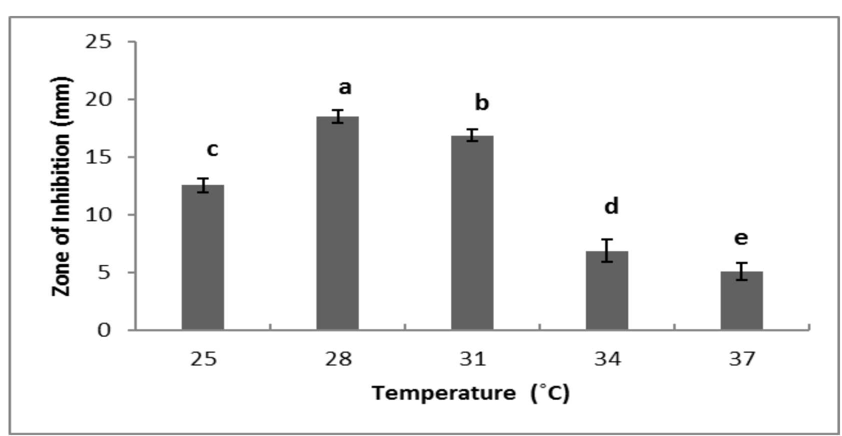


Figure 5: Effect of Temperature on the production of antibiotic by B2 Isolate. Data expressed as mean± SD (n=3) P<0.05

Effect of Incubation time on growth and metabolite production

Optimization of the Incubation period for bioactive metabolite production by B2 isolate was monitored over a period of 20 days. TSB was inoculated with B2 isolate was incubated at 28°C on the rotary shaker for 20 days. The culture broth was analyzed for antimicrobial content after every 24 hrs. Antibiotic production started after 7 days. It reached maximum on the 18th day after incubation (21.6mm) (Figure 6), However, further increase in incubation period showed decline trend in inhibition zone.

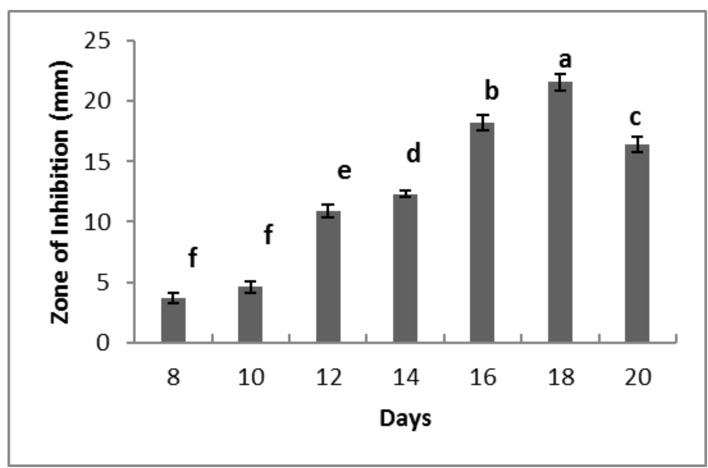


Figure 6: Effect of Incubation period on the production of antibiotic by B2 Isolate. Data expressed as mean± SD (n=3) P<0.05

Effect of Carbon source on growth and metabolite production

The effect of different carbon sources on antibiotic production by the isolate is presented in Figure 7. The effect of Carbon sources on antibiotic production was detected by fermentation medium with selected B2 isolate at different carbon sources (Glycerol, Sucrose, Starch, Glucose, and maltose). Antibiotic production was predicted on the basis of the diameter of zones of inhibition. The result showed that antibiotic production was higher in the medium having Starch as the carbon source as compared to others.

