Screening of Actinomycetes Against Multi Drugs Resistant Human Pathogenic Bacteria Isolated from the Soil of Khyber Pakhtunkhwa and Optimization Studies of Selected *Streptomyces* RMN6

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Abstract: In the present study total 43 actinomycetes RMN1-RMN43 were isolated from the collected soil samples. To screen out active metabolites producing actinomycetes against human pathogenic bacteria, 30 Gram positive and negative bacteria were isolated from hospitalized patients. In the primary screening, 21 actinomycetes showed antibacterial potential against one or more pathogenic bacteria. Total 14 actinomycetes, RMN1-14, showed antibacterial activities through agar well diffusion assay. One isolate of actinomycete, RMN5, produced exclusively narrow spectrum bioactive secondary metabolites against *E. coli* with 13 mm zone of inhibition. In the secondary screening most potent actinomycete, RMN6, produced broad spectrum bioactive metabolites against ATCC bacterial culture and multi antibiotic resistant bacteria such as *E. coli ATCC 25922, P. aeroginosa ATCC 27553,* and *Staphylococci ATCC 25923* activities at the range of 14, 13 and 19 mm zone of inhibition respectively while against isolated bacterial pathogens *E. coli, P. aeruginosa* and *S. aureus* (MRSA) 15, 18 and 23 mm zone of inhibition was recorded, respectively. RMN6 was identified morphologically and biochemically as *Streptomyces*. Different conditions were optimized for RMN6 in batch fermentation in which pH 8, 144 hours incubation period, 29^{o}C, 7.5% inoculum size, 3% glucose concentration and 160 rpm speed of shaking orbital incubator produced maximum activity. Maximum activity was recorded against *M.luteus*ATCC 10240 and *S.aureus* (MRSA) clinical isolate as 32 and 28 mm respectively.

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Introduction

Human pathogenic bacteria cause serious infections and has acquired resistance to commonly available antibiotics creating a serious healthcare threat worldwide [1]. For example *S.aureus,* causes various infections including bacteremia, pneumonia, endocarditis, osteomyelitis and pimples. *S.aureus* is a virulent human pathogen and due to the misuse of antibiotics has become resistant to various groups of antibiotics [2].

For the last two decades doctors' community and public health officials have great concern regarding the methicillin resistant *S. aureus*. Only option was vancomycin for the treatment of (MRSA) during much of this time, but the emergence of Vancomycin resistant pathogens has complicated the matter [3-4]. The emergence of bacterial strains like MRSA, VRSA challenges public health officials and clinicians. The entry of new antimicrobial agents liked aptomycin, linezolid, and a streptogramin combination (quinupristin/dalfopristin) recently in the clinical application for therapy fortunately overcome the problem in the last few years [5-6]. However, due to certain adverse side effects and the acquisition of resistant pathogenic strains against these new drugs, require the strategies to develop new antimicrobial compounds active against such type of Gram positive bacteria [7-8]. Another reason of great hurdle is that Gram negative bacterial opportunistic pathogens are becoming resistant to antibiotics. Gram negative environmental and enteric organisms become the major cause of human infection in the hospital settings and communities with multi drugs resistance and resistant to first second and third generation of cephalosporin and penicillin [9-10]. In immunocompromized patients *P.aeroginosa* cause infections and act as opportunistic pathogen [11].

Filamentous Gram positive bacteria have high G+C contents are categorized in a group of actinomycetes. Biotechnologically important products like enzymes, antibiotics and large numbers of other bioactive compounds are derived from this group of bacteria [12-13]. More than 70 % of total antibiotics have been extracted from actinomycetes especially from *Streptomyces* species. Screening and survey for antibiotic producing actinomycetes in large areas of

the world is nearly exhausted. This and due to the increasing emergence of antibiotic resistant pathogenic strains, an emergency demand for the survey of unscreened and unexplored areas for isolation of new antibiotic producing actinomycetes [14].Actinomycetes have immense importance for the production of bioactive compounds of increased commercial value and nowa day the search and screening for new compounds is continue on routine basis. These routine investigations have been fruitful and approximately two third of naturally found antibiotics, large numbers of these antibiotics have medical value have been extracted from actinomycetes [15].

