SCREENING OF POTENTIAL ACTINOMYCETES ISOLATES AGAINST SELECTIVE PATHOGENIC MICROORGANISM

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ABSTRACT

Objective: *To isolate, evaluate and characterize the potential bioactive compound producing actinomycetes from soil sample of different location of Gwalior.* ***Method:*** *A total of 11 strains of actinomycetes were isolated from soil collected from different location of Gwalior. The isolates were characterized by using morphological and biochemical methods. 11 isolates were identified and subjected to submerged fermentation methods to produce crude extracts. The fermented biomass was extracted by organic solvent extraction method and tested against bacterial strains by agar well diffusion method.* ***Results:*** *All the isolates of this study were gram positive and their colony morphology was studied microscopically. Morphologically Isolates had branched mycelium. All 11 isolates were biochemically characterized. Majority of isolates (90.90%) were able to hydrolysis of starch; 18.18% isolates were produced proteolytic caseinase and utilize casein as carbon source efficiently; all isolates were negative for indole production; 81.81% isolates were positive for citrate utilization test, all isolates were negative for gas and hydrogen sulphide production while 18.18% isolates were found positive for glucose fermentation and 27.2% for sucrose fermentation. In this study it has been found that 27.27% isolates were reported with broad spectrum antibacterial activity. In this study it has been found that GWL 3 and GWL 6 demonstrate highest bactericidal activity against Salmonella typhi with 15mm ZOI. This study also concludes that maximum number of isolates exhibit antibacterial activity against Salmonella typhi.*

Keywords- *Actinomycetes, Bioactive, Biochemical, Submerged.*

I. INTRODUCTION

Actinomycetes are the most extensively distributed groups of microbes in nature which mainly reside in the soil **[1].** Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now they are recognized as prokaryotes. The actinomycete encompasses two different groups of filamentous bacteria; the actinomycetes and the nocardia/streptomycin complex **[2].** Actinomycetes have high G+C (>55%) in their DNA **[3],** which produce branching mycelium of two types: substrate and aerial mycelium. Soil microorganisms provide an excellent source for the isolation and identification of therapeutically important products. Among them, actinomycetes are a vital group of filamentous, gram positive, free living, saprophytic bacteria producing antibiotics of agricultural and medicinal importance **[4].** Actinomycetes can be isolated from soil and marine sediments. Although soils have been screened by the pharmaceutical industry for about 50

years, only a small fraction of the surface of the earth has been sampled, and only a little fraction of actinomycetes taxa has been discovered **[5]**. Actinomycetes are widely dispersed in man-made environments, and play a significant role in the decomposition of organic matter. Actinomycetes hold a major position for their variety and capability to generate novel substances. The terrestrial soil actinomycetes have potential biotechnological applications, and are a new source for structurally different secondary metabolites **[6]**. Actinomycetes are the responsible for the manufacture of about half of the exposed bioactive secondary metabolites, especially antibiotics **[7]** such as, novobiocin, amphotericin, vancomycin, neomycin, gentamycin, chloramphenicol, tetracycline, erythromycin, nystatin, etc., antitumor agents **[8]**, immunosuppressive agents **[8]**, and enzymes **[9]**. Secondary metabolites are also used as herbicides, pesticides, anti-parasitic, and enzyme like cellulase and xylanase used in waste treatment **[1]**. From the 22,500 biologically active compounds that have obtained from microorganisms, 45% are produced by actinomycetes, 38% by fungi, and 17% by unicellular bacteria **[3]**. Actinomycetes have provided several important bioactive compounds of high commercial worth and continue to be routinely screened for new bioactive compounds. Most of the antibiotics in use today are derivatives of natural products of actinomycetes and fungi **[11]**. The antibiotics are extensively distributed in environment, where they play an important role in regulating the microbial population of soil, water, sewage and compost **[12]**. Almost 80% of the world's antibiotics are known to come from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* **[13]**. Antibiotics are used for the several disease treatment caused by parasites, fungi and bacteria. Severe infections caused by bacteria that have become resistant to generally used antibiotics have become a major global healthcare difficulty in the 21st century **[14].** According to the World Health Organization, over-prescription and the improper use of antibiotics has led to the generation of antibiotic resistance in many bacterial pathogens. At the present time, the drug resistant strains of pathogen appear more quickly than the rate of invention of novel drugs and antibiotics. For this reason, many scientists and pharmaceutical industry have vigorously involved in isolation and screening of actinomycetes from diverse untouched habitats, for their production of antibiotics **[10]**. Physicians acquired *Methicillin-resistant S. aureus* (MRSA), which also bears resistance too many antibiotics. During this time, vancomycin has been the therapeutic answer to MRSA, Vancomycin resistant strains have emerged clinically **[15, 16, 17]**. As the human pathogens developed resistance to antibiotic drugs, there is an urgent need for the safe, nontoxic and cost effective antibiotics. Thus, it is crucial that novel groups from unknown or underexploited habitats is the different part of the globe in the last few years may be pursued as resources of new bioactive secondary metabolites. These actinomycetes have been severely studied during last few decades in numerous intact environments. In this connection, this study summarizes the isolation and identification of actinomycetes isolates with the potential activity against bacterial pathogenic organism.

