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Isolation, identification and optimization of fermentation parameters for improved production of antimicrobial compounds from indigenous *Streptomyces* isolates

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In order to find out new antimicrobial compounds producing Stretomycetes from indigneous soil, twenty five *Streptomyces* isolates were purified from hundred soil samples and screened for their antimicrobial potential. Among these isolates, four (SK-1, SK-3, SK-4 and SK-5) were selected for further studies on the basis of antimicrobial compounds produced by them. Morphological, cultural, physiological and biochemical characterization revealed that these isolates belonged to the *Streptomyces* species. Moreover, 16S ribosomal RNA gene analysis confirmed that these isolates showed 99% identity to *Streptomyces variabilis*, *Streptomyces flavomacrosporus*, *Streptomyces levis* and *Streptomyces griseostramineus*, respectively. The antimicrobial compounds produced by SK-1, SK-3 and SK-4 isolates exhibited strong antibacterial activities against *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa and Rhodococcus facians.* While, isolate SK-5 showed strong antifungal activities against *Trichoderma* and *Aspergillus* species. The optimum inoculum size (v/v %), temperature and pH for antimicrobial metabolite production by *Streptomyces* isolates (SK-1, SK-3, SK-4 and SK-5) were found to be 10%, 28-34°C and 8.0-9.0, respectively. Casein starch medium supplemented with 1% starch and 0.2% sodium glutamate proved to be the best carbon and nitrogen sources for the production of antibacterial compounds by isolates SK-1, SK-3 and SK-4. While isolate SK-5 gave maximum antifungal activities when 1% glycerol was used in the fermentation medium along with 0.2% tryptone as nitrogen source.

Key words: Antimicrobial compounds, *Streptomyces*, fermentation, phylogenetic analysis.

INTRODUCTION

There is disquieting shortage of new antibiotics currently under development in the pharaceutical industy (Zhao et al., 2009). Moreover, infections diseases due to bacteria that have deveoped resistance to commonly used antibiotics is the major global healthcare problem of the 21st century (Alanis, 2005). Increasing number of antibiotic-unresponsive infectious diseases, face up to patients world wide (Livermore, 2003). The consensus has been evolved that it is essential to develop novel classes of antibiotic as part of the strategy to overcome the growing nuber of drug resistant pathogens (Abbanat et al., 2003). So, the exploration of new antibiotics effective against drug resistant pathogens is presently an important area of research.

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Still natural microbial products remain the most promising source of novel antibiotics having noval structures, although new approaches are required to improve the efficiency of the discovery process (Thumar et al., 2010). Soil is an extensively exploited ecological nich in which the inhabitants produce many useful biologically active natural products, including clinically important antibiotics (Dancer, 2004). These investigation have been extremely successful and about two-third of naturally occuring antibiotics have been explored from actinomycetes (Olano et al., 2009). Actinomycetes occupied a large proportion of the soil microbial biomass. *Streptomyces* represents an important genera of actinomycetes and comprises the largest number of species and varieties, which differ significantly in their morphology, physiology and biochemical properties. Mainly, the antibiotic-producing actinomycetes are present amongst these *Streptomyces* (Reddy et al., 2011; Panjiar et al., 2013). The species of genus *Streptomyces* constitute 50% of the total soil actinomycetes and about 70-80% of commercially and medicinally valuable antibiotics have been obtained from this genus (Mellouli et al., 2003; Procopio et al., 2012). This group of microorganisms still remains a useful source of antibiotics (Watve et al., 2001), because they are readily biodegradable, specific and generally have low toxicity (Newman et al., 2003). Furthermore, over 6000 antibiotics are obtained by different species of *Streptomyces* and many of these compounds are commercially available as anti-infective (antibiotics, antifungal and antiparasitic), anticancer or immunosuppressant agents (Iznaga et al., 2004). Actinomycete-derived antibiotics constituted around 30% of total antibiotic sales. In a report published in December 2009 (The World Antibacterial Treatments Market 2010-2024) (Vision, 2009), the total annual sales value of the global anti-infectives market was estimated at $79 billion. Of this total, the anti-bacterial market accounted for $37 billion. Applying the 30% market share, actinomycete-derived antibiotics would account for revenues of approximately 11 billion. If *Streptomyces* research supports the development of a substantial number of new anti-bacterial products, new revenue streams will be created in this market. Even a very conservative increase of 1% would lead to additional sales revenue potential of $370 million (£247 million10) per year as a result of *Streptomyces* research.

Moreover, for the production of these antibiotics, different types of fermentation conditions are used. The type and amount of certain components in fermentation media have remarkable effects on the production of antibiotic. The effect of particular nutrients on biosynthesis of antibiotic can be determined by the chemical nature of antibiotic substances (Oskay, 2009). Growth of microorganism is rapidly reduced and antibiotic biosynthesis takes place in the stationary phase, when carbon or nitrogen source is a limiting factor. While, in other cases, antibiotic production may be growth associated and maximum production can be attain in log phase (Osman et al., 2011). The objective of the present investigation was to screen soil samples collected from diverse range of environments which remained unscreened previously, for the isolation of potent and broad-spectrum antibiotic-producing *Streptomyces* isolates against various pathogenic bacteria and fungi. Moreover, the identification, characterization and optimization of fermentation parameters were also carried out to get maximum production of these compounds.

MATERIALS AND METHODS

Isolation of microorganisms

Around 100 soil samples were collected from a variety of diverse habitats for the isolation of *Streptomyces*. These habitats consisted of plants rhizosphere and agricultural soil. After removing approximately 3 cm of the soil surface, the samples were taken up to a depth of 20 cm. Isolation and screening of *Streptomyces* was carried out according to Morakchi et al. (2009).

The collected soil samples were sieved to remove unwanted materials. All the soil samples were treated with $CaCO_3$ (10:1 w/w) and incubated at 37°C for 5 days. They were then perched in sterile distilled water. Test tubes containing a 10^{-2} dilution of the samples were kept in a water bath at 45°C for 16 hrs to detach the spores from vegetative cells. These dilutions were spread on the surface of the starch casein medium. The composition of this medium was (g/l): starch 10.0, casein 0.3, KNO_3 2.0, NaCl 2.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and agar 18.0, which was supplemented with cyclohexamide (50 µg/ml) and nystatin (25 µg/ml) to suppress the growth of fungus. The plates were incubated at 28°C until the appearance of *Streptomyces* colonies. Twenty five colonies of *Streptomyces* were then purified, transferred to starch casein nitrate agar slants and preserved at 4°C. The isolates (25) were maintained as spore suspensions and mycelia fragments in 20% v/v glycerol at -70°C.