Cultural conditions i.e inoculum size, inoculum age of the producing actinomycetes, fermentation medium, carbon source and incubation period as well as temperature have direct or indirect effect on antibiotic production from producing microorganism. Lower concentration of nutrients may led to poor growth and low level secondary metabolites production.Optimum concentration of various nutrients and optimized cultural conditions are required for better growth of microorganism and product formation [16].

In the present study, unscreened areas of Khyber Pakhtunkhwa, for soil sample collections and isolation of actinomycetes were targeted. Innovative strategies were used for screening of novel types of secondary metabolites producing actinomycetes against antibiotic resistant human pathogenic bacteria. Fresh cultures of human pathogenic bacteria were isolated from hospitalized patients and their sensitivity assays were conducted to determine the resistance pattern as sensitive, intermediate and resistant. This strategy became fruitful for screening of most effective secondary metabolites producing actinomycetes from the isolated pool of actinomycetes. In the next step optimization parameters for maximum production of metabolites were determinedfor effective actinomycetes.

Methods and Materials

Area selection and collection of soil samples

Soil samples were collected from Swat and Bunir districts of Khyber Pakhtunkhwa (KPK) during April 2011 to September 2011. All these samples were collected from agricultural and non-agricultural land. Sampling sites were determined by GPS (Global positioning system) coordinate. Soil samples were taken from 5 to 15 cm depth. Samples were kept in sterile polythen bags, brought to the laboratory and dried under shade for seven days,crushed and sieved for isolation of actinomycetes [17].

Isolation and enrichment

Soil samples were serially diluted up to 10^{-5}, selected diluted samples were used for plating on Actinomycetes Isolation Agar (AIA) and Nutrient agar media with and without antibiotics. Nystatin and Cyclohexamide (50 µg/ml) was added to inhibit bacterial and fungal growth, respectively. One gram of each soil samples sieved and pretreated with 0.1 gram $CaCO_3$ was added to 99 ml sterile distilled water and kept in orbital shaker at 150 rpm for half hour to mix completely. Serially diluted samples were plated in duplicate using AIA media with and without Nystatin and Cyclohexamide. Another soil sample was plated on Nutrient Agar media in duplicate. All plates were incubated at 28-30 ^{o}C for 2 to 3 weeks. During incubation period, growth of actinomycetes was observed intermittently. Selected colonies of actinomycetes were sub cultured on AIA and Nutrient Agar media for screening of antibiotic producing actinobacteria.

Test pathogenic bacteria

Thirty Gram positive and negative pathogenic bacteria were isolated from human blood, urine, pus and sputum samples at Lady Reading Hospital Peshawar. These isolated bacteria were *S.aureus, P.aeroginosa, E. coli* and *S.typhi* ATCC bacterial culture, provided by Pakistan Council of Scientific and Industrial Research(PCSIR) laboratories Peshawar, were *P.aeroginosa* ATCC# 27853, *S. aureus* ATCC# 25923, *E. coli* ATCC# 25922, *E.faecalus* ATCC# 29212, *K. pneumonia* ATCC# 700603 and *M.leuteus* ATCC # 10240.

Biochemical tests for clinical isolates identification

After isolation of pathogenic bacteria, they were morphologically identified using MacConkey agar, Manitol Salt Agar (MSA), Eosine Methylene Blue agar (EMB), Blood Agar media, Gram staining and microscopy. For biochemical identification different tests were also performed including Urease vogesprokauser, Beta galactosidase, Lactose, Mannitol, Glucose, Sucrose utilizations, Oxidase, Citrate, Indole, Lysine decarboxylase and H_2S up to species level identification [18].

Antibiotic sensitivity assay

Antibiotic sensitivity assay was conducted through Kirby-Bauer method[19]. Twenty four hours fresh culture of pathogenic bacteria was inoculated into 5 ml nutrient broth and then incubated at 37 ^{o}C for 24-48 hours. The turbidity of indicator strains was adjusted with McFarland solution adding 0.89% saline solution in culture tube of indicator pathogenic strain. Uniform lawns were prepared on Muller Hinton agar for each pathogen using sterile cotton swabs. Antibiotic discs were gently placed on the prepared lawn at equal distance using sterile forceps. After incubation at 37 ^{o}C for 24-48 hours, zone of inhibition was measured for determination of sensitivity towards different antibiotics.