II. MATERIAL AND METHODS

2.1. Soil Sampling and Pretreatment

Soil samples were collected from different niche habitats soil of Gwalior (geographical location: 26.221521°N 78.178024°E). For Isolation of microorganism from soil, soil was to be taken 6-12 inches depth (Fungi are found 3-4 inches of soil, where as bacteria and actinomycetes were concentrated much deeper. Soil samples were pretreated with heat at 45^0C in hot plate 3-4 hours, for the removal of moisture from the soil samples. Soil pretreatment was required for inhibiting or eliminating unwanted microorganisms **[18]**.

2.2. Isolation Of Pure Actinomycetes Colony From Mixed Microbial Population

For the isolation of actinomycetes, 1gm. soil was dissolved in 9ml. distilled water from each area. Soil samples were serially diluted in sterile distilled water up to 10^{-6}. An aliquot of 0.1 ml of each dilution was taken and spread evenly up to over the surface of actinomycetes isolation agar (AIA) medium **[19]** supplemented with cycloheximide (50 µg /ml) and Plates were incubated at 30°C for 7 days. After 7 days observation, mixed populations of microorganisms were seen. The selected colonies were transferred into actinomycetes isolation agar plates, and pure isolates of actinomycetes were seen.

2.3. Characterization Of Isolates

2.3.1. Gram Staining

Gram staining was done for all pure isolates and the stained slides were viewed under the microscope (100X) **[20]**.

2.3.2. Biochemical characterization

Biochemical characterization tests were done for selected actinomycetes isolates, which were found to be positive. Some biochemical tests were performed such as indole test, starch hydrolysis test, casein hydrolysis test, citrate utilization test, and triple sugar iron test for study **[21, 22]**.

2.3.2.1. Indole Test

The Purified isolate was inoculated in trypton broth, containing trypton amino acid and incubated it for overnight at 30°C. After incubation, few drops of Kovac's reagent were added. Formation of red or pink colored ring at the top is taken as positive test.

2.3.2.2. Starch hydrolysis Test

Make a spot of each isolates with the help of sterile loop on Starch agar medium plates and were incubated for 24 to 48 hours at 28±2°C. After proper inoculation and incubation, flood the starch agar plates with Gram's iodine solution for 30 sec. The blue-black colors appear due to formation of starch-iodine complex. Appearance of clear zone surrounding the bacterial growth indicates starch hydrolysis by microorganism due to its production of the extracellular enzymes, as positive result.

2.3.2.3. Casein hydrolysis Test

Actinomycetes isolates were spotted on Skim milk agar plates by sterile inoculation loop and incubated for 24 to 48 hours at 28±2°C. After incubation period, clear zone around the isolate culture showed the hydrolysis of casein as positive result.

2.3.2.4. Citrate utilization Test

Isolates were picked up from a sterile inoculation loop and inoculated into slop of Simmon's Citrate agar slants. The slants were incubated for overnight at 30°C. The color was changed from green to blue indicate utilization of citrate as positive result.

2.3.2.5. Triple sugar iron Test

Stab the needle containing the pure culture of actinomycetes into the TSI agar slants, from up to the bottom of the TSI tube, and then streak the needle back and forth along the surface of the slants. The slants were incubated at 28^{o}C for 24 hours.

2.4. Preservation of actinomycetes isolates

For long term preservation of purified actinomycetes isolates were made 40% glycerol stock with ISP1 media and stored at 4^{o}C.

2.5. Test organism

The test organisms (*Staphylococcus aureus*, *Shigella flexneri*, and *Salmonella typhi)* used for this study was procured from IMTECH, Chandigarh.