Antimicrobial activity

Primary screening was carried out using the modified method of Kirby Bauer antibiotic susceptibility test (Bauer et al., 1966; Singh et al., 2012). Antimicrobial activity was determined on yeast extract-malt extract agar media inoculated with test organisms. The test organisms included different bacteria (*Bacillus subtilis*, one drug (chloramphenicol, amoxicillin and trimethoprim) resistant strain of *Salmonella typhi* and one drug (azteronam, amoxicillin, ofloxacin and streptomycin) resistant strain of *Escherichia coli*, *Pseudomonas aeruginosa*, *Rhodococcus facians*) and fungi (*Trichoderma* and *Aspergillus species*). The *Streptomyces* isolates were lawn-cultured by dense streaking on starch casein nitrate medium plates and incubated at 30°C for seven days. The 6 mm agar discs were prepared using sterile cork borer from well grown cultures and placed on fresh lawn culture of test organisms. The plates were then kept at 4°C for 30 min for the diffusion of the culture broth, and then incubated at their respective optimum temperature (37°C for bacteria and 30°C for the fungus). The zones of inhibition for bacteria were determined after 18-24 h and for fungi after 3 days. Four isolates which showed broad spectrum activity against test organisms in primary screening were subjected to secondary screening by agar well diffusion method (Shahrokhi et al., 2005). Erlenmeyer flasks (250 ml) containing 50 ml of starch casein nitrate

broth were inoculated with 7 days old culture and incubated at 28°C for 5 days at 180 rpm. The culture broth was centrifuged at 4°C and 10000 rpm and the activity of the supernatant was determined against test organisms by adding 50 µl to wells (6 mm) bored into freshly inoculated plates. The plates were then kept at 4°C for 30 min for diffusion of the antibiotic. The plates were incubated at 37°C overnight for bacterial cultures and 3 days for fungal cultures. The formation of zone of inhibitions showed antimicrobial activity. Each test was repeated for three time and the activities were shown as the mean of diameter of the inhibition zone.

Morphological and cultural characteristics

Morphological and cultural characteristics of four potent isolates were studied in different media following the instructions given by the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966; Singh et al., 2012). Different media used were: Starch nitrate agar medium, tryptone yeast extract agar (ISP-1) (tryptone 5 g; yeast extract 3g; agar 20g; H_2O 1000 ml, pH 7.2), ISP-2 agar (yeast extract 4 g; malt extract 10g; glucose 4g; agar 20g; H_2O 1000 ml, pH 7.2), ISP-3 agar (meals 20g; $MnCl_2.4H_2O$ 0.1g; $FeSO_4.7H_2O$ 0.1g; $ZnSO_4.7H_2O$ 0.1g; agar 18 g, H_20 1000 ml, pH 7.2), ISP-4 agar (L-asparagine 1g; glycerol 10g; K_2HPO_4 1g; $MnCl_2$. $4H_2O$ 0.1g; $FeSO_4.7H_2O$ 0.1g; $ZnSO_4.7H2O$ 0.1g; agar 20g; H_2O 1000 ml, pH 7.2), ISP-5 (starch soluble 10g; K_2HPO_4 1g; $MgSO_4.7H_2O$ 1g; NaCl 1g; $(NH_4)_2SO_4$ 2g; $CaCO_3$ 2g; agar 20g; H_20 1000 ml, pH 7.2), ISP-6 (peptone yeast extract iron agar) and ISP-7 (tyrosine agar medium) at 28°C for 7 to 14 days (Shirling and Gottlieb, 1966).

Physiological and biochemical studies

Other characteristics like production of lecithinase were tested on egg-yolk medium (Nitsh and Kutzner, 1969). Lipase activity was determined by the method described by Elwan et al. (1977), pectinase (Hankin et al., 1971) and catalase activity was checked by the method of Jones (1949). Melanin pigment production was studied according to the method described by Pridham et al. (1957). Degradation of esculin and xanthine was tested by the method of Gordon et al. (1974). Nitrate reduction (Gordon, 1966), hydrogen sulphide production and oxidase test was also performed (Cowan and Steel, 1974). The cultural behaviors were studied according to the guidelines available in International *Streptomyces* Project (Shirling and Gottlieb, 1966). Colours characteristics of colonies, mycelia and pigments among others were studied by the method of Kenneth and Deane (1955). The use of different carbon and nitrogen sources by these isolates was investigated with the help of method described by Shirling and Gottlieb (1996).

Amplification and sequencing of 16S rRNA gene and phylogenetic analysis

The molecular identification of these isolates was carried out on the basis of sequence analysis of 16S rDNA. Polymerase chain reaction (PCR) amplification of 16S rRNA gene of these four potent antimicrobial compounds producing local isolates was conducted using two universal primers, forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (5′-ACGG(ACT)TACCTTGTTACGACTT-3′). The amplification of 16S rRNA gene by PCR was performed with DNA polymerase using 20ng of the template DNA, 0.4 µM of each primer, 0.3 mM each of deoxyribonucleotide triphosphate (dNTPs) mix (Invitrogen), 2.0 mM $MgCl_2$ and 10X NH_4 buffer with a final 1X concentration. Amplification was obtained with 1 cycle of 5 min of denaturation at 94°C, 30 cycles of 0.45 min of denaturation at 94°C, 0.45 min of annealing at 50°C and 1.30 min of extension at 72°C with a final additional extension for 10 min at 72°C. The resulting 16S rRNA gene fragment (1500bp) was sequenced partially by Macrogen, Korea. The sequences of 16S rRNA genes were compared with others sequences available in GenBank database using the NCBI BLAST (www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences of related organisms were taken from GenBank and aligned with the partial sequences of antimicrobial agents producing local *Streptomyces* isolates used in current study with CLUSTALX program (Thompson et al., 1997). The aligned sequences were used to make a distance matrix, after the generation of 100 bootstrap sets that was consequently used to build a phylogenetic tree, by neighbor-joining method in TREECON software.

Optimization of culture conditions for the production of antibiotics

The inocula of all the four isolates were prepared in enrichment medium containing (g/l) K_2HPO_4 0.5g; casein 3.0g; starch 10g; peptone 1.0g; yeast extract 1.0g and malt extract 10g. From different production media, starch casein medium with potassium nitrate was selected as basal medium for further optimization studies. To investigate the effect of various carbon and nitrogen sources, basal medium was supplemented with different types of carbon (starch, glycerol, lactose, fructose, maltose and glucose at 1% concentration) and nitrogen (potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, sodium glutamate, peptone, tryptone at 0.2% concentration) sources. The environmental conditions such as temperature (28-40°C), incubation period (1-10 days), initial pH of the medium (5.0-10.0) and size of inoculum (4-12% v/v) were also studied to get maximum production of these compounds. Maximum production was indicated by showing the diameter of zone of inhibition against *B. subtilis* for antibacterial producing isolates (SK-1, SK-3 and SK-4) and against *Aspergillus* species for antifungal producing isolates SK-5.