Antibacterial activity

Primary screening of isolated Actinomycetes was carried out through cross agar streaking to assess its antimicrobial activity. For this purpose all isolated Actinomycetes were selected for primary screening. Actinomycetes were streaked in single line at the center of Nutrient agar and incubated for 2 to 3weeks at 28°C. All isolated pathogenic bacteria were then perpendicularly streaked and incubated for 24-48 hours at 37°C.

Secondary screening through agar well diffusion assay

All positive actinomycetes at primary screening were subjected for secondary screening. Positive actinobacteria were inoculated in the starch casein broth and incubated in the orbital shaker at 28°C for 2-3 weeks. Samples were taken during incubation period after every 24 hours, centrifuged at 10000 rpm for 10 minutes at 4°C and crude extract was tested through agar well diffusion method against indicator bacterial lawn prepared on Muller Hinton agar.

Identification of active actinomycetesRMN6

Most potent *Actinomycetes* was identified on the basis of colony morphology, different pigmentation on ISP media, light microscopy at 100 X, and biochemical tests. Morphological characteristics of isolate RMN6 was determined as proposed in the Bergey's manual of determinative bacteriology and biochemical tests according to the methods [20].

Biochemical tests conducted were: formation of melanin pigments, reduction of nitrate, proteolytic properties, liquefaction of gelatin, hydrolysis of starch, assimilation of carbon, production of acid and production of H_2S.

Optimization of parameters

All batches were optimized in duplicates in orbital shaker incubator. 250 ml Starch casein broth media was prepared in the 500 ml flasks and sterilized. 10% inoculum was prepared in sterilized starch casein broth media in 250 ml flasks. Production media was inoculated and placed in the orbital shaker at 28 °C for one week. Samples were taken at every 24 hours interval, centrifuged, pellets was discarded and supernatant had been used against indicator strains at the range of 80 µL per well. Various parameters optimized for maximum antibiotic productions were: pH, temperature, inoculum size, incubation period, D-glucose concentration and speed of orbital inrpm. Different values selected for different parameters were: pH, 5, 6, 7, 8 and 9,Temperature 27, 28, 29, 31, 32 and 37°C; Inoculum size 5%, 7.5%, 10%, 12.5%, 15% and 17.5%; Incubation period 24, 48, 72, 96, 120, 144,168 and192 hours. For speed of orbital shaking incubator 100, 120, 140, 160, 180, 200 rpm were optimized. All these parameters were adjusted at mentioned values for batch fermentation; samples were taken centrifuged at 4 C^0 and 10000 rpm for 10 minutes. Antibacterial potentials at different parameters were checked through agar well diffusion assay against *S. aureus* (MRSA) and *M. leuteus* ATCC 10240.

Results

Isolated Actinomycetes

A number of actinomycetes were isolated from these unexplored areas of Khyber Pakhtunkhwa. Out of 43 isolated actinomycetes, 14 were active against some Gram positive and some Gram negative pathogenic bacteria in secondary screening. Active actinomycetes were 55% different colony morphology from each isolates it means that isolated bioactive actinomycetes were from diverse groups of filamentous bacteria.

Isolation of Human pathogenic bacteria

Total 30 bacterial pathogens were isolated from blood, urine, pus and sputum samples of hospitalized patients. The isolated pathogens were *S.aureus, P.aeroginosa, E.coli, S.typhi, Enterococcus faecalus* and *K. pneumonia*.

Biochemical identification of bacterial pathogens

Biochemical tests were performed for all isolated pathogenic strains after Gram staining and morphological characteristics. Clear results were observed which it make feasible to identify the pathogenic strains up to species level (Table 1).

