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ISOLATION AND SCREENING OF POTENT ACTINOMYCETES FROM THE SOIL SAMPLES OF SOUTHERN PART OF UTTAR PRADESH, INDIA

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ABSTRACT

Many bacterias are having the potential to produce antibiotics. Among them, actinomycetes are best known for their ability to produce antibiotics. The present study was carried out for the discovery of novel drugs and also new species of actinomycetes which have the potential to produce antimicrobial metabolites. A total of 50 soil samples were collected from different locations of southern part of Uttar Pradesh, India. 12 actinomycetes strains were isolated and screened for their ability to produce antibiotics by spektra-plak method against test organisms. Out of 12 strains, 5 isolates (TAA04, TAA10, TAA12, TAA33 and TAA46) showed potent activity against test organisms. These isolates were further characterized for temperature tolerance and NaCl tolerance tests. Biochemical tests were performed for the identification of the isolates. Based on the findings, isolates were found to be of genus *Streptomyces.* Further, these isolates were evaluated for antimicrobial activity against test organisms i.e. *S. dysenteriae* (MCCB-0043), *S. aureus* (MCCB-0045), *K. pneumoniae* (MCCB-0026), *B. subtilis* (MCCB-0222), *P. vulgaris* (MCCB-R035), *P. aeruginosa* (MCCB-0034), *E. coli* (MCCB-0017), and *E. feacalis* (MCCB-161) by determining the zones of diameter using well diffusion method. Isolate TAA04 showed no zone against *P. vulgaris.* Isolate TAA12 showed no activity against *S. aureus* and *E. coli.* TAA46 was found inactive against *S. aureus* and *P. vulgaris*. TAA46 showed highest activity against *K. pneumonia*, *B. subtilis* and *E. faecalis*; TAA10 was highly active against *S. aureus* and *E. coli*; TAA04 and TAA12 showed maximum zone for *S. dysenteriae* and *P. vulgaris* respectively. The antibacterial activity of the isolates showed potential as a source of antibiotic producers.

KEYWORDS: Actinomycetes, antibacterial activity, antibiotics, soil sample.

INTRODUCTION

Actinomycetes are unique, notable, free living species mostly found in soil, water and colonizing plants but their growth pattern is known to be lethargic. Earlier they were characterized as an intermediate organism to bacteria and fungi but now they are grouped as bacterial species. They are gram positive, aerobic prokaryotes with high GC content in their DNA. Percentage of GC in DNA of actinomycetes were estimated nearly to be 57-75%.[1] On the surface of agar medium, such type of microorganisms appears to be powdery, slimy, hard colonies.

Scientists have been vitally pursuing the screening of microorganisms for the production and utilization of novel antibiotics in various fields' viz. agriculture, pharmaceutical, and veterinary to name but few. Among various microorganisms of soil population, actinomycetes act as a masterpiece for the production of antibiotics and various other secondary metabolites. Antibiotics play an important role in therapeutic and commercial importance.[2,3,4,5,6] Natural metabolites derived from microbes still appear as the most promising sources of antibiotic production.[7] It has been estimated that roughly two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes family mainly from genus *Streptomyces*.[8]

Isolation and screening of promising potential microorganisms to combat infection and diseases are still areas of search by many scientists till date. Screening for

antimicrobial property by various methods can reveal species that produce novel antibiotics. Thus this study was aimed to isolate and screen species of actinomycetes that can act as antagonist against pathogenic organisms. This study will be useful in the discovery of novel drugs and new species of actinomycetes which has the potential to produce antimicrobial metabolites.

MATERIALS AND METHODS

This study was carried out in the Department of Microbiology and Fermentation, Technology, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad, India. 50 soil samples (approximately 5 gms) were collected from the upper crust of the soil using clean, dry and sterile polythene bag and transported immediately to the laboratory. It was crushed and filtered to remove the detritus and stowed for further processing.