RESULTS

Isolation and screening

Twenty five pure colonies of *Streptomyces* were isolated from around hundred soil samples and screened for the significant antimicrobial activities against Gram's positive and Gram's negative bacteria and some fungi. Only 4 isolates (isolate SK-1, SK-3, SK-4 and SK-5) exhibited wide spectrum antimicrobial activity. First three isolates have shown good antibacterial activities against Gram's positive and Gram's negative bacteria including drug resistant *S. typhi* and *E. coli*. Isolate SK-5 was found to be very active against some indigenous fungal isolates of *Trichoderma* and *Aspergillus* species. Maximum antimicrobial activities by these isolates against all the test organisms are given in Table 1.

Morphological, cultural and biochemical characteristics

A great variety of morphological, cultural, physiological and biochemical characteristics of the four potent isolates were studied in different media reported for *Streptomyces*.

Table 1. Maximum antimicrobial activity of indigenous *Streptomyces* isolates.

Test organism	Zone of inhibition (mm)			
Isolates	SK-1	SK-3	SK-4	SK-5
Bacillus subtilis	38±1.00	36±0.96	34±1.00	00±0.00
E. coli	28±0.75	22±0.52	20±0.53	00±0.00
Rhodococcus faciens	30±0.80	30±0.92	28± 0.52	00±0.00
Salmonella typhi	28±0.55	20±0.67	00±0.00	00±0.00
Pseudomonas aeroginosa	25±0.50	22±0.53	20±0.50	00±0.00
Aspergillus species	00±0.00	00±0.00	00±0.00	48±1.55
Aspergillus species	00±0.00	00±0.00	00±0.00	48±1.55
Trichoderma species	00±0.00	00±0.00	00±0.00	40±1.30

Mean ± standard deviation.

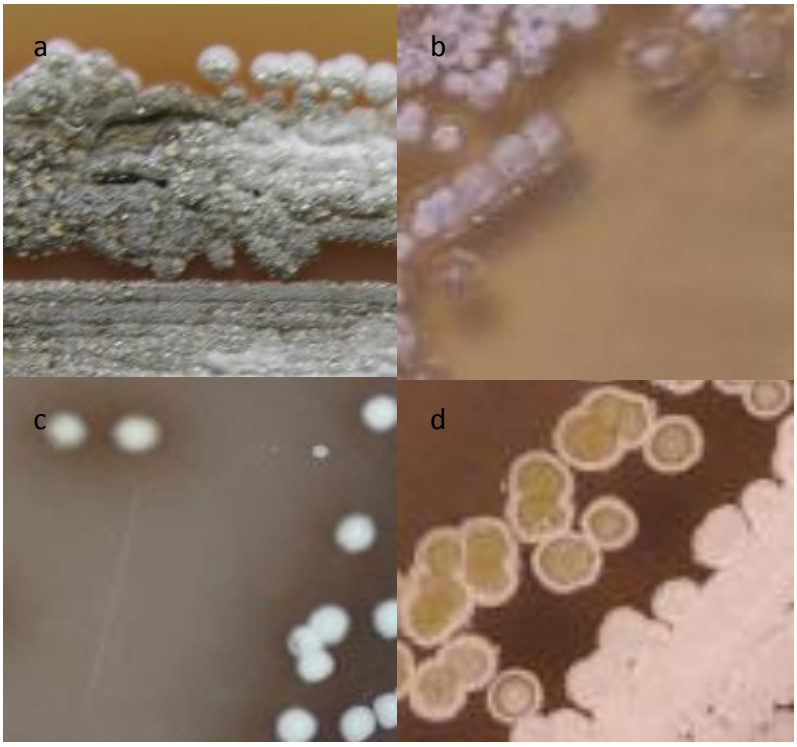

Figure 1. Colony morphology and pigment production of antimicrobial compounds producing indigenous isolates of *Streptomyces*, a (SK-1), b (SK-3), c (SK-4) and d (SK-5) on starch casein nitrate agar.

All the four isolates were identified as members of *Streptomyces* species on the basis of these characteristics. Most favourable growth and pigment production by all the isolates were observed in starch casien nitrate agar medium (Figure 1). Therefore, different morphological and cutural chracteristics of these isolates including growth, aerial and substrate mycelium colour and formation of soluble pigments on starch casein agar medium are presented in Table 2. All of these isolates were Gram's positive filamentous bacteria with extensively branched aerial mycelia, aerobic, mesophilic and grew readily on most of the nutrient media reported for *Streptomyces* (Sembiring and Goodfellow, 2008). Colonies of isolate SK-1 and SK-4 have grey and white aerial mycelium and brown substrate mycelium with the production of brown soluble pigment on agar plates. Isolate SK-3 has pink aerial and light pink substrate mycelium showing the production of light pink soluble pigment. The antifungal compound producing isolate SK-5 exhibited white aerial and yellow substrate

Table 2. The morphological and cultural characteristics of antimicrobial compounds producing indigenous *Streptomyces* isolates.

Characteristic	Isolate			
Morphological (starch nitrate agar medium	SK-1	SK-3	SK-4	SK-5
Vegetative Growth	+++	+++	+++	+++
Aerial Mycelium	Grey	Pink	White	White
Substrate Mycelium	Brown	Light pink	Brown	Yellow
Soluble pigment	Brown	Light pink	Dark brown	Yellow
Motility	Non-motile	Non-motile	Non-motile	Non-motile
Cultural	Growth			
Tryptone yeast extract agar medium (ISP-1)	++	+++	++	+
Glycerol asparagines agar medium (ISP-4)	++	++	++	+++
Yeast extract-malt extract agar medium (ISP-2)	+++	++	+++	++
Oatmeal agar medium (ISP-3)	++	++	+	+++
Inorganic salts starch agar medium (ISP-5)	+++	++	+++	+
Peptone yeast extract iron agar (ISP-6)	+++	+++	++	++
Tyrosin agar medium (ISP-7)	+	++	++	+++

+++, Good; ++, medium; +, weak; - negative.

mycelium. It produced soluble yellow pigment on starch casein agar which was associated with its antifungal activity. These isolates produced substrate based extracellular enzymes such as lecithinase, xylanase and amylase. Two of these isolates SK-4 and SK-5 reduced nitrates. H_2S was also produced by isolates SK-1 and SK-4 whereas elastin and xanthine were degraded by all the isolates. Temperature for growth of these isolates ranged from 20 to 40°C and an optimal temperature was found between 28 to 34°C. Other phenotypic and biochemical characteristics are given in Table 3. These characteristics strongly suggested that these isolates belonged to the genus *Streptomyces*. It has been indicated that all of these isolates could utilize a wide range of carbon source such as xylose, glucose, glactose, arabinose, lactose, maltose, fructose, sucrose, starch and glycerol with slight variation in growth. Some differences also occurred in the utilization of different amino acid sources including valine, histidine, lysine, arginine, serine and tyrosine among others (Table 4).