2.6. Production and Extraction of secondary metabolites

The selected actinomycetes isolates was inoculated in 100 ml starch casein medium and kept at 37^{o}C for 7 days at 150rpm. After incubation the broths were filtered through Whatman No.1 filter paper, bioactive compound was extracted from the solvent phase. The culture filtrate was centrifuged at 10,000 rpm for 10 min. Equal volume of ethyl acetate was added at 1:1 ratio **[20]**. The filtrate with bioactive components were concentrated and checked for antimicrobial activity.

2.7. Determination of the antibacterial activity.

Antibacterial activity of purified extract was determined by agar well diffusion method. Concentration of all test microorganisms was adjusted at 0.5 McFarland turbidity standards and the bacterial test organisms were transferred into MHA plates and spread over the plate surface by using sterilized cotton swabs. MHA plates were bored by using sterilized 1ml micro tip and 100 µl each extract was poured into wells. MHA plates were incubated at 37^{o}C for 48 hours. After incubation, zone of inhibition were measured with the help of antibiotic zone scale **(Himedia laboratories Pvt. Ltd., Mumbai)** and recorded in millimeter **[23]**.

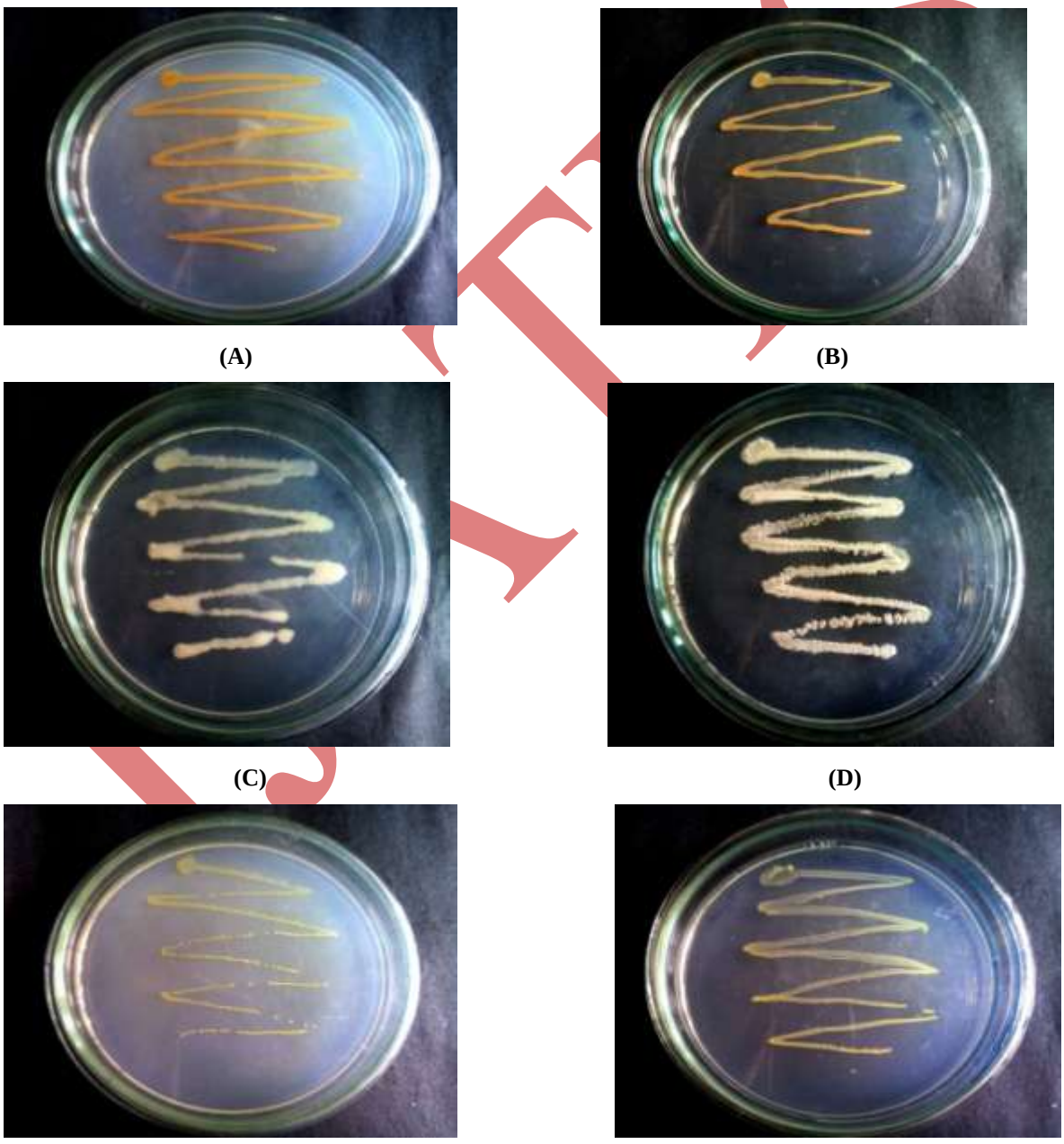
III. RESULTS

3.1. Isolation From Soil Sample

Soil samples were collected from 4 different location of Gwalior region for isolation of actinomycetes. The samples were serially diluted and spread over actinomycetes isolation agar plates. Mixed culture was seen **(Fig. 1)** which further transferred into other AIA plates. Pure colonies of actinomycetes were obtained after repeated streaking **(Fig. 2)**. Morphologically same isolates were discarded and different were selected for the next process. The 11 pure actinomycetes isolates were used for further study. All 11 isolates were transferred to glycerol stock and stored at 4^{o}C.

(A) (B)

Figure 1. Mixed microbial population on petri-plates by serial diluton.



(A) (B)

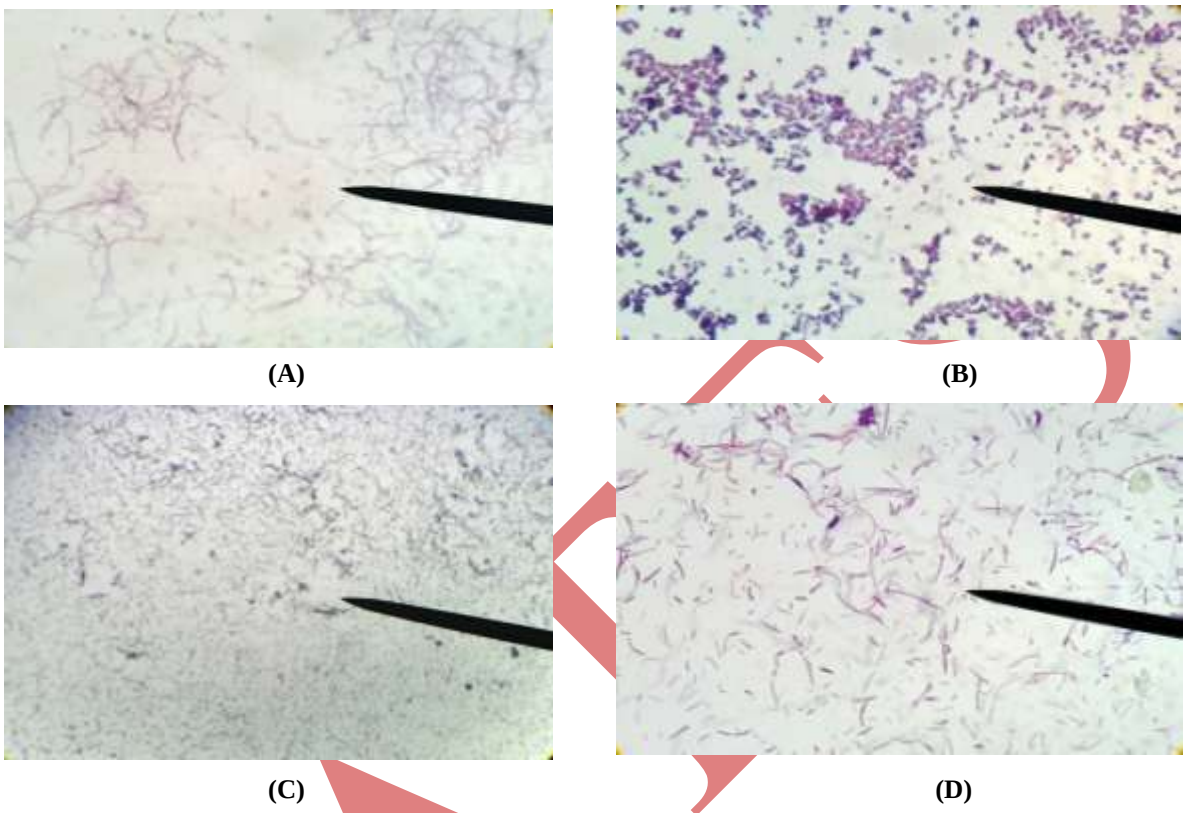
(C) (D)

(E) (F)

Figure 2. Pure colonies of actinomycetes isolates from different location of Gwalior.