Isolation of actinomycetes from soil

Soil suspension was prepared by dissolving 1g of the soil sample in 10 ml of distilled water. Isolation of actinomycetes was done by serial dilution and pour plating method using Glycerol-Yeast Extract Agar media (GYEA) (glycerol 5g, yeast extract 2g, dipotassium phosphate 0.1g, peptone 25g, agar 15g, distilled water 1000ml, pH 6.0 ± 0.8) supplemented with nystatin (25-50µg/ml) to minimize the fungus contamination.[6] 1ml of soil sample was taken from soil suspension and serially diluted up to 10^{-7} in sterile condition. Different dilutions (10^{-4} and 10^{-5}) were poured in sterile petri plates followed by glycerol yeast extract agar media. The plates were incubated at 27ºC for 7 to 14 days. Colonies of actinomycetes were selected as rough textured, hard and chalky in appearance.[9] Selected colonies were inoculated on Yeast-Malt Extract Agar Media (YMEA) (yeast extract 4g, malt extract 10g, dextrose 4g, agar 15g, distilled water 1000ml, pH 7.0 ± 0.3) to procure the purified culture of actinomycetes. Plates were incubated for 7-14 days at 27ºC.[10] Isolates were sub cultured onto respective slants for short time preservation at 4°C.

Test organisms

The test organisms included in the *in vitro* study were obtained from Microbial Culture Collection Bank (MCCB), Department of Microbiology and Fermentation Technology (SHUATS). These organisms were *Shigella dysenteriae* (MCCB-0043), *Staphylococcus aureus* (MCCB-0045), *Klebsiella pneumoniae* (MCCB-0026), *Bacillus subtilis* (MCCB-0222), *Proteus vulgaris* (MCCB-R035), *Pseudomonas aeruginosa* (MCCB-0034), *Escherichia coli* (MCCB-0017), and *Enterococcus feacalis* (MCCB-161).

Screening of actinomycetes for antibacterial activity

Primary screening was done by spektra-plak method.[11] A total of 12 isolates were screened for antibacterial activity against test organisms. Mueller Hinton Agar (MHA) (beef infusion 300g, casamino acids/acid hydrolysate of casein 17.5g, starch 1.5g, agar 17g, distilled water 1000ml, pH 7.0 ± 0.4) plates were prepared and inoculated with actinomycetes culture by single streak of inoculum in the centre of the petri plate and incubated at 37ºC for 3-4 days. Later the plates were seeded with test organisms by single streak at 90º angle to the inoculated strains. Clear zone formation between isolate and the test organisms were considered positive for antimicrobial activity. The isolates were then selected based on a wide spectrum activity against tested microorganisms for further testing.

Characterization and identification of actinomycetes

The following tests were performed for characterization and identification of actinomycetes, i.e., Gram Staining, temperature tolerance, growth with sodium chloride and biochemical analysis.

(i) Gram staining- The microscopic examination was carried out by standard gram's staining method.[12] A smear of culture was taken in a clean glass slide and heated gently over a flame. The smear was covered with a thin film of crystal violet for 1 minute and washed gently in slow running tap water. Gram's iodine solution was flooded over the smear for 2 minute and washed with tap water. Alcohol was used to decolorize the smear until the violet color ceased to flow away**.** Then the slide was washed with water and counter stain safranine was flooded over the smear for 2 minute then the slide was washed, drained, air, dried and viewed under microscope. The culture retaining the violet color indicated Gram positive organism.

(ii) Temperature tolerance test- Actinomycete isolates were inoculated in nutrient broth and uninoculated tubes were taken as a control. All the tubes were provided with different incubation temperatures i.e. 20ºC, 28ºC, 35ºC and 45ºC for 2-3 days. Bacterial growth was observed at different temperatures by recording the optical density at 540 nm.

(iii) Growth with sodium chloride- Actinomycete isolates were inoculated in the nutrient broth provided with different salt concentrations, i.e. 4%, 8%, 12%, 16% under sterile condition. All the inoculated tubes along with uninoculated (control) tubes were incubated at 28ºC for 2-3 days. Bacterial growth was observed by measuring the optical density at 540 nm.