Phylogenetic analysis

The identification of these antimicrobial agents producing local isolates was also performed using 16S rRNA gene sequence analysis. These sequences were submitted to GenBank and accession numbers were obtained. Phylogenetic tree was constructed to predict the species level characterization of the studied isolates and their relation with some other already reported *Streptomyces* species by neighbour-joining method, using TREECON software (Figure 2). Partial sequence profiles of the closely related microbial strains and other antibiotic producing strains have been compared with new isolates to establish the novelty of these antibiotic producing isolates. Sequence comparison study of isolate SK-1, SK-3, SK-4 and SK-5 showed 99% identity with 16S rRNA gene sequences of *Streptmyces variabili*s, *Streptomyces flavomacrosporus*, *Streptomyces levis* and *Streptomyces griseostramineus*, respectively. It is evident from the phylogenetic tree that isolates SK-1, SK-3 and SK-5 formed a separate branch (a branch well supported by bootstrapping) far away from the *Streptomyces variabilis*, *S. flavomacrosporus* and *S. griseostramineus* respectively for which they showed 99% 16S rRNA gene sequence identity in BLAST N. Nevertheless, SK-4 formed a separate branch in the phylogenetic tree however; it was close to the *Streptomyces levis*. It is clear from the phylogenetic tree that these isolates are not only forming a separate branch but, are also far away from the already reported antibiotic producing *Streptomyces* strains like *Streptomyces rimosus*, *Streptomyces fradiae*, *Streptomyces kanamceticus*, *Streptomyces hygroscopicus and Streptomyces aureofaciens*. Such differences impact the novelty of these isolates from the previously reported antimicrobial producing strains of *Streptomyces*.

Optimization of cultural conditions for maximum antibiotic production

Cultural characteristics and media components were optimized by using various carbon and nitrogen sources. All the isolates were subjected to grow on different media

Table 3. The physiological and biochemical characteristics of antimicrobial compounds producing indigenous *Streptomyces* isolates.

Characteristic	Isolate			
	SK-1	SK-3	SK-4	SK-5
Hydrolysis				
Starch	+++	++	+++	+++
Xylane	+++	++	++	++
Catalase test	+++	++	+++	++
Production of melanin				
Tryptone yeast extract agar medium (ISP-1)	++	+++	+++	+++
Yeast extract-malt extract agar medium (ISP-2)	+++	++	++	-
Oatmeal agar medium (ISP-3)	++	++	++	-
Glycerol asparagines agar medium (ISP-4)	+++	++	++	+++
Inorganic salts starch agar medium (ISP-5)	++	+++	+++	+
Peptone yeast extract iron agar (ISP-6)	+	-	++	+++
Tyrosin agar medium (ISP-7)	-	-	++	+
Degradation				
Lecithin	++	++	+++	++
Esculin	++	++	+++	++
Xanthin	+++	+++	++	+++
Nitrate reduction	-	-	++	++
H2S production	++	-	+++	-
NaCl tolerance	+++	++	+++	++
Urea test	++	+	++	+++
Growth temperature	28°C	28°C	30°C	34°C
Optimum pH ranges	8-9	8-9	8-9	8-9

+++, Good; ++, medium; +, weak; - negative.

Table 4. Utilization of carbon and amino acid sources by antimicrobial compounds producing indigenous *Streptomyces* isolates.

Utilization	Isolate			
	SK-1	SK-3	SK-4	SK-5
Carbon source				
D-Xylose	+++	++	++	++
D-Glucose	++	++	+++	++
D-Glactose	+++	++	++	+
L-Arabinose	+	-	-	-
Lactose	+++	++	++	++
Maltose	+++	++	++	++
D-fructose	+	++	+	++
Sucrose	-	+	-	-
Starch	+++	+++	+++	++
Glycerol	++	++	++	+++
Amino acids				
L-Valine	++	++	+++	+
L-Histidine	+	+	+	-

Table 4. Continued.

L-Lysine	+++	++	++	++
L-Arginine	++	++	+	+++
L-Serine	+	++	+	+++
L-Tyrosine	-	+	+	+

+++, Good; ++, medium; +, weak; - negative.

