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Bioactive potential of actinobacteria against drug resistant pathogens

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ABSTRACT

The present investigation was focused to study the antagonistic potential of actinobacterial strain TK2 isolated from Thirukurungudi Hills (Western Ghats, Tamil Nadu), against selected drug resistant bacterial pathogens. Of the 9 clinical bacterial strains, *S. aureus* was found to be resistant against methicillin and vancomycin while the remaining 8 gram negative pathogens were confirmed as extended spectrum beta lactamase (ESBL) producers. Strain TK2 showed good antagonistic activity against all the bacterial pathogens, except the three isolates of *P. aeruginosa*. Strain TK2 produced the antimicrobial activity on 4^{th} day of incubation when growing on ISP2 agar medium whereas the same strain showed activity only on 8^{th} day of incubation when it was grown on ISP2 broth. Of the various solvents tested for extraction, bioactive compound was extracted only in ethyl acetate. The crude extract showed 14-18 mm inhibition zone in disc diffusion method. The crude extract produced two spots in thin layer chromatography (TLC) in chloroform: methanol (30:60) solvent system. In bioautography, the second spot (Rf value 0.685) showed activity. The active compound was purified by preparative TLC, which showed maximum activity (15-20 mm inhibition) against test pathogens. Based on the results of chemical screening, the active compound was identified as sugar containing substance. Strain TK2 showed good growth on various growth media and culture conditions. Based on the studied phenotypic characteristics strain TK2 was identified as the species of the genus *Streptomyces*. Of the various growth parameters tested, ISP2 medium, glucose, pH 7, 1% NaCl and temperature 30^0C was influenced the antagonistic activity of strain TK2. Strain TK2 will be a potential for the isolation of bioactive compound(s) which will be a candidate for the development of antibiotic against drug resistant bacterial pathogens.

Keywords: Antagonistic activity, resistant pathogens, optimization, taxonomy, actinobacteria.

INTRODUCTION

The history of infectious diseases is as old as the human civilization, and need of protection against these infections are always remains one of the prime concerns. The development of resistance among the pathogenic microorganisms to multiple drugs is a major problem in the treatment of infectious diseases. Among the various drug resistant pathogens *Staphylococcus aureus* resistant to methicillin and vancomycin, extended spectrum beta lactamases (ESBL) producing pathogens and multi- and extensively-drug resistant *Mycobacterium tuberculosis* are in major concern.

Infections caused by methicillin resistant *S. aureus* (MRSA) are associated with a mortality rate of 15-60%. Extended Spectrum Beta Lactamases (ESBLs) are mutant plasmid mediated broad spectrum beta lactamases and confer resistance to penicillin, first-, second-, and third generation cephalopsporins and aztreonam, except cephamycins and carbapanems (Hemachandran *et al*., 2011). ESBLs are most commonly encountered in gram negative bacteria such as *Escherichia coli, Klebsiella sp, Pseudomonas aeruginosa, Enterobacter, Citrobacter, Salmonella sp* and *Proteus sp.* Antibiotic resistance is a complex and truly global problem that requires global solutions through effective antibiotics (McCallum *et al*., 2011)

Actinobacteria is a class/phylum which includes more than 245 Gram positive filamentous bacterial genera with high G+C content in their DNA. The diversity of actinobacteria in various natural including extreme and man-made environments is well documented. They are the most economically valuable prokaryotes which are well known to produce chemically diverse metabolites with wide range of biological activities (Balagurunathan and Radhakrishnan, 2010). Out of 22,500 microbial bioactive metabolites reported, about 10,100 are reported to synthesize by actinobacteria in which most of them are from terrestrial origin (Berdy, 2005). The rate of discovering new compounds from terrestrial actinobacteria has decreased but the rate of re-isolation of known actinobacteria and antibiotics is on the increase (Lam, 2006). This precluded the study of normal terrestrial sources particularly for actinobacteria and has led researchers to explore unique and extreme habitats like deep sea, desert, forest and mountain environment for potentially new biosynthetic diversity. With this view, the present study is attempted to study the bioactive potential of an actinobacterium TK2 isolated from Western Ghats region, South India against the drug resistant bacterial pathogens.

MATERIALS AND METHODS

Description of actinobacterial strain TK2

Actinobacterial strain TK2 was isolated from soil sample collected from Thirukurungudi Hills (Western Ghats; Lat., 8° 25'E; Long., 8° 53'N) in Tamil Nadu, South India, using starch casein agar medium. Growth of strain TK2 was maintained on yeast extract malt extract (YEME) agar (ISP2 medium) slants as well as in 30% glycerol broth (Radhakrishnan *et al.,* 2007).