Disc diffusion assay

Table 2 is showing the resistance pattern of *E. coli, S. aureus, P. aeruginosa* and *S.typhi* towards different antibiotic. *E. coli* was resistant to Cep, Cip, Ceph, Co, Pi, Na, Ce, Cef, Cefu, Of, Sp, Le, No while *S. Aureus* was resistant to Pe, Co, Ge,Me, Im, Ce, Cip, Of, Kl, Ox, Cef, Ce, Le, Sp, Fu, Cot and Ch.*P. aeruginosa* was resistant to Ge, Ceph, Cept, Ce, Cefu,Cefi, Im and *S.typhi*was resistant to Amo, Ge, Cep, Cefu and Cot.

Primary screening

Screening of potent producers of antimicrobial compounds was pointed out through cross streak agar method against all isolated human pathogenic bacteria. Twenty oneactinomycetes were found active against at least one indicator strain which indicated that 48.8% isolated strains of actinomycetes had bioactive potential against human bacterial pathogens.Some producing strains were found active against all pathogenic strains while some have narrow spectrum activity against only Gram positive or negative isolated pathogens.

Secondary screening

Primary screened antibiotic producers were confirmed through agar well diffusion assay. Excellent results were observed against selected indicator ATCC and isolated selected human pathogenic strains. Inhibitory activity towards ATCC bacterial culture and

human pathogenic bacteria was at the range of 9 to 25 mm. RMN 4 isolate was active against only Gram positive bacterial strain, while RMN6 have most potent activity against all bacterial indicator strains which was in the range of 13 to 25 mm zone of inhibition against *P. aeroginosa* ATCC 27553and *M. luteus* ATCC 10240, While against pathogenic bacterial isolates 13, 15, 18 and 23 mm zone of inhibition was recorded against *S. typhi, E. coli, P. aeroginosa* and *S. aureus* respectively. RMN7 have bioactive potential against *M. leuteus* ATCC 10240 and *S. typhi*(clinical isolate) showing 11 and 10 mm zone of inhibition. Actinomycetes RMN 8 have broad spectrum activity against *E. coli* ATCC 25922 and *S. aureus*with 11 and 19 mm zone of inhibition (Table 3).

Identification of selected actinomycetes RMN6

Morphological characteristics of RMN6 revealed filamentous growth on ISP 5 to ISP 7 media, pigmentation was also observed on ISP 6 media. Microscopic observations at 100X magnification, after Gram staining, revealed that strain RMN6 was rod shaped, Gram positive. Colony morphology, growth of aerial hyphae and spore development showed similar characteristics with Streptomyces. RMN6 was designated as Streptomyces RMN6 in accordance with *Bergey's Manual of Systematic Bacteriology* [21]. Biochemical results of RMN6 revealed brown pigments on the prepared slants of Waksman media for 96 hours incubation at 28^{o}C.Nitrate reduction testwas positive in organic nitrate broth and red pink color was observed when Naphthalene solution and sulphonic acid solution was added after one week incubation period at 28^{o}C. For proteolytic activity pasteurized skimmed milk was utilized by actinomycetes RMN6 and acidic reaction was observed.RMN6 was positive for starch hydrolysis by the action of amylolytic enzymes.Nutrient gelatin medium inoculated through stab of isolate and incubated for two weeks at 28^{o}C nutrient gelatin medium was liquefied by RMN6 by extracellular enzyme secretion indicate positive results. Carbohydrate utilization agar ISP 9 was prepared along with bromocresole dye, inoculated with 1 ml RMN6 in the sterile Petri plates. Discs having 3% of carbon sources were starch, dextrose, sucrose, maltose andlactose,were placed on the medium surface and incubated at 28^{o}C for one week. Purple yellow colors around the disc of lactose indicate positive result for the utilization of lactose in the presence of oxygen. Inoculated glucose nutrient broth media incubated for two weeks at 28^{o}C revealed blue to yellow color production positive for acid production. Prepared slants of hydrogen sulfide production media was inoculated and incubated for one week at 37^{o}C. Rotten egg smell and medium color changed to black indicate positive result. Biochemical results of selected actinomycte revealed that it belongs to streptomyces and designated as streptomyces RMN6 (Table 4).