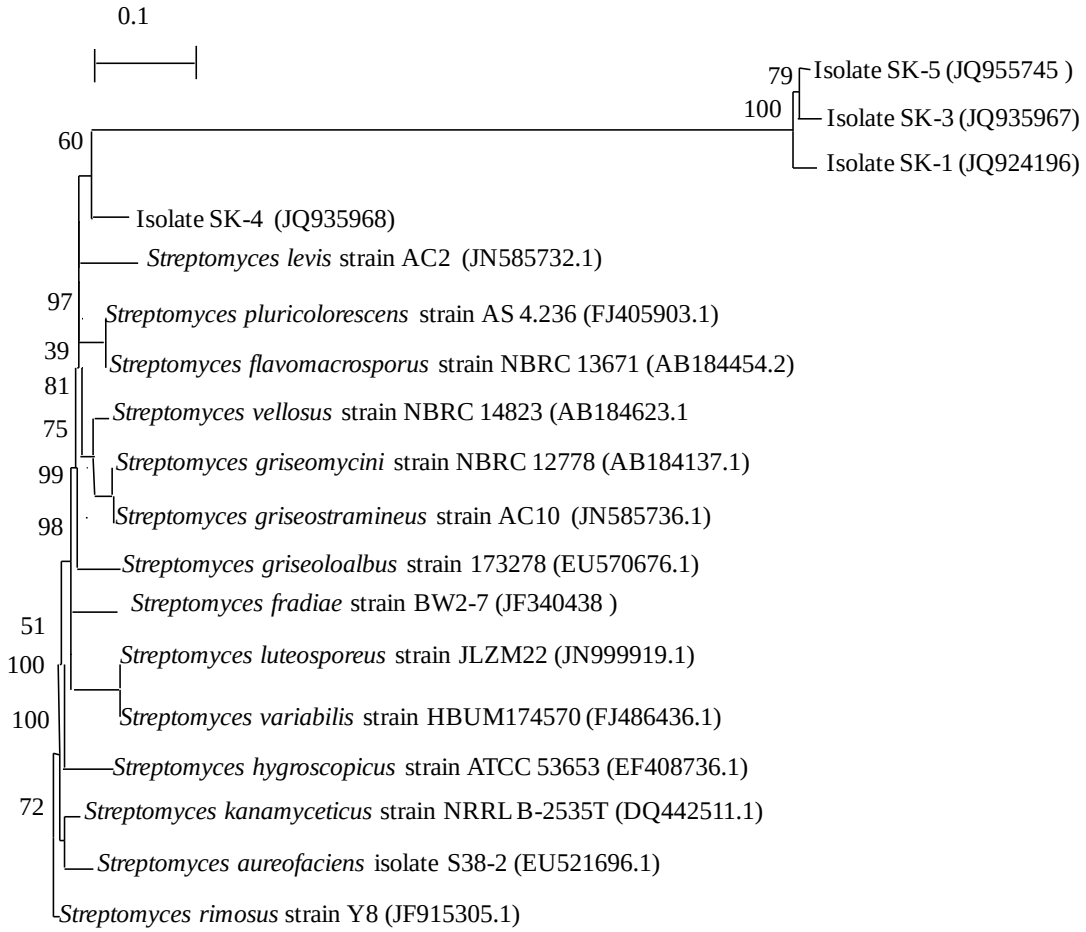

Figure 2. Phylogenetic tree obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of indigenous *Streptomyces* isolates producing antibacterial (SK-1, SK-3 & SK-4) and antifungal (SK-5) compounds, among their phylogenetic neighbors. Numbers at branch nodes are bootstrap values, expressed as percentages of 100 replicates (only values >50% are shown). Bar, 0.1 substitutions per nucleotide position.

as per International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). The culturing characteristics of all potent isolates are given in Table 3. All the potent *Streptomyces* isolates have shown excellent growth when grown onto the starch nitrate medium and also produced high amount of antimicrobial metabolites as measured by the zones of inhibition (Table 1). Therefore, this medium was selected to optimize the other fermentation parameters like incubation period, inoculum size, temperature, pH, media nutrients (carbon and nitrogen sources) etc.

Incubation period

The effect of incubation period on the production of

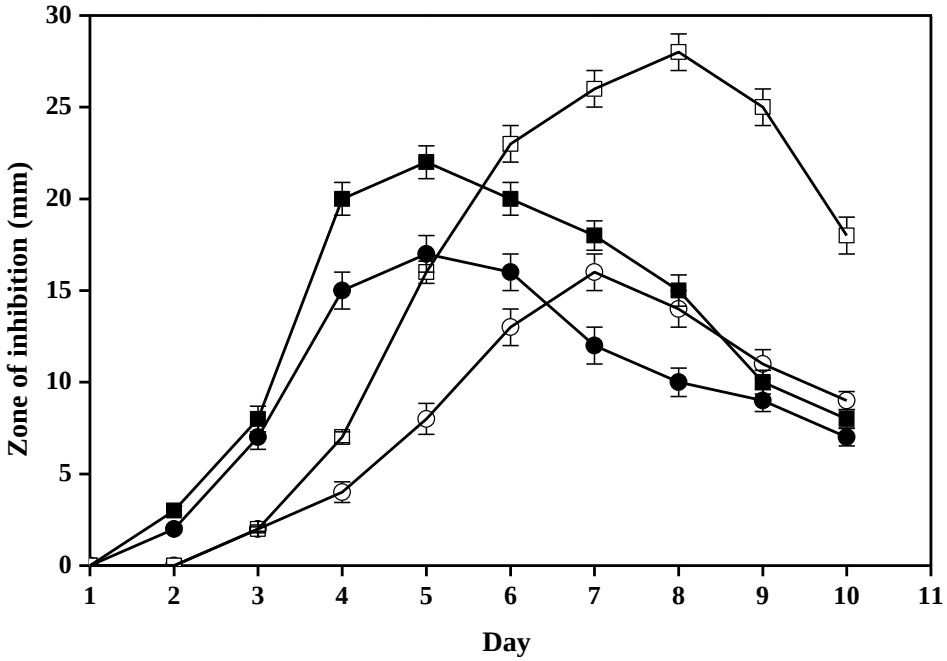

Figure 3. The effect of incubation period on production of antibacterial compounds by indigenous *Streptomyces* isolates SK-1(■), SK-3(●), SK-4(⊄) and antifungal compound by SK-5(ϒ).

antimicrobial compounds by these isolates was studied using 5% inoculum size, initial pH of the medium 7.2 at 30°C. The production of antimicrobial compounds was started at day 4th of incubation by all the isolates measured by zone of inhibition. It was found that the production of antibacterial compounds increased up to day 5th of incubation by SK-1 (22±0.99mm) and SK-3 (17±1.0mm) while isolate SK-4 showed maximum antibacterial activity (16±1mm) at day 7th. The antifungal producing isolate SK-5 gave maximum zone of inhibition activity at 8th day of incubation (Figure 3).

Inoculum size

The effect of inoculum size on the production of antimicrobial compounds by four potent isolates of *Streptomyces* was also studied. The maximum production of antimicrobial compounds was obtained using 10% (v/v) inoculums in the fermentation medium by isolates SK-1 (31±0.90mm), SK-3 (27±0.80mm), SK-4 (23±0.86mm) and SK-5 (35±1.2mm) (Figure 4).

Temperature

The effect of temperature on the production of antimicrobial compounds by four potent isolates was studied (Figure 5). The growth and production of antimicrobial compounds by these isolates were studied at different temperatures (28-40°C). The maximum growth and antimicrobial activities were observed at 28°C for the isolates SK-1 (31±0.90mm), SK-3 (27±0.80mm) and 32°C for SK-4 (26±0.80mm). However, isolate SK-5 gave the maximum production (42±1.3mm) of antifungal compound at 34°C.

pH

Different initial pH values (5.0-11.0) of the media were used for the growth and production of antimicrobial compounds by all the four isolates. It was observed that all isolates exhibited rich growth and production of antimicrobial compounds in pH range of 8.0-9.0 (Figure 6).