3.2 Microscopic characterization

Gam staining was subjected to pure actinomycetes isolates. Micrograph (100X) of eleven isolates were examined **(Fig. 3)** and morphological patterns were obsereved and recorded in **TABLE 1**. Eleven isolates were found Gram positive.



(A) **(B)**

(C) **(D)**

Figure 3. Gram staining results of isolates under microscope (100X).

TABLE 1. Microscopic examination and Gram staining observation of purified isolates.

Actinomycetes isolates	Gram staining	Structure under microscope (100X)
GWL1	+ve	Mycelium
GWL2	+ve	Cocci
GWL3	+ve	Cocci
GWL4	+ve	Star shape
GWL5	+ve	Rod
GWL6	+ve	Cocci
GWL7	+ve	Filament shape
GWL8	+ve	Filament shape
GWL9	+ve	Cocci
GWL10	+ve	Cocci
GWL11	+ve	Rod

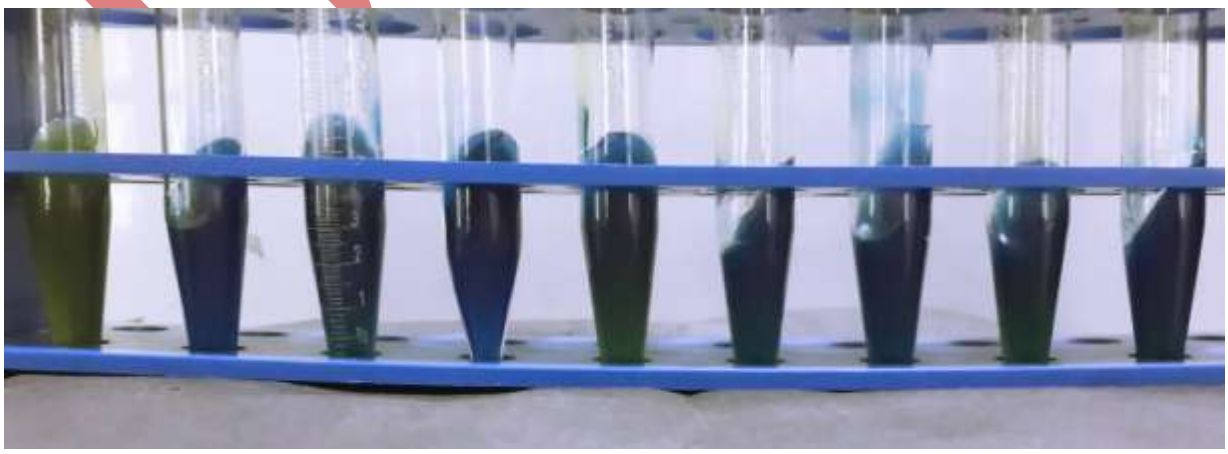
3.3. Biochemical characterization

Some biochemical test such as Indole test, Starch hydrolysis test, Casein hydrolysis test, Citrate utilization test, and Triple sugar iron test were performed for the all selective isolates. The results of test are shown **(Fig. 4)** and summarized **(TABLE 2).**