(iv) Biochemical tests- Biochemical tests were performed for the identification of isolates, viz. catalase, indole, urease, starch hydrolysis, casein hydrolysis, nitrate reduction, motility, ONPG, H_2S, methyl red, voges prauskouer, gelatin, carbohydrate fermentation (glucose, fructose, sucrose, maltose, xylose, mannitol, raffinose, mannose, galactose, trehalose, arabinose). Results of these tests were compared with the Guide to the Classification and Identification of the Actionomycetes and their Antibiotics.[13] Also, the isolates were characterized and identified by following the directions of Bergey's Manual of Systematic Bacteriology.[14]

Determination of antibacterial activity

Secondary screening of final selected isolates was done by well diffusion method using Nutrient agar media plates.[15] Nutrient agar plates were prepared and test organism was swabbed on it by using sterile swab sticks. By using sterile cork borer, wells (6 mm in diameter) were punctured in fresh test microbial lawn cultures. Isolated culture were grown in nutrient broth and inoculated at 37ºC for 24-48 h in shaking incubator. After incubation, the broth was centrifuged at 8000 rpm for 15 min and supernatant (100 µl) was collected in vials. The supernatant culture broths were then filled in each well. The bacterial plates were then incubated at 37ºC for 24 hours. An uninoculated plate was used as media control. Test organism swabbed plates were used as organism control. Bioactivity was determined by measuring the zones of diameter (mm).

RESULTS

Total 12 isolates were recovered from rhizospheric soils of plants and terrestrial land taken from different locations of southern part of Uttar Pradesh, India. These isolates were selected based on appearance, growth pattern and color of the colonies and were isolated to obtain purified culture. These selected colonies were observed as rough, chalky colonies with irregular growth. The colors of the selected colonies varied as creamy, whitish and pale yellow. All the isolates were found to be gram positive on performing Gram's staining.

Further these selected isolates were subjected to primary screening for antimicrobial activity by spektra-plak method against above mentioned test organisms. The isolate designated as TAA04, TAA10, TAA12, TAA33 and TAA46 were found active against test organisms and were considered as good antibiotic producers as shown **[Table 1, figure 1]**.

Table 1: Screening of actinomycetes species against test organisms.

Isolates	TAA 01	TAA 02	TAA 04	TAA 10	TAA 12	TAA 17	TAA 20	TAA 28	TAA 33	TAA 37	TAA 42	TAA 46
***S.dysenteriae* (MCCB-0043)**	-	+	++	+++	++	-	+	-	+	-	-	+++
***S.aureus* (MCCB-0045)**	-	-	+++	++	-	-	-	-	+	-	-	-
***K.pneumoniae* (MCCB-0026)**	-	-	++	+++	++	-	-	-	++	-	-	++
***B.subtilis* (MCCB-0222)**	-	-	+++	++	++	-	-	-	+	-	-	++
***P.vulgaris* (MCCB-R035)**	-	-	-	+	+	-	-	-	+	-	-	-
***P.aeruginosa* (MCCB-0034)**	-	-	+++	+++	++	-	-	-	+++	+	-	++
***E.coli* (MCCB-0017)**	-	-	+	+++	-	-	-	-	++	-	-	+++
***E.faecalis* (MCCB-161)**	-	-	+++	++	+	-	-	-	+++	-	-	++

+++, ++, + *Degree of inhibition under plate observation;* - *No activity*

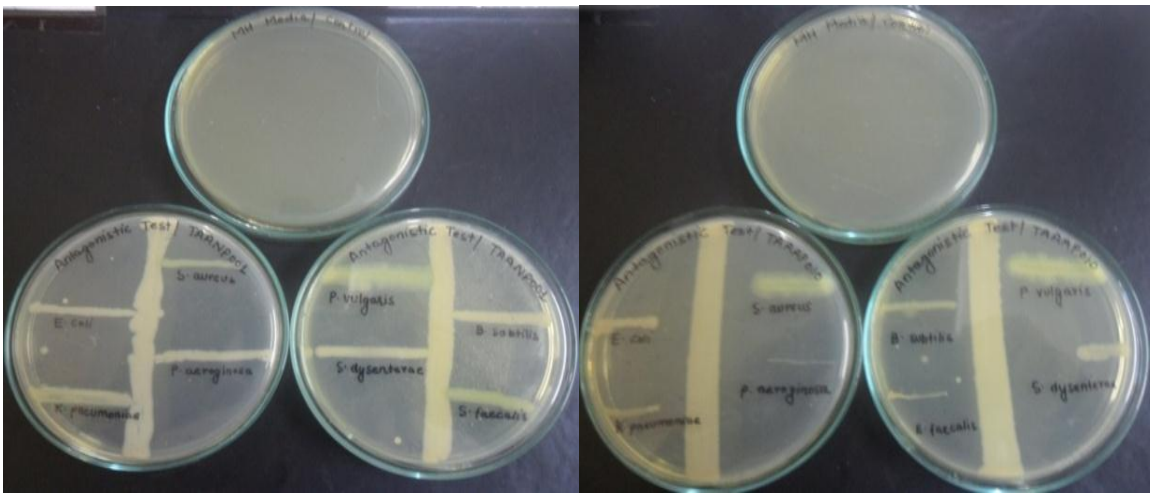

Figure 1: Screening of actinomycetes species against test organisms using spektra-plak method.