Test organisms

Standard bacterial strains such as *Staphylococcus aureus* NCIM 2079, *Bacillus pumilus* NCIM 2327, *Bacillus subtilis* NCIM 2063, *Bacillus cereus* NCIM 2106, *E. coli* NCIM 2256, *P.aeruginosa* NCIM 5031 and *Klebsiella pneumoniae* MTCC 432 are used for bioactivity studies.

Bacterial pathogens, each one strain of *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus mirabilis* and each three strains of *E. coli* and *P. aeruginosa* are used. The antibiotic resistant pattern of selected clinical bacterial pathogens was confirmed by Kirby-Bauer antibiotic susceptibility test (Bauer *et al.,* 1966) on Muller-Hinton agar. Standard antibiotic discs (Hi media) used include methicillin, vancomycin, meropenem, cefotoxime, ciprofloxacin, cephadroxil, cefoperazone, ceftazidime and clavulanic acid.

Preliminary screening for antagonistic activity

Strain TK2 was screened for antagonistic activity against bacterial pathogens by adopting cross streak method (Radhakrishnan *et al*., 2007) and agar plug method (Eccleston *et al*., 2008). In cross streak method, strain TK2 was inoculated into modified nutrient glucose agar (MNGA) as a straight line. After incubation at 28^0C for 5 days, 18 hours old test bacterial pathogens were inoculated at right angle to the original streak of the actinobacterial strain TK2. Inhibition of bacterial pathogens was observed after incubation at 37^0C for 24 hours. In agar plug method, test organisms were inoculated into Muller Hinton Agar (MHA) plates using sterile cotton swab. Actinobacterial strain TK2 grown on YEME agar plates (20 ml/plate) at 28^0C for 10 days was taken. After scraping the mycelial growth, 5 mm diameter agar plug were taken and placed over MHA plates seeded with test organisms. All the plates were incubated at 37^0C for 24 hours.

Effect of liquid and solid culture on growth and bioactivity of strain TK2

Growth of strain TK2 was inoculated into YEME agar plates and YEME broth. All the YEME agar plates were incubated at 28^0C whereas all the flasks were incubated in rotary shaker with 95 rpm at 28^0C. For every 24 hours, agar plugs from YEME agar and the cell free supernatant from YEME broth cultures were tested against *S. aureus* NCIM 2079 by agar plug method and well diffusion method, respectively. The crude extract from YEME agar and YEME broth was extracted using ethyl acetate. The concentrated crude extract was tested against the bacterial pathogens at 250µg/disc concentration by disc diffusion method (Radhakrishnan *et a*l., 2007).

Production and extraction using different solvents

Actinobacterial strain TK2 was inoculated in to ISP2 agar medium (20 ml of medium/ plates) and incubated at 28^0C for 7 days. After removing the mycelial growth aseptically from the agar surface, the antibiotic containing agar medium was cut in to pieces and extracted using different solvents (1:2 ratio) such as methanol, chloroform, ethyl acetate, dichloromethane, diethyl ether and n-hexane for overnight at room temperature. The solvent extracts were concentrated under reduced pressure and quantified. Antibacterial activity of different solvent extracts was tested by disc diffusion method against *S. aureus* and *E. coli* (Yilmaz *et al*., 2008). Solvent extract which showed good activity was tested against all the standard and clinical pathogens used in the preliminary screening.

Purification of crude compound by thin layer chromatography

The crude compound was purified by using silica gel thin layer chromatography (Merck; silica gel 60-F254nm). To find out

the best solvent system to separate the crude compound, solvents such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water were used in different proportions. After running, the sheet was kept at room temperature for the complete drying of the plate and the separated spots were visualized in iodine chamber. Rf value of the spots on the TLC plate was determined (Marston, 2011).

Detection of active compound by bioautography

The bioautography method described by Irena *et al*., (2011) is followed for the detection of active compound separated in TLC. Chromatogram developed as described above was placed in a sterile bioassay petri dish containing nutrient agar medium inoculated with *S. aureus*. Active compound from crude ethyl acetate extract was further purified from preparative TLC and tested for antimicrobial activity by disc diffusion method as described earlier.

Chemical Screening

Chemical screening of partially purified compound was performed to determine the functional group present in the bioactive compound (Fiedler, 1993). Commercially available readymade TLC sheets (Merck; silica gel 60-F254nm) spotted with crude compound, and chloroform: methanol (30:60) was used as a solvent system. Spots were obtained after the solvent gets eluted. After drying, the sheets were sprayed with reagents such as ninhydrin, vanillin, and napthoresorcin-sulphuric acid. The sheets were kept in hot air oven at 120 ºC to observe the colour change.