Parameters optimization studies

pH and Incubation Temperature

The pH effects the growth and production of secondary metabolites during batch fermentation process. (Jain P, Pundir RK 2011). The effect of different pH was studied at 5, 6, 7, 8 and 9 (Table 5). The effect of pH from 5 to 9 revealed that the antimicrobial activity increased and attained maximum with gradual increase of pH from 6 to 8. No activity was observed at pH 5 against both indicator strains. Further increase of pH to 9, antibacterial activity decreases against indicator strains. Best activity was observed at pH 8.Initial incubation temperature ranges was adjusted from 25 to 37 ^{o}C. Antibacterial activities increased from 25 to 29 ^{o}C and then decreases up to 37 ^{o}C. Optimum activity was observed at 29 ^{o}C (Table 5).

Glucose concentration and incubation period

The glucose concentration from 1 to 6% was investigated for maximum production of antibacterial metabolites. Each concentration of glucose was added to the production medium for maximum production of metabolites. Results indicate that 3% glucose concentration was optimum for maximum production of metabolites and exceeding the 3% glucose concentration decreases the antimicrobial metabolite production. The batch fermentation was carried out for 8 days. Initially no activity was observed for 24 hours of incubation period. The activity was recorded after 48 to 192 hours of incubation. Maximum antibacterial activity was observed after 144 hours of incubation (Table 6). Decrease in antimicrobial activity after 144 hours of incubation might be due to the depletion of nutrients and accumulation of toxic nitrogenous waste products in the medium.

Inoculum size and speed of shaking incubator

Inoculum size percentage volume/volume was 5 to 17.5% inoculum. The difference between two ranges of inoculums was 2.5%. Inoculum volume of 7.5% was the best inoculum size for the maximum growth and antibacterial activity for RMN6 against two indicator strains. Speed of orbital shaking incubator was adjusted at 100 to 200 revolutions per minute (rpm). Best results were noted at 160 rpm (Table 7).

Optimization parameters adjusted at optimized values revealed that selected actinomycetes isolate Streptomyces RMN6 have maximum antimicrobial activity against *M. luteus* ATCC 10240 (32 mm) and *S. aureus* MRSA (28 mm) (Table 8).

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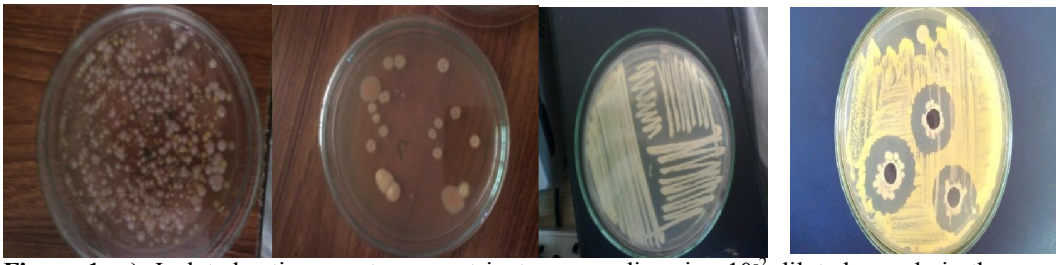

Figure 1: a). Isolated actinomycetes on nutrient agar media using 10^{-2} diluted sample in the presence of antifungal and antibacterial compounds. b). Isolated actinomycetes on actinomycetes agar media using 10^{-5} diluted soil sample in the presence of antibacterial and antifungal compounds. c). Pure culture of selected actinomycetes RMN6 on nutrient agar media d). Antibacterial activities of Isolate RMN 6 actinomycte tested against *M. luteus*ATTC#10240 through agar well diffusion assay.

Table 1. Biochemical Tests for Identification of Pathogenic Bacterial Strains Used in the Primary Screening and Secondary Screening of Bioactive Actinomycetes.

S#	Pathogens	Ur	Vp	Nopg	Lac	Glu	Suc	Man	Ox	Cit	Ind	Ldc	H_2S
1	*E. coli*	-	-	+	+	+	+	+	-	-	+	+	-
2	*S. typhi*	-	-	-	-	+	-	+	-	-	-	+	+
3	*K. pneumonia*	+	+	+	+	+	+	+	-	+	+	+	-
4	*S. aureus*	nt	+	nt	nt	+	+	+	nt	nt	+	nt	+
5	*p. aureginosa*	nt	nt	nt	nt	nt	nt	nt	+	nt	+	nt	+
6	*E. faecalus*	-	+	+	+	+	+	+	+	+	+	+	+

Key: Ur: Urease, Vp: Vogesprokauser, Nopg: Beta galactosidase, Lac: Lactose, Glu: Glucose, Suc: Sucrose, Man: Mannitol, Ox: Oxidase, Cit: Citrate, Ind: Indole, Ldc: Lysine decarboxylase, H_2S = Hydrogen sulphide, nt: Not tested.