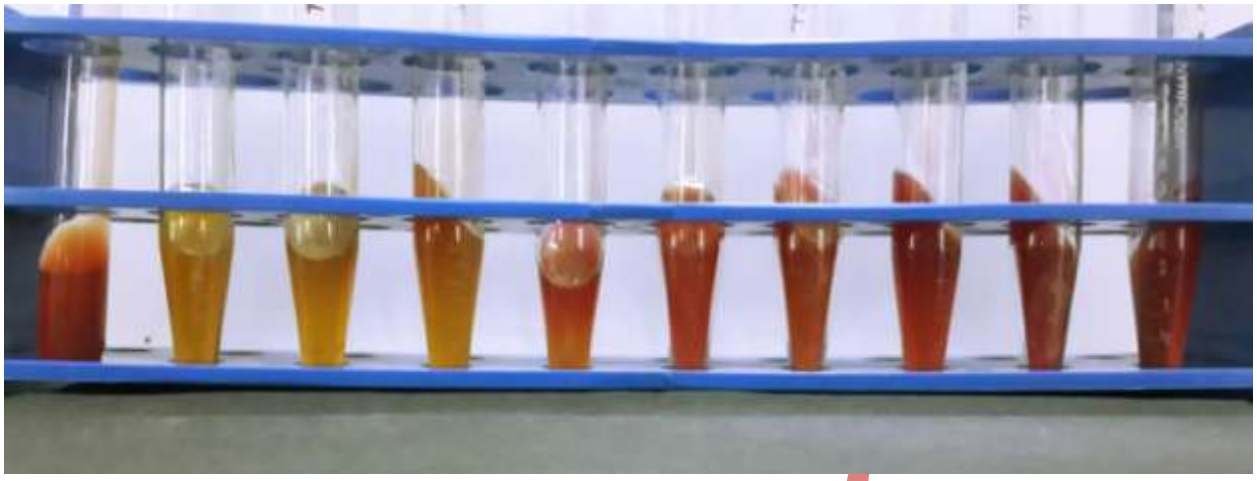
Indole Test

Starch Hydrolysis Test

Casein Hydrolysis Test



Citrate Utilization Test



Triple Sugar Iron Test

Figure 4. Results of biochemical characterization of isolates.

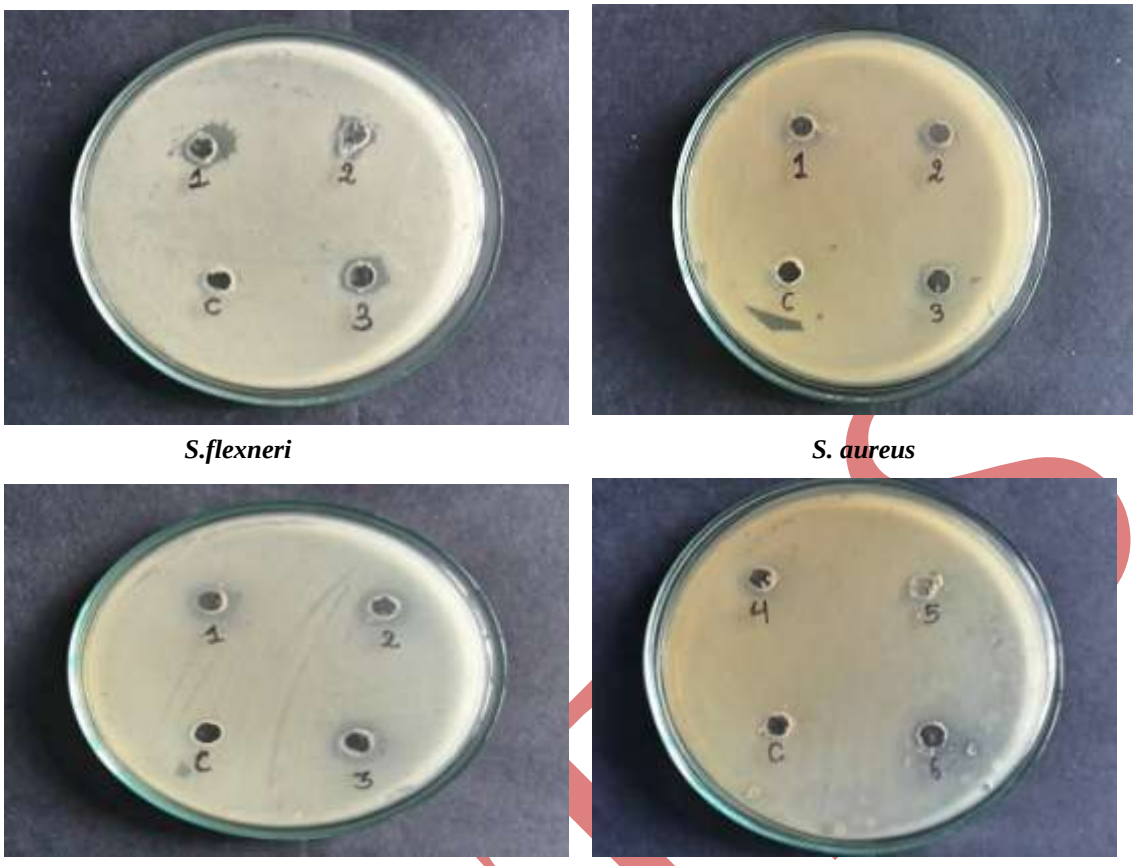
TABLE 2. Biochemical characterization of actinomycetes isolates.