Taxonomy of potential actinomycete strain

Phenotypic characteristics such as micromorphology, cultural characteristics, carbon utilization and other physiological characteristics of strain TK2 were studied by adopting standard procedures (Shirling and Gottileb, 1966; Balagurunathan *et al*., 2010). Effect of different culture medium and physiological conditions on antimicrobial activity of strain TK2 was studied using agar plug method.

RESULTS AND DISCUSSION

The present investigation was focused on novel bioactive compound from actinobacterial strain TK2. The actinobacterial strain TK2 was well grown on ISP2 agar with good aerial mycelium and substrate mycelium. Antibiotic resistant pattern of clinical pathogens was given in table 1. Of the 9 clinical isolates tested, *S. aureus* was found to be resistant against methicillin and vancomycin. The remaining 8 gram negative isolates were showed sensitivity to meropenem, except *P.aeruginosa* 3, with varying susceptibility pattern to other antibiotics. All the 8 Gram negative bacterial isolates were identified as ESBL producers. MRSA has become a worldwide concern because it is highly prevalent, capable of developing new clones, resistant to almost all currently available antibiotics except vancomycin and teicoplanin, and can potentially cause death. Recently, the susceptibility of MRSA to vancomycin has decreased; thus, increases in vancomycin-intermediate *S. aureus* and resistant *S. aureus* (VISA and VRSA, respectively) have been reported in several countries. The emergence of MRSA resistant to the 'last resort' antibiotics (vancomycin and teicoplanin) has created an urgent need in the discovery of alternative antibiotics (Isnansetyo and Kamei, 2009). Infections caused by ESBL producers complicate therapy and limit treatment options. In addition, Antimicrobial resistance has a significant negative impact on the outcome of therapy and increases the risk of cross-infection in hospitals. Resistance leads to inappropriate empirical therapy, delay in starting effective treatment and the use of less effective, more toxic and more expensive drugs (French, 2010). This has led to the parallel increase in the usage of beta lactamase inhibitor/ beta lactams combinations, monobactams and carbapanems. However last few years have witnessed resistance to these drugs as well. So there is an urgent need to discover antibiotics against these resistant pathogens.

Antagonistic activity of strain TK2 against standard and drug resistant clinical bacterial pathogens was given in table 2. Except the standard and clinical isolates of *P. aeruginosa*, strain TK2 was found to be active against all the test organisms by both cross streak and agar plug method. In agar plug method, strain TK2 produced 12-16 mm zone of inhibition. There are numerous reports are available for the anti MRSA activity of actinobacteria (Thakur *et al.*,2007; Anima Nanda *et al.*, 2009; Namita *et al*.,2010) whereas the reports on the activity of actinobacteria against ESBL producing strains are very few (Farhat *et al.*, 2009; Hemachandran *et al*., 2011). In addition, the agar plug used in this study is very simple and effective and economic method for the preliminary screening of actinobacteria for antagonistic activity when compared to cross streak method.

Table. 1: Antibiotic resistant pattern of clinical bacterial pathogens.

Test Pathogens	Zone of inhibition (diameter in mm)								
	M	V	MR	CTX	CF	CQ	CS	CAZ	CAC
S.aureus	0 (R)	10 (R)	-	-	-	-	-	-	-
E.coli 1	-	-	12 (S)	13 (S)	16 (S)	0 (R)	0 (R)	0 (R)	0 (R)
E.coli 2	-	-	12 (S)	21 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
E.coli 3	-	-	12 (S)	15 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
K. pneumoniae	-	-	10 (R)	15 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
P. mirabilis	-	-	13 (S)	16 (S)	20 (S)	0 (R)	0 (R)	0 (R)	0 (R)
P.aeruginosa 1	-	-	15 (S)	14 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
P.aeruginosa 2	-	-	16 (S)	16 (S)	20 (S)	15 (S)	16 (S)	18 (S)	15 (S)
P.aeruginosa3	-	-	10 (R)	10 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)

S- Sensitive; R-Resistant; - - not tested; 0 – no activity

M-Methicillin, V- Vancomycin, M-Mropenem, CTX- Cefotoxime, CF-Ciprofioxacin, CQ-Cephadroxil, CS- Cefoperazone, CAZ- Ceftazidime, CAC- Clavulanic acid

Table. 2: Antagonistic activity of actinobacterium strain TK2.