Table 2. Resistance Pattern of Selected Isolated Pathogenic Bacterial Strains Towards Antibiotics.

S#	Clinical Isolate	Resistance pattern
1	*E. coli*	Cep, Cip, Ceph, Co,Pi,Na, Ce, Cef, Cefu, Of, Sp, Le, No
2	*S. aureus (MRSA)*	Pe, Co, Ge,Me, Im, Ce, Cip, Of, Kl, Ox, Cef, Ce, Le, Sp, Fu, Cot, Ch
3	*P. aeroginosa*	Ge, Ceph, Cept, Ce, Cefu,Cefi, Im
4	*S. typhi*	Amo, Ge, Cep, Cefu, Cot

Key: Cep = Cephradine, Cip = Ciprofloxacin, Ceph = Cephtriaxone, Co = Coamoxiclave, Pi = Pipemedic acid, Na = Nalidixic Acid, Ce = Cefotaxime, Cef = Ceftazidime, Cefu = Cefuroxime, Of = Ofloxacin, Sp = Sparfloxacin, Le = Levofloxacin, No = Norfloxacin, Pe = Pencillin G, Ge = Gentamicin, Me = Meropenem, Im = Imipenem, Kl = Klarithromycin, Ox = Oxacillin, Fu = Fucidic Acid, Cot = Cotrimaxazole, Ch = Chloramphenicol, Cept = Cephtriaxone, Cefu = Cefuroxime, Cefi = Cefipime, Am = Amikacin, Amo = Amoxicillin.

Table 3. Antimicrobial Activity of Primarily Screened Active Actinomycetes Against Various Bacteria by Agar Well Diffusion Method, Secondary screening.

Activity against *ATTC* culture						Activity against human pathogens				
Test bacteria zone of inhibition in mm										
Code of isolates	*E.coli 25922*	*P.aeroginosa 27553*	*S.aureus 25923*	*K.pneumonia* 700603	*E.faecalus* 29212	*M.luteus 10240*	*P.aeroginosa MDR*	*S.aureus MRSA*	*E.coli MDR*	*S.typhi MDR*
RMN1	0	0	14	10	11	15	0	13	0	0
RMN2	10	12	17	16	10	19	14	15	0	13
RMN3	0	0	12	0	0	0	10	10	0	0
RMN4	0	0	15	0	0	13	0	0	0	0
RMN5	13	0	0	0	0	0	0	0	0	0
RMN6	14	13	19	16	11	25	18	23	15	13
RMN7	0	0	0	0	0	11	0	0	0	10
RMN8	11	10	15	13	9	16	12	19	10	0
RMN9	0	0	12	0	0	17	13	14	12	13
RMN10	11	9	13	14	0	18	12	11	0	11
RMN11	0	0	0	11	12	11	0	0	0	0
RMN12	0	0	14	12	0	18	0	0	0	0
RMN13	0	0	12	0	0	13	0	0	0	0
RMN14	10	11	18	10	9	15	13	0	0	0

Table 4. Biochemical Characteristics of Isolate RMN6.

Isolated Code	Biochemical Tests	Characteristics
RMN6	Melanin production	Brown pigment
	Nitrate Reduction	+
	Proteolytic properties	Acidic reaction
	Liquefication of Gelatin	+
	Hydrolysis of Starch	+
	Carbon assimilation	Lactose
	Acid Production	+
	H_2S Production	+

Table 5. Optimization of Temperature and pH

Parameter ranges for temperature optimization	Zone of inhibition in (mm)		Parameter ranges for pH optimization	Zone of inhibition in (mm)	
Temperature (^oC)	*S. aureus MRSA*	*M. luteus 10240*	pH	*S. aureus MRSA*	*M. luteus 10240*
25	**15**	**21**	**5**	**0**	**0**
29	**25**	**29**	**6**	**12**	**13**
31	**22**	**24**	**7**	**18**	**21**
34	**14**	**20**	**8**	**23**	**27**
37	**0**	**13**	**9**	**20**	**26**