On performing temperature tolerance test, the growth of the isolates decreased with the increase in temperature. Least growth was observed at temperature range of 45ºC. The most favorable temperature for all the isolates was found to be 20 ºC **[Table 2, Figure 2]**.

Table 2: Temperature tolerance of actinomycetes isolates.

S. No.	Isolates	Absorbance recorded at different temperatures (540 nm)			
		20 °C	28 °C	35 °C	45 °C
1	TAA04	0.43	0.36	0.30	0.24
2	TAA10	0.32	0.30	0.27	0.18
3	TAA12	0.37	0.28	0.22	0.16
4	TAA33	0.52	0.43	0.37	0.30
5	TAA46	0.38	0.32	0.21	0.12

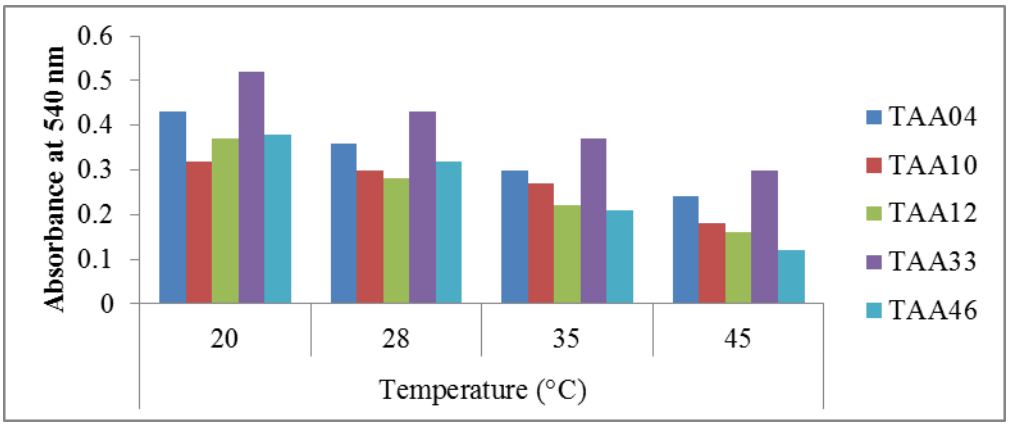

Figure 2: Temperature tolerance of actinomycetes isolates.

All the isolates were grown at different concentration of sodium chloride. Isolate TAA12 and TAA46 were best grown at 4% concentration of NaCl while TAA04, TAA10 and TAA46 were best grown at 8% concentration. Least growth was observed at 16% salt concentration **[Table 3, figure 3]**.

Table 3: Sodium chloride tolerance of actinomycetes isolates.

S. No.	Isolates	Absorbance recorded at different concentrations of NaCl (540 nm)			
		4%	8%	12%	16%
1	TAA04	0.38	0.47	0.40	0.32
2	TAA10	0.52	0.63	0.57	0.42
3	TAA12	0.56	0.43	0.36	0.30
4	TAA33	0.43	0.48	0.37	0.29
5	TAA46	0.63	0.56	0.50	0.48