Standard and clinical resistant bacterial strain	Activity of strain TK2 (cross streak method)	Activity of strain TK2 agar plug method [zone of inhibition in diameter in mm]
S.aureus NCIM 2079	+	12
B.pumilus NCIM 2327	+	16
B.subtilis NCIM 2063	+	13
B.cereus NCIM 2106	+	14
E.coli NCIM 2256	+	16
K.pneumoniae MTCC 432	+	13
P.aeruginosa NCIM 5031	-	-
S. aureus [VRSA, MRSA]	+	14
E.coli1 [ESBL producer]	+	13
E.coli2 [ESBL producer]	+	14
E.coli 3 [ESBL producer]	+	15
K. pneumoniae [ESBL producer]	+	13
P.mirabilis [ESBL producer]	+	12
P.aeruginosa 1 [ESBL producer]	-	-
P.aeruginosa 2 [ESBL producer]	-	-
P.aeruginosa 3 [ESBL producer]	-	-

Table. 3: Effect of medium consistency and incubation period on the antimicrobial activity of strain TK2.

Incubation period [Days]	Antimicrobial activity (zone of inhibition in diameter in mm)			
	S.aureus		*E.coli*	
	ISP2 agar	ISP2 broth	ISP2 agar	ISP2 broth
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	17	-	15	-
5	20	-	21	-
6	21	-	19	-
7	20	-	16	-
8	23	10	13	10
9	18	14	13	12
10	15	16	13	15

Strain TK2 produced the antimicrobial activity on 4th day of incubation when it was grown on ISP2 agar medium whereas the same strain showed activity only on 8th day of incubation when it was grown on ISP2 broth (Table 3). From the 8th-9th day of incubation, the agar plug prepared from YEME agar started to produce decreased zone of inhibition. The pH of the fermentation medium was found to be around 7.0 throughout the fermentation period. The morphology of actinobacterial mycelium is well suited for invasive growth on solid medium the hyphal mode of growth gives the filamentous organism the power to penetrate into the solid substrates. In addition, their ability to grow at low water activity (a^w) and high osmotic pressure conditions (high nutrient concentration) makes actinobacterial efficient (Barrios-Gonzlez and Mejia, 1996). But the filamentous morphology of actinobacteria is responsible for the considerable difficulties in large submerged culture. These include shear forces, increasing viscosity due to metabolic secretion, and reduction in the metabolic stability. This results in high requirements for mixing and oxygen transfer efficiency and can lead to problems during product recovery. Therefore, Solid State Fermentation technology can be exploited as an alternative, allowing better oxygen circulation, especially when large quantities of secondary metabolites are required to be produced in short fermentation periods with minimal expenditure of media and downstream processing (El-Naggar *et al.*, 2009). In the present study also, the strain TK2 produced good growth on ISP2 agar medium and also produced antimicrobial activity on early incubation period.

Among the various organic solvents tested for extraction, only the crude compound extracted in ethyl acetate showed 15 mm and 16mm zone of inhibition against drug resistant isolate of *S. aureus* and *E. coli 3*, respectively. The crude ethyl acetate extract prepared from strain TK2 showed 14-18 mm inhibition against the drug resistant pathogens tested (table 4).

Table. 4: Antimicrobial activity of crude ethyl acetate extract prepared from strain TK2.

Standard and clinical resistant bacterial strains	Antimicrobial activity [zone of inhibition in diameter in mm]
S.aureus NCIM 2079	17
B.pumilus NCIM 2327	18
B.subtilis NCIM 2063	14
B.cereus NCIM 2106	15
E.coli NCIM 2256	18
K.pneumoniae MTCC 432	15
S. aureus [VRSA, MRSA]	17
E.coli1 [ESBL producer]	14
E.coli2 [ESBL producer]	16
E.coli 3 [ESBL producer]	17
K. pneumoniae [ESBL producer]	15
P.mirabilis [ESBL producer]	14

Among the different solvent systems tested in various proportions, chloroform: methanol in 30:60 showed good separation and two spots were observed when exposed into iodine vapor. The Rf value of the separated spots were calculated as 0.465 and 0.685, respectively. In bioautography, the second spot (Rf value 0.685) showed activity against the test organisms. Bioautography allows localizing the antimicrobial activity of an

extract on the chromatogram, it supports quick search of new antimicrobial agent through bioassay guided fractionation. This method also avoids the previous purification of the substance and reducing the cost of initial screening (Scorzoni *et al.*, 2007). The active substance purified by preparative TLC showed maximum activity (15-20 mm) against test pathogens when compared to crude extract. The strategy for the discovery of new lead structure was described by Zahner (1985) who developed a non target screening method based on TLC combined with different spraying reagents for the detection of new secondary metabolites. This, so called chemical screening led to the isolation of a variety of new bioactive compounds (Fiedler, 1993). In chemical screening, among the different spraying reagents used, blue colouration was observed while using napthoresorcin-sulphuric acid. Based on the observation, the active compound was identified as a sugar containing substance

Cultural, microscopic (fig. 1), physiological characteristics and antibiotic susceptibility pattern of strain TK2 was given in table 5. Strain TK2 was produced good to moderate level of growth on different ISP media as well as under different culture conditions. Strain TK2 was sensitive to most of the antibiotics tested. Molecular characterization will leads to the identification of this strain at species level.