Isolates	Indole	Starch	Casein	Citrate	TSI		
					A	B	C
GWL1	-ve	+ve	-ve	+ve	-ve	-ve	-ve
GWL2	-ve	+ve	-ve	+ve	-ve	-ve	-ve
GWL3	-ve	+ve	-ve	+ve	-ve	-ve	Glucose & Sucrose
GWL4	-ve	+ve	-ve	+ve	-ve	-ve	Glucose
GWL5	-ve	+ve	+ve	+ve	-ve	-ve	Glucose & Sucrose
GWL6	-ve	+ve	-ve	+ve	-ve	-ve	-ve
GWL7	-ve	+ve	-ve	+ve	-ve	-ve	-ve
GWL8	-ve	-ve	-ve	-ve	-ve	-ve	-ve
GWL9	-ve	+ve	+ve	-ve	-ve	-ve	-ve
GWL10	-ve	+ve	-ve	+ve	-ve	-ve	Glucose
GWL11	-ve	+ve	-ve	+ve	-ve	-ve	Glucose & Sucrose

Where **A:** Gas production, **B:** H_2S production, **C:** Sugar fermentation.

3.4. Antibacterial activity of isolates

Antibacterial activity of crude bioactive extract of isolates was screened on MHA media against selective pathogens by agar well diffusion method and zone of inhibition against bacterial **(TABLE 3)** pathogens were recorded **(Fig. 5).**



S.flexneri *S. aureus*

S. flexneri *S. typhi*

Figure 5. Extract of different isolates shows antibacterial activity against selective pathogenic strains.

TABLE 3. Antibacterial activity (ZOI in mm) results against selective pathogenic strains.

Isolates compound	PS1	PS2	PS3
GWL 1	11	10	11
GWL 2	10	10	12
GWL 3	10	10	15
GWL 4	-	-	-
GWL 5	-	-	-
GWL 6	-	8	15
GWL 7	7	7	7
GWL 8	7	7	7
GWL 9	7	12	7
GWL 10	-	11	-
GWL 11	-	-	-

Pathogenic strains (PS) are listed below respectively as in Table 3.

PS1. *Staphylococcus aureus*.

PS2. *Shigella flexneri*.

PS3. Salmonella typhi.

IV. DISCUSSION

Total 11 isolates were isolated and purified from 4 soil samples from different geographical region of Gwalior district, MP. The sample locations were highly rich in microbial diversity. All the isolates of this study were gram positive and their colony morphology was studied microscopically. About, 45.45% isolates were white or dirty white in colour, 36.36% were light orange in colour and 18.85% were brown in colour. Morphologically Isolates had branched mycelium. All 11 isolates were biochemically characterized. Majority of isolates (90.90%) were able to hydrolysis of starch; 18.18% isolates were produced proteolytic caseinase and utilize casein as carbon source efficiently; all isolates were negative for indole production; 81.81% isolates were positive for citrate utilization test, all isolates were negative for gas and hydrogen sulphide production while 18.18% isolates were found positive for glucose fermentation and 27.2% for sucrose fermentation. Although there are various biochemical test were perform but it was unable to identify other actinomycetes due to limitation of these test. After fermentation starch casein nitrate broth was found suitable and used for production of bioactive compounds from actinomycetes. Solvent extraction is generally used for the extraction of bioactive compounds form culture filterate obtained from media. In this study ethyl acetate was used to extract the bioactive compound. Ethyl acetate is a good solvent due to its high polarity and high volatility. Sufficient yield of crude extract was obtained from 100 ml fermented broth of isolate. The obtained bioactive compound was extracellular in nature and exhibit potent antibacterial activity. In this study it has been found that 27.27% isolates were reported with broad spectrum antibacterial activity. 45.45% isolates were not active against any test- organism. In this study it has been found that GWL 3 and GWL 6 demonstrate highest bacteriocidal activity against *Salmonella typhi* with 15mm ZOI. This study also concludes that maximum number of isolates exhibit antibacterial activity against *Salmonella typhi*. Pathogenic strain *Staphylococcus aureus* and *Shigella flexineri* found to be least sensitive towards actinomycetes isolates.

V. CONCLUSION

Gwalior has good region of biodiversity and has been adequately acceptable by the affluence of its microbial diversity. Actinomycetes represent the biggest possibility to obtain further medically, agriculturally and industrially useful compounds which may serve as direct drugs or indirectly as lead compounds for structural modifications and templates for the rationale drug design and other derivatives. So, further intensive studies are required for search of antimicrobial compound of microbial origin from Gwalior district. Potentially biotopes exclusively from Gwalior may be form an important input into pharmaceutical industries. As long as the major challenges in biotechnology and biomedicine remain (e.g. emerging diseases, established diseases, antibiotic resistance, and environmental pollution and need for renewable energy) microbial resources will be of interest to mankind providing sustainable and environmentally friendly solutions. Some new screening programs have been already developed for discovering of new species or unknown bioactive substances. One of the modern approaches is isolation and screening of microorganisms from relatively unknown or unstudied areas. Among microorganisms isolated from such areas actinomycetes are the least frequently met. In the recent years using molecular genetics methods it was found that the majority of the isolated microorganisms are new species. This expands the possibilities for their use in biotechnology.