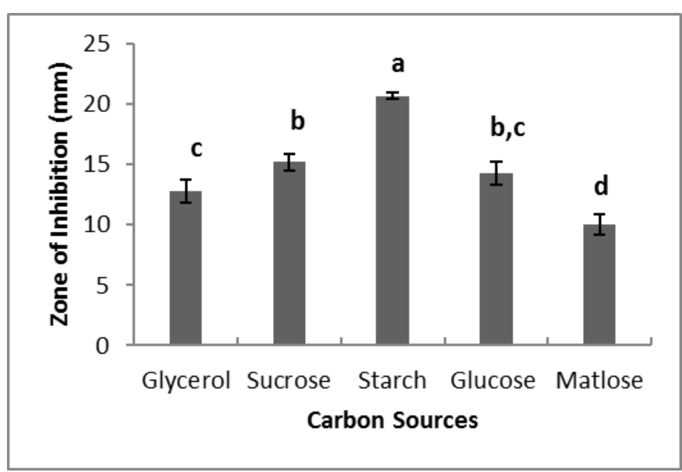


Figure 7: Effect of Carbon source on the production of antibiotic by B2 Isolate. Data expressed as mean± SD (n=3) P<0.05

Effect of Nitrogen source on growth and metabolite production

The effect of Nitrogen sources on antibiotic production was detected by fermentation medium with selected B2 isolate at different nitrogen sources (Yeast extract, Oatmeal, Casein, peptone, and Cornmeal). The result showed that casein was optimum nitrogen source for the antibiotic production with an inhibition zone of 18.3mm (Figure 8).

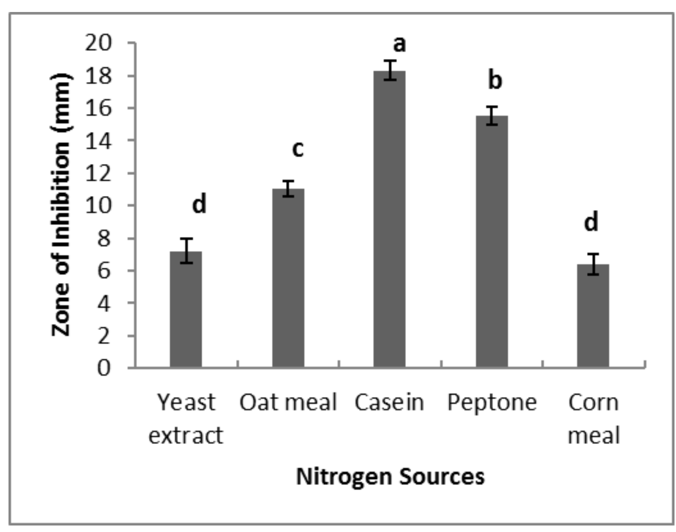


Figure 8: Effect of Nitrogen source on the production of antibiotic by B2 Isolate. Data expressed as mean± SD (n=3) P<0.05

Effect of NaCl on growth and metabolite production

Antibiotic production was predicted on the basis of the diameter of zones of inhibition. The result showed that 2% NaCl was the optimum concentration for the production of antibiotic with an inhibition zone of 19.4mm (Figure 9).

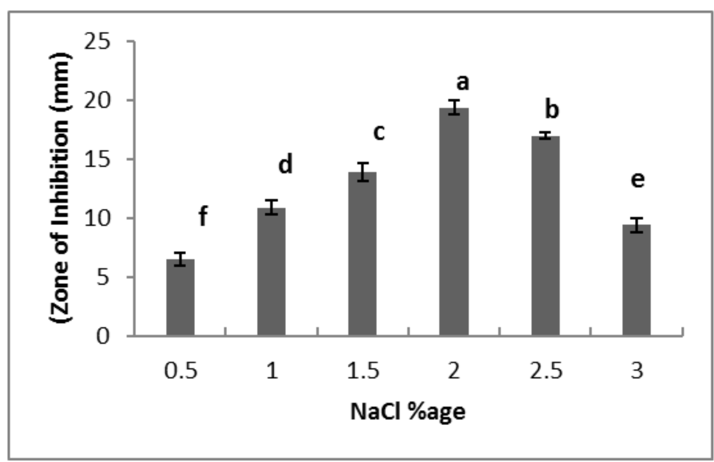


Figure 9: Effect of NaCl % on the production of antibiotic by B2 Isolate. Data expressed as mean± SD (n=3) P<0.05