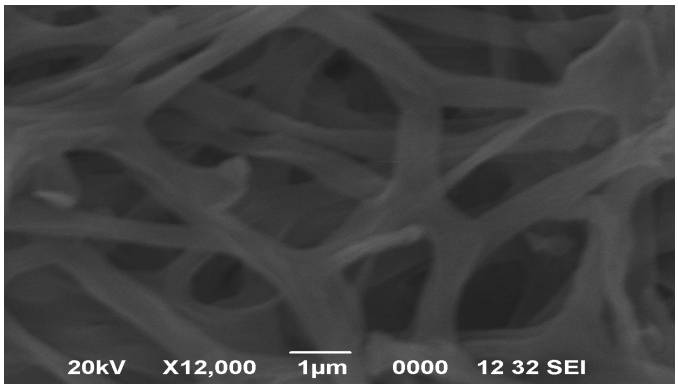

Fig. 1: Scanning Electron Micrographs of the actinobacterium TK2.

Effect of culture conditions on antagonistic activity of strain TK2 was given in table 5. Of the various conditions tested, ISP2 medium, glucose, pH 7, 1% NaCl and temperature 30^0C was influenced the antagonistic activity of strain TK2, it showed effective zone of inhibition against *S.aureus* and *E.coli 3* (table 6). This result is based on the classical one-factor-at-a-time experiment. The effect of interaction between the above variables on the antimicrobial compound production will be determined by adopting statistical based methods like Response Surface Methodology (RSM).

Table. 5: Phenotypic characteristics of actinobacterium TK2.

Parameters	Variables	Growth
Media	ISP1	+
	ISP2	+++
	ISP3	++
	ISP4	++
	ISP5	++
	ISP6	+
	ISP7	++
Carbon source	Glucose	+++
	Arabinose	++
	Sucrose	++
	Xylose	++
	Inositol	++
	Mannitol	++
	Fructose	++
	Rhamnose	+
	Raffinose	+
	Cellulose	+
Nitrogen source	Asparagine	++
	Glutamine	++
	Tyrosine	++
pH	5	-
	7	+++
	9	++
	11	++
Temperature (^{0}C)	20	++
	30	+++
	40	++
	50	-
NaCl (%)	0	++
	1	+++
	2.5	++
	5	++
	7.5	++
	10	-
Antibiotic susceptibility pattern	Penicillin G	Sensitive
	Tetracycline	Sensitive
	Meropenem	Sensitive
	Amikacin	Sensitive
	Kanamycin	Sensitive
	Chloramphenicol	Resistant
	Streptomycin	Sensitive

	Vancomycin	Sensitive
	Clavulanic acid	Resistant
	Methicillin	Sensitive
	Gentamycin	Resistant
	Bacitracin	Sensitive
	Erythromycin	Sensitive

+++ = Good, ++ =Moderate, + = poor

Table. 6: Effect of culture conditions on the antimicrobial activity of actinobacterium TK2.

		Antimicrobial activity	
Parameters	**Variables**	***S.aureus***	***E.coli 3***
Media	ISP1	12	10
	ISP2	16	14
	ISP3	15	14
	ISP4	12	13
	ISP5	10	7
	ISP6	10	8
	ISP7	12	13
Carbon source	Glucose	17	16
	Arabinose	13	-
	Sucrose	12	13
	Xylose	10	8
	Inositol	13	-
	Mannitol	12	-
	Fructose	13	-
	Rhamnose	10	-
	Raffinose	10	-
	Cellulose	10	-
Nitrogen source	Asparagine	12	10
	Glutamine	13	10
	Tyrosine	10	12
pH	5	-	-
	7	17	14
	9	14	13
	11	10	12
Temperature	20	10	-
	30	16	15
	40	13	12
	50	-	-
NaCl (%)	0	12	12
	1	16	16
	2.5	13	13
	5	10	-
	7.5	11	9
	10	-	-

CONCLUSION

Strain TK2 will be a potential for the isolation of bioactive compound(s) which will be a candidate for the development of antibiotic against drug resistant bacterial pathogens.

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