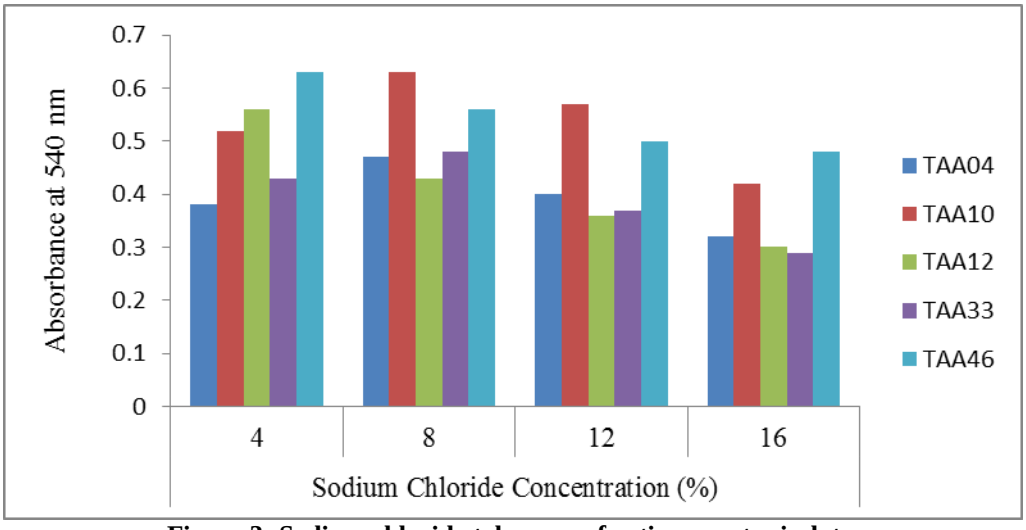

Figure 3: Sodium chloride tolerance of actinomycetes isolate.

Biochemical tests were performed to identify the isolates and the results were recorded as shown in Table 4.

Table 4: Biochemical analysis of actinomycetes isolates.

S.No.	Test name	TAA04	TAA10	TAA12	TAA33	TAA46
1	Catalase	+	+	+	+	+
2	Indole	-	-	-	-	+
3	Urease	+	-	+	+	+
4	Sarch hydrolysis	-	+	+	+	+
5	Casein hydrolysis	-	+	-	-	-
6	Nitrate reduction	+	+	+	+	+
7	Motility	+	+	+	+	+
8	ONPG	+	+	-	-	+
9	H_2S	-	+	+	-	-
10	Methyl red	+	+	-	+	+
11	Voges prauskouer	-	-	-	-	-
12	Gelatin hydrolysis	+	-	+	+	-
13	Glucose	+	+/A	+	+	+
14	Fructose	+	+/A	+	+	+
15	Sucrose	+	+/A	+	+/A	+/A
16	Maltose	+	+/A	+	+	+
17	Xylose	+	+/A	-	-	+
18	Mannitol	+	+/A	+	+	+
19	Raffinose	+	+/A	-	-	-
20	Mannose	+	+/A	+	+	+
21	Galactose	+	+/A	-	-	-
22	Trehalose	-	+/A	-	-	-
23	Arabinose	-	+	-	-	-

+ is Positive reaction; - is Negative reaction; A is Acid formation

On comparing the results with the Guide to the Classification and Identification of the Actionomycetes and their Antibiotics, it was found closely related to the species of *Streptomyces*.[13] Further the results were compared with Bergey's Manual of Systematic Bacteriology, and the isolates were found to be of the *Streptomyces* genus.

Based on the primary screening results, the selected isolates were subjected for secondary screening for antibacterial properties using well diffusion method and their potency was tested by measuring its zone diameter (mm) **[Table 5, figure 4]**.

Table 5: Antibacterial activity of actinomycetes species against test pathogens.

Species	Zone diameter (mm)				
	TAA04	TAA10	TAA12	TAA33	TAA46
***S. dysenteriae* (MCCB-0043)**	25	23	17	20	22
***S. aureus* (MCCB-0045)**	20	22	-	15	-
***K. pneumoniae* (MCCB-0026)**	18	20	20	15	22
***B. subtilis* (MCCB-0222)**	26	24	25	28	30
***P. vulgaris* (MCCB-R035)**	-	26	30	22	-
***P. aeruginosa* (MCCB-0034)**	20	15	18	18	10
***E. coli* (MCCB-0017)**	23	30	-	28	20
***E. faecalis* (MCCB-161)**	25	20	20	18	30