Effect of carbon sources

The effect of different carbon sources on the production of antimicrobial compounds by above mentioned isolates was studied (Figure 7). Different carbon sources at the concentration of 1% were tested in the fermentation medium to achieve maximum production of antimicrobial compounds by these isolates. For isolates SK-1, SK-3 and SK-4, starch favored the maximum production of antibacterial compounds by giving zone of inhibition

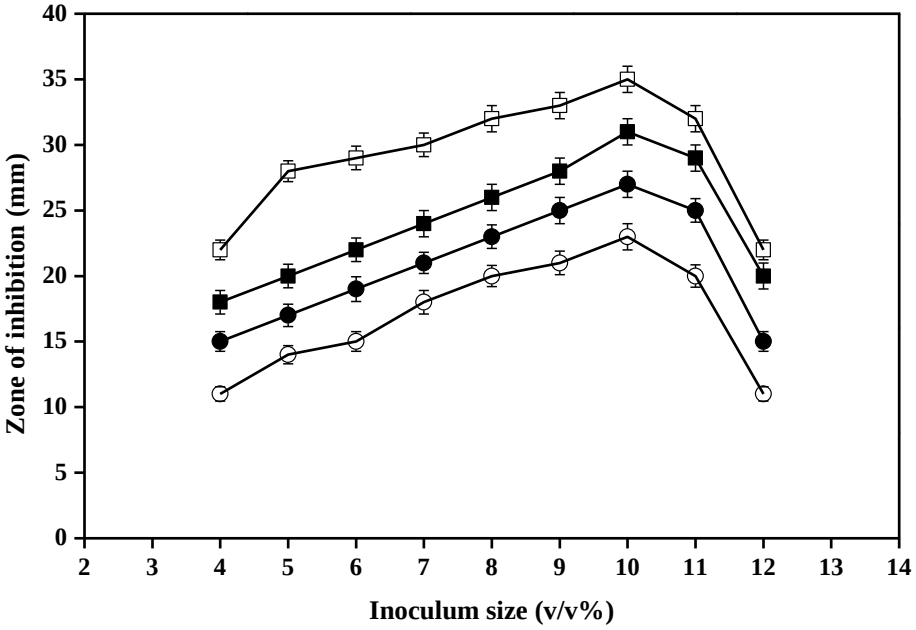

Figure 4. The effect of inoculum size (v/v%) on production of antibacterial compounds by indigenous *Streptomyces* isolates SK-1(■), SK-3(●), SK-4(⊄) and antifungal compound by SK-5(ϒ).

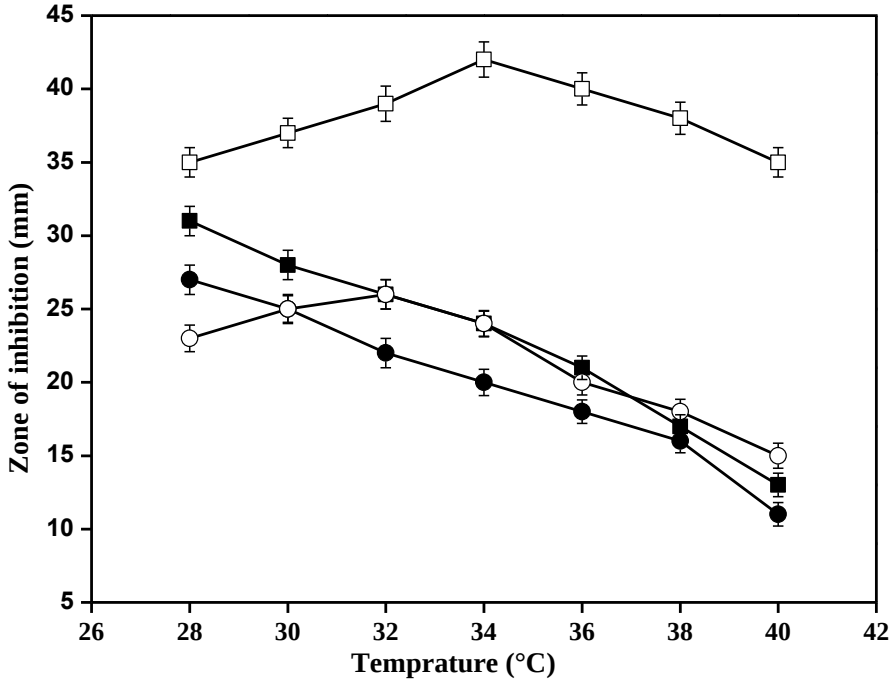

Figure 5. The effect of temperature on production of antibacterial compounds by indigenous *Streptomyces* isolates SK-1(■), SK-3(●), SK-4(⊄) and antifungal compound by SK-5(ϒ).

35±1.5 mm, 33±1.2 mm and 30±1.0 mm respectively. Isolate SK-5 showed maximum antifungal activity (44±1.8mm) when glycerol was used as sole carbon source in the fermentation medium. Very low antibiotic

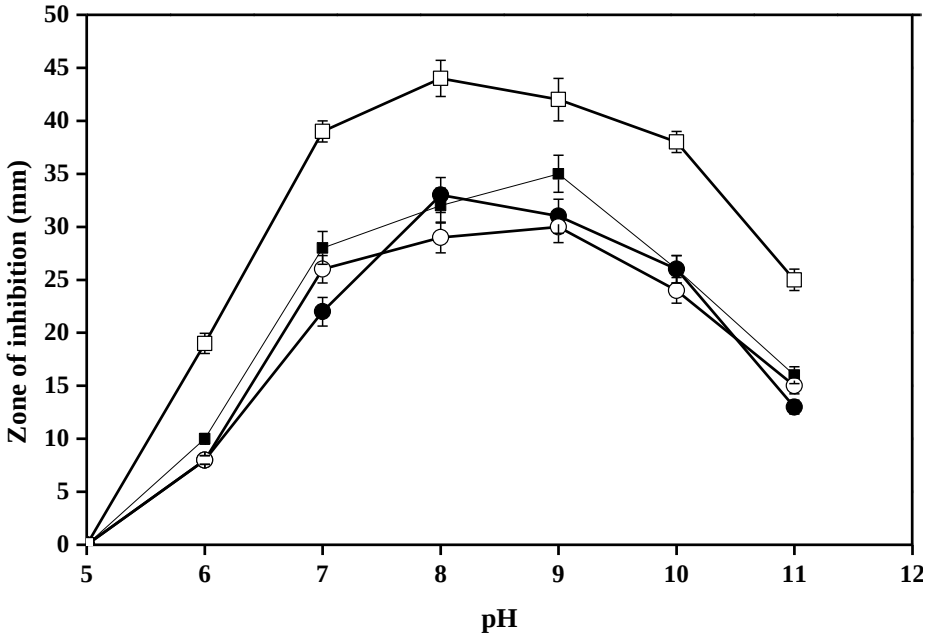

Figure 6. The effect of pH on production of antibacterial compounds by indigenous *Streptomyces* isolates SK-1(■), SK-3(●), SK-4(○) and antifungal compound by SK-5(□).