Table 6. Optimization of D-glucose Concentration and Incubation Period

Parameter ranges for glucose concentration optimization	Zone of inhibition in (mm)		Parameter ranges for incubation time optimization	Zone of inhibition in (mm)	
D-glucose concentration (%)	*S. aureus* MRSA	*M. luteus* 10240	Incubation time	*S. aureus* MRSA	*M. luteus* 10240
1	20	21	24	0	0
2	22	24	48	0	11
3	**23**	**27**	72	13	15
4	21	23	96	16	17
5	18	22	120	20	22
6	13	15	**144**	**23**	**25**
			168	22	23
			192	19	21

Table 7. Optimization of Inoculum Size and Speed of Shaking Incubator.

Parameter ranges for optimization of size of inoculum	Zone of inhibition in (mm)		Parameter ranges for optimization of rpm in shaking incubator	Zone of inhibition in (mm)	
Size of inoculums (%)	*S.aureus MRSA*	*M. luteus 10240*	Speed of shaking incubator in rpm	*S.aureus MRSA*	*M. luteus 10240*
5	25	26	100	22	24
7.5	**27**	**29**	120	23	26
10	23	27	140	25	27
12.5	22	24	**160**	**26**	**29**
15	22	23	180	24	28
17.5	20	21	200	24	26

Table8.Antimicrobial Activity of RMN6 at Optimized Parameters against *M.luteus* and *S.aureus*

Indicator strains	pH (8)	Temperature (29 °C)	Inoculum size (7.5%)	Speed of shaker (160rpm)	Incubation period (144 hours)	D-Glucose concentration (3%)
Micrococcus luteus ATCC 10240	**32 mm**					
Staphylococcus aureus (MRSA)	**28 mm**					

Discussion

Due to the lake of education and health regulations for sale of antibiotics, without prescription, antibiotic resistance increases especially in Khyber Pakhtunkhwa at alarming rate. In the last few decades several unexplored habitats in the world for isolation of bioactive compounds producing microorganisms were extensively screened[22]. A study conducted in Baida area of Jordan, Streptomyces strains isolated from soil were active against antibiotic resistant bacteria [23].Selection of unexplored diverse range of habitats in KPK for soil sampling was targeted to isolate actinomycetes flora active against wide range of indicator bacterial strains. Target objective was achieved by successful isolation of 14 bioactive compounds producing actinomycetes active against some Gram positive and negative antibiotic resistant bacteria and ATCC bacterial strains. Research investigations carried out in our study also correlates with another study conducted in Turkey in which actinomycetes were isolated from 11 soil samples collected from terrestrial environment. Isolated Streptomyces had antibacterial activity against 7 indicator bacterial strains including multi drugs resistant bacteria. Five isolated Streptomyces had potent activities against *Methicillin Resistant Staphylococcus aureus* [24].

At optimized condition, 28 and 32 mm zones of inhibition was noted against indicator bacterial strains. Similar optimum range of pH was reported by (Ripa1 F. A*, et al* 2009), for the antimicrobial activity by *Streptomyces sp.* RUPA-08PR isolated from soil. The results of this study also nearly correlates with another study for optimized parameters conducted for Amycolatopsis Alba var. nov. DVR D4 specie in which the optimized conditions were: 2% D- glucose concentration, 4% malt extract, 5% inoculum size, 28 °C incubation temperature, 220 rpm of orbital shaker speed and 96 hours incubation period for maximum antimicrobial activities [16].

Conclusion

RMN6, a *Streptomyces,* showed maximum activity at pH 8, 144 hours incubation period, 29°C, 7.5% inoculum size, 3% glucose concentration and 160 rpm speed of shaking. Maximum activity was recorded against *M.luteus* ATCC 10240 and *S. aureus* (MRSA) clinical isolate as 32 and 28 mm respectively.

Competing interests:

The authors has no competing interests.

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