- *No inhibition zone*

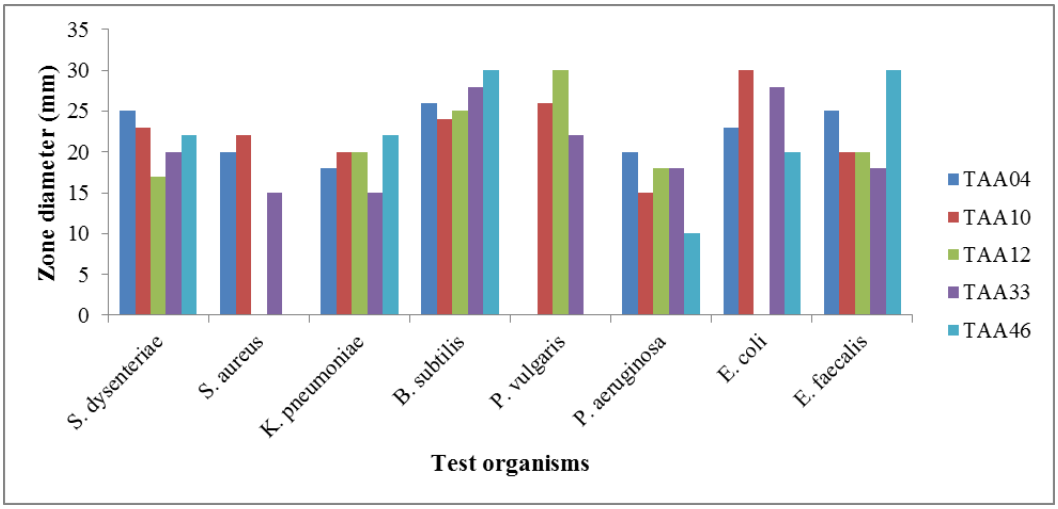

Figure 4: Antimicrobial activity of actinomycetes against test organisms.

DISCUSSION

The present study was aimed to find out potential actinomycetes species which acts as antagonists against pathogenic organisms. Actinomycetes colonies were isolated and screened primarily against gram-negative and gram-positive bacteria to select the potential isolates. The ability of actinomycetes to grow at different temperatures were evaluated and found that it grows well at temperature ranging from 20-30 ºC.[16,17] It is also reported the growth of actinomycetes at different temperatures and found that all the isolates grew well at temperature of 28 ºC.[18] The findings a study reveals that actinomycetes grow at temperature of 37 ºC and 42 ºC;[19] however, negative result was obtained at temperature of 50 ºC. Thus, it is clear from the above findings that actinomycetes can best tolerate lower temperature as on increasing the temperature its growth gets reduced. In general the optimal condition for their growth are temperatures of 20-30 ºC (50ºC is for thermo-actinomycetes). Growth with sodium chloride was studied and the best growth observed was at 7% concentration.[9] One of the study recorded growth at various salt concentrations and reported the best growth at 5-7%, also found no growth at 9% concentration.[19] These findings were very similar with the present study. The ability of actinomycetes to grow at different NaCl concentrations was found. At 2.5% concentration the growth was high while at concentration 5% and 7 % growth was less.[16] Different concentrations of sodium chloride (0%, 2%, 5%, 7%, 10% and 20%) solution were added to the medium to observe the presence or absence of growth. The tested isolate was growing well at 7% of NaCl, and no growth was recorded above 7% NaCl.[20] The results of the above studies are nearly similar to the present work.

Biochemical analysis was done which showed bubbles of oxygen during catalase test for all the isolates which indicates a positive result. Isolate TAA46 was found positive for indole production. TAA04, TAA12, TAA33 and TAA46 produced urease enzyme while TAA10 gave a negative result for urease production. Starch hydrolysis was observed by all the isolate except TAA04. Only isolate TAA10 gave a positive result for extracellular caseinase secretions. All the isolates were found to produce nitrate reductase enzyme that hydrolyses nitrate to nitrite. The motility test was positive for all the isolates. TAA04, TAA10 and TAA46 were found to be lactose fermentor by ONPG test. TAA10 and TAA12 showed blackening of the culture medium thus indicating the positive result for hydrogen sulphide production. All the isolate was negative for voges-prauskouer test and isolate TAA12 was negative for methyl red test. TAA04, TAA12 and TAA33 produced gelatinase. Glucose, Fructose, Sucrose, Maltose, Mannitol and Mannose was utilized by all the organisms resulting as carbohydrate fermentor for these sugars. TAA33 gave negative result for Xylose, Raffinose, Galactose, Trehalose and Arabinose. TAA12 and TAA46 were negative for Raffinose and Galactose. Trehalose and Arabinose was fermented only by TAA10. Isolate TAA10 was found as distinct acid producing bacteria as it positively formed bubbles in durhams tube except for Arabinose. Isolate TAA33 and TAA46 showed positive result for acid production. Biochemical characterization of *Streptomyces sps.* viz. starch hydrolysis, gelatin hydrolysis, nitrate reduction, catalase production indole test and MRVP test recorded in a study is in agreement of the present result.[21] The results of MRVP, indole test, hydrogen sulphide, starch hydrolysis, glucose and sucrose fermentation was found similar to the present study.[22]