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DISCUSSION

Actinomycetes are the richest source of secondary metabolites and have been proven as a potential source of bioactive compounds (Suthindhiran K et al., 2009). Almost 70% of all recognized antibiotics have been isolated from *Actinomycetes* of which 75% and 60% are used in medicine and agriculture respectively (Pachaiyappan Saravana Kumar et al., 2012). Antibiotic resistance is one of the most pressing public health issues worldwide. Presently, extensive emergence and distribution of antibiotic-resistant organisms are developing and compromising the treatment of a number of infectious diseases. Antimicrobial resistance poses major challenges for current clinical care. As a result, there has been increasing interest in searching valuable antibiotics with the novel mechanism of action from soil *Actinomycetes* in diversified ecological niches (Abo-Shadi M et al., 2010). To isolate novel bioactive *Actinomycetes* from the freshwater environment is in advance arena. There is less information regarding *Actinomycetes* isolated from freshwater lake as compared to marine water. In Ethiopia Tana Lake, showed the broad

range of inhibition zone against tested bacterial strains (Gebreselema et al., 2013). Three freshwater *Actinomycetes* isolated from the Eastern Cape Province of South Africa showed antibacterial activities against both gram-negative and gram-positive test bacteria with inhibition zones ranging from 8 to 28 mm (Sibanda et al., 2010). The present study was aimed to isolate *Actinomycetes* from soil sediment of Dal Lake and screened them for the antimicrobial metabolites. Total 52 *Actinomycetes* were isolated from soil sediments of Dal Lake. The maximum numbers of colonies were isolated on AIA media. In 2012, Kumar et al also reported AIA as the best media for isolation of *Actinomycetes*. The 52 isolates were subjected to primary screening against the selected group of human pathogens, among them only 14 showed antibacterial activity. During the secondary screening, 8 crude extracts showed the wide range of inhibition zone against tested bacterial strains. Many researchers have shown that isolates possessing good activity in primary screening failed to manifest in secondary screening (Pickup et al., 1993). It was found that the isolates had broad-spectrum antimicrobial activity against Gram positive and Gram negative bacterial cultures. Although, the maximum potential was found in isolate B2 with the maximum zone of inhibition of 21 mm against *E.coli, P.fluorescens, and P.aeruginosa*. The results showed that B2 secretes a broad spectrum antimicrobial agent which inhibits the growth of gram-positive bacteria, gram-negative bacteria, Penicillin resistance *Staphylococcus aureus* (PRSA) and clinical isolates of *S.aureus*.

The capability of a microbe to form antibiotic is not a permanent property but can be greatly increased or totally lost under different conditions of nutrients and cultivation (Krassilnikov, 1960). Conditions of incubation influenced quantitatively the biosynthesis of antibiotics from *Streptomyces* Sp. (Al-zahrani., 2007). Carbohydrates and nitrogen play important roles in the structural and energy component in the cell. Also, a number of cultivation parameters like pH, incubation period and temperature play a foremost role in the production of antibiotics (Kiranmayi et al., 2011). The development of an efficient fermentation process for the production of the antimicrobial compound by B2 requires optimization of fermentation conditions. The culture medium is a major factor as for the growth of microorganisms and production of metabolites.Isolate B2 showed maximum bioactive metabolite production in Tryptone soya broth medium by showing 21mm zone of inhibition (Fig 3). Hamza et al., 2013 also showed that maximum metabolite production by *Streptomyces* spAH11.4 in TSB medium.