VI. ACKNOWLEDGEMENTS

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REFERENCES

[1] M Oskay, A.U. Tamer and C. Azeri, Antibacterial activity of some actinomycetes isolates from farming soils of Turkey, *African J Biotechnol, 3*, 2004, 441-46.

[2] E Kuster and S.T. Williams, Selective media for the isolation of *Streptomycetes*, *Nature, 202*, 1964, 928-9.

[3] E Kuster, Taxonomy of soil actinomycetes and related organisms. In: Gray S, Parkinson T, editors. Ecology of soil bacteria. Liverpool University press; 1968.

[4] J Berdy, Bioactive microbial metabolites: a personal view, *J Antibiot, 58*, 2005, 1-26.

[5] R H Baltz, Antimicrobials from Actinomycetes: Back to the Future, *Microbe, 2*, 2007, 125-31.

[6] Y Okami and K. Hotta, Search and discovery of new antibiotics. In M. Goodfellow, S. T. Williams, M. Mordarski, Actinomycetes in Biotechnology, San Diego: Academic Press, Inc;1988;37-67.

[7] W R. Strohl, Antimicrobials. In: A. T. Bull, editor, Microbial Diversity and Bioprospecting, Washington DC: ASM press; 2004;366-55.

[8] G M Cragg and D.J. Newman, Plants as a source of anticancer agents, *Journal of Ethnopharmacology*, 100, 2005, 72-9.

[9] J Mann, Natural products as immunosuppressive agents, *Nat Prod Rep, 18*, 2001, 417-30.

[10] C W Pecznska and M. Mordarski, In: M. Goodfellow, S. T. Williams, M. Mordarski, Actinomycetes in Biotechnology, London: Academic press, UK; 1988;219-83.

[11] M S Butler and A.D. Buss, Natural products: The future scaffolds for novel antibiotics?, *Biochem Pharmacol, 71*, 2006, 919-29.

[12] Zinsser, Antimicrobial Agents, In: Joklik et al. editors. Zinsser Microbiology, 20th ed. Appleton and lang publishers, UK 1988;128-16.

[13] B Pandey, P. Ghimire and V. P. Agrawal, International Conference on the Great Himalayas: Climate, Health, Ecology, Management and Conservation, Kathmandu, Organized by Kathmandu University and the Aquatic Ecosystem Health and Management Society, Canada 2004.

[14] A J Alanis, Resistance to antibiotics: are we in the post-antibiotic era?, *Archives of Medical Research*, *36*, 2005, 697-705.

[15] K Hiramatsu, Vancomycin resistance in *Staphylococci*, Drug Resist Updates, 1, 1998, 135-150.

[16] B Bozdogan, D. Esel and C. Whitener, Antibacterial susceptibility of a vancomycin-resistant Staphylococcus aureus strain isolated at the Hershey Medical Center, *Journal of Antimicrobial Chemotherapy*, *52*, 2003, 864-68.

[17] Anonymous, Brief report: vancomycin-resistant *Staphylococcus aureus*-New York, *MMWR, 53*, 2004, 322-23.

[18] Saadoun, K. M. Hameed and A. Moussauui, Characterization and analysis of antibiotic activity of some aquatic Actinomycetes, *Microbios*, *99*, 1999, 173-9.

[19] L Pine and S. J. Watson, Evaluation of an isolation and maintenance medium for Actinomyces species and related organisms, *J Lab Clin Med.*, *54(1)*, 1959, 107-14.

[20] N Kumar, R.K. Singh, S.K. Mishra, A.K. Singh and U.C. Pachouri, Isolation and screening of soil *Actinomycetes* as source of antibiotics active against bacteria, *International Journal of Microbiology Research*, *2(12)*, 2010, 12-16.

[21] K R Aneja, Biochemical activities of Microorganisms, *Experiments in microbiology plant pathology & biotechnology*, 4th edition. New Delhi, New age international (p) Ltd publisher, 2003;245-74.

[22] A Sazci, A. Xradford and K. Erenle, Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method, *Journal of Applied bacteriology*, *61*, 1986, 559-62.

[23] M E Umashankar, G. Kumar, L. Kartik and K. V. Bhaskera Rao, Exploration of antagonistic actinobacteria from amirthy forest, *Int J Curr Pharma Research*, *1(2*), 2010, 16-9.