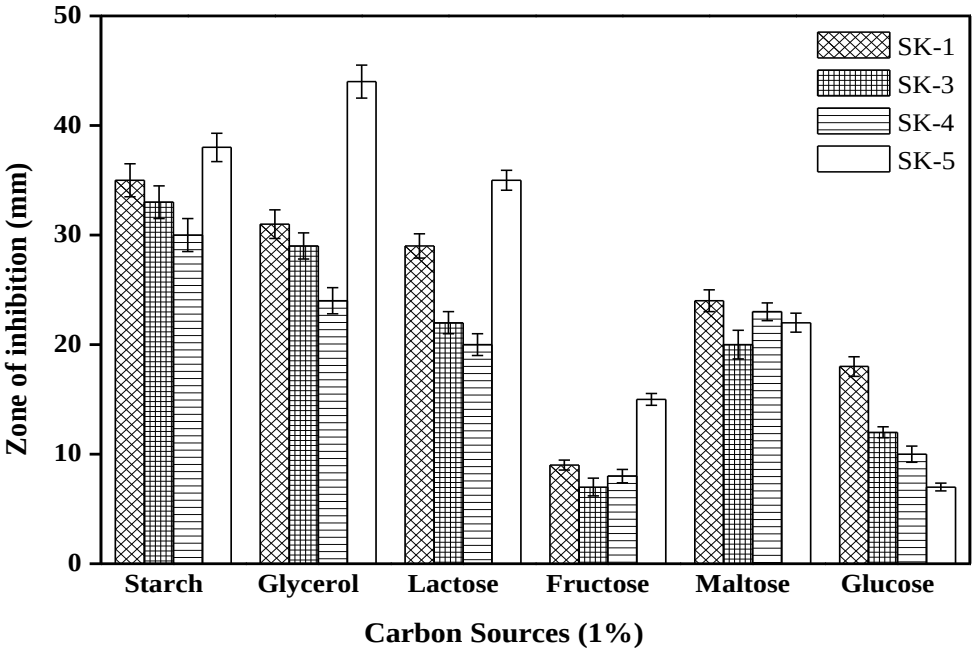

Figure 7. The effect of carbon sources on production of antibacterial compounds by indigenous *Streptomyces* isolates SK-1, SK-3, SK-4 and antifungal compound by SK-5.

production was observed when the medium was supplemented with fructose as a sole carbon source by isolate SK-1and SK-4.

Effect of nitrogen sources

Antimicrobial productivity of all the isolates (SK-1, SK-3,

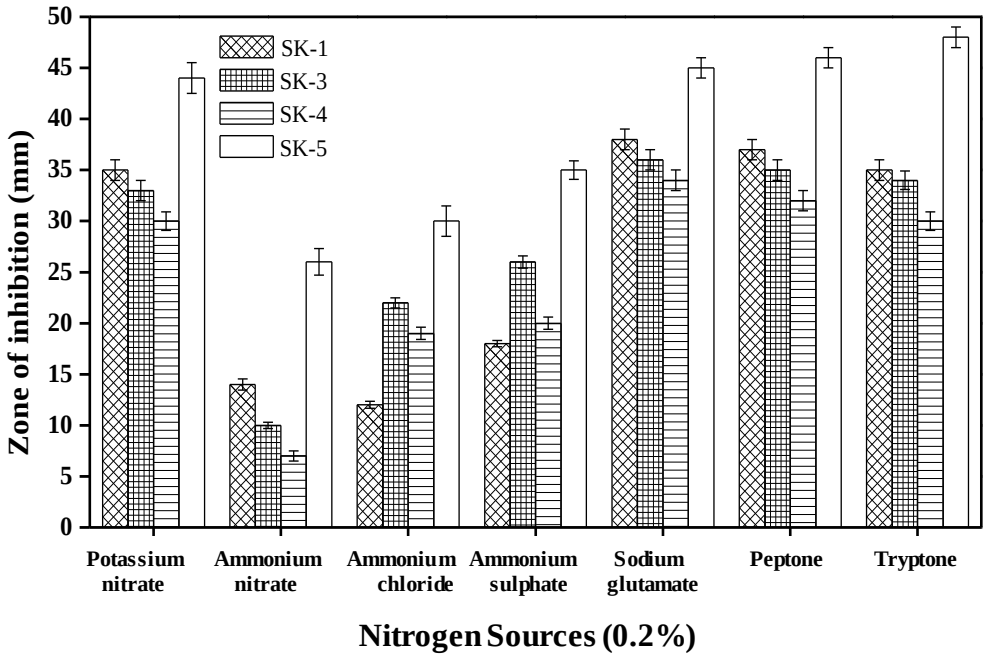

Figure 8. The effect of nitrogen sources on production of antibacterial compounds by indigenous *Streptomyces* isolates SK-1, SK-3, SK-4 and antifungal compound by SK-5.

SK-4 and SK-5) was studied using different nitrogen sources (0.2% w/v) at 1% starch for antibacterial compounds from isolate SK-1, SK-3, SK-4 and at 1% glycerol for antifungal production by isolate SK-5 as carbon source (Figure 8). Tryptone, peptone and sodium glutamate enhanced the antimicrobial activities by all the isolates while, ammonium nitrate, ammonium chloride, ammonium sulphate were observed to suppress the antimicrobial activities as compared to already used nitrogen source that is potassium nitrate. The use of sodium glutamate in the fermentation medium supported maximum antibacterial compounds formation by isolate SK-1 (38±1.6mm), SK-3 (36±1.2mm) and SK-4 (34±1.0mm). However, isolate SK-5 gave maximum antifungal activity (48±1.5mm) when tryptone was used in the fermentation medium as nitrogen source.

DISCUSSION

Currently, widespread use of antibiotics caused the development of antimicrobial agent's resistance in microorganisms all over the world. Therefore, investigations regarding the discovery of new antimicrobial compounds have been led to look at natural sources of antimicrobial agents to find new pharmaceutical products (Ebrahimipour et al., 2011). The taxonomic and ecological positions of antibiotic producing microorganisms are an integral part in antimicrobial agents' development programme. Complete understanding of the organisms give useful information about the secondary metabolites being produced by them and other activities carried out by them in their habitat (Adegboye and Olubukola, 2012). Actinomycetes particularly genus *Streptomyces* are prolific producers of antibiotics and other industrially useful secondary metabolites such as antibiotics, herbicides, pesticides and anti-parasitic (Saadoun and Gharaibeh, 2003). Criteria for the identification of *Streptomyces* include morphological, physiological, ecological and molecular characterization. It is vital to identify the organism up to species level, since this will give an indication whether the antimicrobial agent being produced is novel or not. The suborder and habitat also act as pointers for possible secondary metabolites production and bestowed the need for further exploration (Adegboye and Olubukola, 2012).

It has long been known that some of the *Streptomyces* strains of the same species could generate different antibiotics, whereas some other strains belonging to different species generated the same antibiotics (Lechevalier, 1975). The production of antibiotics by *Streptomyces*, therefore, may not be species-specific, but rather strain-specific. Antibiotics of *Streptomyces* origin evidence a wide variety of chemical structures, including aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyketides, actinomycins, and tetracyclines (Baltz, 1998).