The incubation temperature also was found to have an effect on growth as well as antibiotic production. In this study, 28˚C was observed to be the optimum temperature for the growth as well as antibiotic production (Fig 5). Maximum biomass and antimicrobial production were observed at 28°C by *Streptomyces erumpens* and *Streptomyces antimycotics* isolated from rhizosphere and non-rhizosphere soil (El-Mehalawy et al., 2005). The temperature at a range of 25-30°C is usually used for the majority of Streptomyces sp. for metabolite production (Muhammad Ali Akond et al., 2016). Antibiotic production generally starts on the sixth or seventh day (Sunita Bundale et al., 2015). It has been observed that B2 showed the progressive increase of biomass during the first 4- 7 days of incubation. Inhibition zone was increased with the incubation period but maximum antibiotic activity was achieved on the 18th day of incubation (Fig 6), However, further increase in incubation period showed turn down in inhibition zone. Many Streptomyces are known to produce maximum metabolite after 11-20 days. The isolate in our study also produced the metabolite within the same range of incubation days (Ganesan et al., 2016; Kumari et al., 2013). The antibiotics acquired from *Streptomyces* are optimally produced in pH close to 7.0 (Saadoun, Momani, Malkawi, & Mohammad, 1999) because *Streptomyces* are neutrophilic in nature (Yu V Zakalyukina et al., 2004). Generally, in most published literature, the pH of the culture medium for metabolite production is one of the most important environmental factors, optimum pH for antibiotic production in *Streptomyces* cultures has been reported to be near neutral (Oskay M et al., 2011; Singh V et al., 2009). For isolate B2, this was found to be in agreement that it showed maximum activity at pH 7 (Fig 4), it was found to be optimum both for growth as well as bioactive metabolite production. In this study, Isolate B2, showed maximum cell growth in medium amended with glucose, Sucrose, and maltose but very poor bioactive metabolite production (Fig 7). On the other hand, maximum bioactive metabolite production was observed in medium amended with Starch as carbon sources. For gentamicin production and growth rate, starch was found to be best carbon source (Himabindu and jetty, 2006). Many studies suggested that polysaccharides such as glycerol and starch are generally the best carbon sources as they sustain a slow growth rate that is suitable for antibiotic production (Jonsbu et al., 2002). Fermentation conditions for metabolite production are significantly affected by nitrogen sources. From the results, it was clear that the growth of the isolates B2 was greatly affected by the nature and type of nitrogen source supplemented in the medium. In this

study maximum, bioactive metabolite production resulted with casein. Sunita Bundle reported that maximum bioactive metabolite production was observed in the medium amended with casein for isolate R3 (Sunita Bundale et al., 2015). Due to the osmotic pressure of the medium, salt concentration influence the production of the metabolite from the microorganism (Pelczer et al., 1993).In case of isolate, B2 NaCl exerted a stimulatory effect on its growth and metabolite production upto2% NaCl and thereafter growth of B2 and metabolite production by the isolate B2 declined sharply at higher concentration of NaCl. Hence the isolate is considered as not a halotolerant strain. It was observed that Streptomyces species SRDP-TK-07 isolated from Talakaveri, Western Ghats of Karnataka, India produced higher bioactive metabolites at the salinity of 2% (K. N. Rakesh et al., 2014). The study showed that medium, pH, temperature, incubation time, carbon source, nitrogen source and NaCl concentration directly affected the production of antibiotic. The B2 Isolate requires definite conditions for highest growth as well as antibiotic production. Bioprospecting of *Actinomycetes* especially *Streptomyces* for medical science is done by humans from ancient times. This study identified a set of optimizing parameters such as a period of 18 days of incubation, with optimum temperature of 28°C and pH of 7 and TSB medium containing starch 1% (w/v), casein 1% (w/v), 2%(w/v) NaCl, higher yield of antibiotic production with a maximum zone of inhibition against clinical isolated *S.aureus* strain and penicillin-resistant *S.aureus*. It also highlights the need to screen soil of freshwater lakes of Northwestern Himalayas region of Kashmir soil actinobacteria against such rising harmful human pathogen and obtain potential antibiotics that could also serve as targets to MRSA like pathogens.

CONCLUSION

In conclusion, this study has shown that freshwater environments could serve as potential reservoirs for *Actinomycetes* of antimicrobial importance with varying spectra of activities. The widest activity spectrum and the largest inhibition zones were shown by strains B2.Thus there is definite scope for bioprospecting of antagonistic *Actinomycetes* from Dal Lake ecosystem once appropriate further studies are undertaken. The purpose of this study is to employ a new technology to discover the next generation of effective antibiotics. These antibiotics will also provide the public with a stronger defense against the threat of bioterrorism. In India, no studies

have been conducted so far to isolate and evaluate *Actinomycetes* from different freshwater habitats located in Kashmir region. Therefore, the present study provides us two valuable *Actinomycetes* strain that can be explored at industrial scale to produce antibiotic against emerging drug resistance.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

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