The previous studies demonstrates identification of the isolated actinomycetes by various morphological and biochemical tests which relates to the species of *Streptomyces* genera and *Streptomycetaceae* family.[6,14,23,24,25,26] On comparing the physiological and biochemical characteristics indicated that isolates are closely related to species *Sreptomyces antibioticus, Streptomyces rimosus, Streptomyces lavendulae, Streptomyces lydicus, Streptomyces albus, Streptomyces halstedii and Streptomyce phaeochromogenes*.[9] Various

biochemical tests viz. catalase, nitrate, gelatin, urease, indole, sugar fermentation, hydrogen sulphide, indole etc. were performed having results closely related to the present study.[11,16,17,19,27,28,29,30,31] The present study is in agreement with the previous works relating to the study of actinomycetes.

Further, antimicrobial activity was determined by well diffusion method. All the isolates showed high activity against *B. subtilis* and few showed good activity against *S. aureus* and *E. coli* which is similar to the findings of one of the study.[11] The activity of *E. coli* and *S. aureus* were recorded high with few isolates while few showed no acivity. The antimicrobial activity of *S. dysenteriae* was found good against all the isolates and also activity with S. aureus which is quite similar to the study which reported antibacterial activity against *P. aeruginosa*, *P. vulgaris*, *S. aureus*, *E. coli* and *B. subtilis* which is in agreement with the present study.[17,32] Also, antimicrobial activity recorded against *K. pneumoniae*, *B. subtilis*, *E. coli*, *P. aeruginosa* , *S. aureus*, *S. typhi*, *Salmonella species*, etc was nearly similar to the present work.[10,18]

Isolate TAA04 showed no zone against *Proteus vulgaris* while TAA12 showed no activity against *Staphylococcus aureus* and *Escherichia coli.* TAA46 was found inactive against *Stahylococcus aureus* and *Proteus vulgaris*. Isolate TAA04 possesed antibacterial activity against all the test organisms except *P. vulgaris*. Highest activity was shown against *B. subtilis* with zone diameter of 26 mm. It also showed good activity against *S. dysenteriae* and *E. faecalis* with zone diameter of 25 mm. TAA10 was active against all the isolates but highly active against *E. coli*, *P. vulgaris* and *B. subtilis* with zone diameter of 30 mm, 26 mm and 24 mm respectively. TAA12 showed good activity for *P. vulgaris* and *B. subtilis* with zone of 30 mm and 25 mm. Isolate TAA33 showed antibacterial effect against *E. coli* and *B. subtilis*. Also, TAA46 was highly active against *B. subtilis*, *E. faecalis*, *S. dysenteriae* and *K. pneumoniae*. They were found active against organisms like *E. coli*, *S. aureus*, *S. typhi*, *B. subtilis*, *P. vulgaris*, *E. faecalis*, *K. pneumoniae*, *S. dysenteriae*, *K. oxytoca*, *P. mirabilis*, *P. aeruginosa*, *V. cholera*, and many more in the previous studies which is comparable with the present findings.[9,11,33,34,35]

CONCLUSION

This study established a rich actinomycetes diversity of the region, especially the various niche habitats of southern part of Uttar Pradesh, India which could help to conserve and utilize them in the bio-industry. Since, soil is a potential source of antibiotic producing actinomycetes useful in various fields such as agricultural industries, pharmaceutical industries and biotechnology industry, therefore this study was based on soil actinomycetes.

On the basis of the findings it is recommended that identification at species level should be done. Further, purification and characterization of secondary metabolites can be carried out. Also, large scale production on potent antibiotic producing actinomycetes isolates can be done.

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