This study was also designed to explore the nature of

indigenous *Streptomyces* isolates and their potential to produce new antibiotics effective against wide range of pathogenic bacteria and fungi. For this study twenty five *Streptomyces* isolates were purified from around hundred soil samples and screened for their potential to produce antimicrobial compounds. Only four *Streptomyces* isolates (SK-1, SK-3, SK-4 and SK-5) were found to exhibit wide spectrum antimicrobial activities and one of the isolate SK-5 produced reasonably high quantities of antifungal compounds (Table 1). For the purpose of identification of these isolates, the morphological, physiological and biochemical characterizations were carried out and the results are given in Table 2.

The composition of fermentation media nutrients is very important during metabolite synthesis process (Yu et al., 2008). Among different nutritional requirements, carbon and nitrogen sources are considered as key factors of metabolism, and numerous examples of the maximum production of metabolites in media with optimized contents of these components are illustrated in the literature (Yuan et al., 2008). So, consumption of different carbon and nitrogen sources was also studied (Table 3). Analysis of the morphological and cultural characteristics of local *Streptomyces* isolates allowed us to verify their probable taxonomic classification in genus *Streptomyces*, according to the directions given by Williams et al. (1983). Such an observation also established a varied affinity of different isolates towards changing substrates. One of the more important ways of find out novel metabolites from microorganisms is by the isolation of new microbial species (Thumar et al., 2010). So for species level identification, 16S rRNA gene analysis of these *Streptomyces i*solates was done. The resulted sequences were aligned with available almost complete sequence of type strains of family Streptomycetaeae. These potent isolates SK-1, SK-3, SK-4 and SK-5 showed 99% identity with 16S rRNA gene sequences of various strains of *S. variabilis*, *S. flavomacrosporus*, *S. levis* and *S. griseostramineus*, respectively. It is also clear from the phylogenetic analysis (Figure 2) that these isolates are not only forming a separate branch but, are also away from the already reported *Streptomyces* strains like *S. fradiae*, *S. hygroscopicus*, *Streptomyces kanamyceticus*, *Streptomyces aureofaciens* and *S, rimosus* which have been documented for antimicrobial activities earlier (Yang and Yueh, 2001). Nevertheless, SK-4 formed a separate branch in the phylogenetic tree however; it was close to the *Strptomyces levis*. Such differences impact the novelty of these isolates from the previously reported antimicrobial producing strains of *Streptomyces* apart from the fact that they fortify other observations made for these isolates as mentioned above.

The designing of appropriate medium is essential for each fermentation process. It is obligatory to optimize every constituent of the fermentation media by changing the amount of media components in order to get maximum antibiotic yield. The purpose of media optimization is to support efficient growth of microorganisms. Different combinations of medium components along with optimized fermentation parameters need to be explored to find growth conditions that produce biomass for optimum antibiotic production (Nanjwade et al., 2010). Maximum antimicrobial activity can be achieved by using different inoculum sizes and incubation period (Mu et al., 2009), pH (Atta, 2010), temperature best carbon and nitrogen (Atta et al., 2011) sources. All of above mentioned parameters like incubation period (1-10 days) (Figure 3), inoculums size (5-12% v/v) (Figure 4), fermentation temperature (28-40°C) (Figure 5), and initial pH of the medium (5.0-11.0) (Figure 6) were obtained to get maximum antimicrobial compounds production by the four potent isolates. The production of antimicrobial compounds was started by all the isolate after 3rd day of incubation but the maximum production of antimicrobial compound by the isolate SK-1 (22±0.99mm), and SK-3 (17±1.0mm) was achieved at day 5^{th} of incubation. While, isolate SK-4 and SK-5 gave the highest level of antibiotic production at day 7^{th} and 8^{th} of incubation and then production was declined gradually (Figure 3) indicating growth independent production of antibiotics by these isolates rendering them secondary metabolites of the isolates.

Antibiotic production was greatly influenced by the use of suitable carbon and nitrogen sources in the fermentation medium (Hobbs et al., 1990). Different media were used for the production of antimicrobial compounds from the four potent isolates of *Streptomyces*. It was found that the starch casein medium with potassium nitrate produced highest amount of antimicrobial metabolites by all the isolates. For this reason, this medium was used to optimize the conditions of other parameters and nature of carbon and nitrogen source used for maximum production. Different carbon sources were tested in the fermentation medium, it was noted that polysaccharides were proved to be the most suitable for the production of antimicrobial compounds by these isolates as compared to mono, di, and tri-saccahrides. For isolates SK-1, SK-3 and SK-4 predominantly producing antibacterial compounds, starch favored the maximum production. Isolate SK-5 also showed maximum antifungal activities when glycerol was used as sole carbon source (Figure 7) followed by starch, maltose, lactose, glucose, fructose, and galactose. Previously it was found that starch supported maximum antibiotic production by *Streptomyces violatusat* (El-Naggar et al., 2003).

Antimicrobial productivity of all isolates was also greatly affected by the use of different nitrogen sources. Use of different types of nitrogen sources was reported to have considerable effect on growth and secondary metabolite production by diverse *Streptomyces* species (Mansour et al., 1996). Use of complex nitrogen sources such as sodium glutamate, tryptone and peptone in the

fermentation medium positively affected the antimicrobial production by these isolates. However, medium supplemented with ammonium nitrate, ammonium chloride, ammonium sulphate reduced the antibiotic production except potassium nitrate on which reasonable amount of antibiotics was produced by all the isolates. The literature referred that the nature and concentration of carbon and nitrogen plays an important role in controlling the process of antibiotic biosynthesis in *Streptomyces* (Sanchez et al., 2010). Sanchez and Damian (2002) reported that elevated level of nitrogen affected the synthesis of enzymes involved in the production of primary and secondary metabolites as well as the consumption of different media nutrients. It was also illustrated that complex nitrogen sources could enhance the production of antibiotics. These sources could maintain high antibiotic titer due to the slow release of nitrogenous components during the course of the fermentation. More generally, it has been studied that nitrogen assimilation is vital for the regulation of antibiotic synthesis but the mechanisms involved is not clearly known (Voelker and Altaba, 2001). In the light of above mentioned results, it could be concluded that the use of starch casein medium along with the optimized carbon and nitrogen sources could maximally support the antibiotic production by these isolates. In conclusion, our findings strongly supported that the rhizospheric soil from indigenous habitat provided a rich source of novel and diverse species of *Streptomyces* for broad spectrum antibiotic production that were not previously explored. A very high level of antimicrobial activity shown by these local isolates after optimizing cultural conditions seems likely to lead to the discovery of potentially beneficial secondary metabolites against pathogenic and drug resistant microorganisms. Moreover, further studies are warranted including identification and characterization of these compounds using various analytical techniques.

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