# Isolation and characterization of antibiotic producing bacterial strains from red soil of Himalayan region of Pakistan

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**Abstract**: The emergence of multi drug resistant microbial pathogens has become a global health challenge and set a dire requirement of searching new effective antimicrobials. Soil is an ultimate reservoir of biologically active micro flora, which harbors trillions of microbial strains producing compounds of commercial interest. Hence aim of the present study was an attempt to isolate and identify the antibiotic producing microbial strains from the red soil of Himalayan an unexplored region of Pakistan. In this study from 10 different soil samples only one bacterial strain was isolated capable of antimicrobial activity. Strain was identified by biochemical characteristics and final identification was done by API 20 NE kit which showed 99% homology with *P. aeruginosa.* Hence the strain was identified as *P. aeruginosa* S2. Antibacterial and antifungal activity of the *P. aeruginosa S2* showed that *Staphylococcus aureus* was extremely sensitive to it with a zone of inhibition of 42mm. *Staphylococcus epidermidis, Enterobacter aerogenes, Aspergillus fumigatus* and *Candida albicans* were also inhibited by the isolated strain. Effect of Glycerol, Copper sulphate (CuSo4), Sodium sulphate ($Na_2SO_4$) and Glycerol on antibiotic production was also evaluated by supplementing growth media with these chemicals. *Pseudomonas aeruginosa* was grown in bulk quantity using solid state fermentation and crude extract was prepared using organic solvents and subjected to silica gel column chromatography for purification of active compound. Purified compound showed antibacterial against human pathogens. The unexplored Kashmir Himalayas are of great significance because of its richness in biodiversity and need to be explored for isolation and characterization of native microbes for biologically active secondary metabolites. This un touched region may be considered as hub of new antimicrobials and may have applications in natural product-based drug discovery.

**Keywords**: Kashmir Himalays, antimicrobial activity, solid state fermentation, *Pseudomonas aeruginosa*, crude extract.

## INTRODUCTION

Antibiotics are also known as "antimicrobial compounds", are the low molecular-weight and non-protein molecules produced by the living microorganisms as secondary metabolites (Sethi *et al*., 2013; Eman and Noor, 2014). In the nature, antibiotics are widely distributed where they play a fundamental role in regulating the microbial population of soil, water, sewage, and compost. Human development was revolutionized by the antibiotic drugs in such a way that only some of the other scientific discoveries have. Antibiotics have not only enabled human beings to save lives of patients but they have also played a key role in achieving major advances not just in infections but also in medication and surgical treatment and in live stock (Gould and Bal, 2013). One of the most important public health concerns of the 21$^{st}$ century is microbial resistance to antibiotics (Woolhouse and Farrar, 2014; Laxminarayan *et al*., 2016). The promotion of resistance in bacteria is associated with the inaccurate prescription, over use or misuse of antibiotics, their extensive use in agriculture, poor hygiene and sanitation practices etc. To surmount the serious crisis of evolving pathogens, resistance bacterial and fungal species and multi drug resistance (MDR) amongst common bacterial strains, there is a continuous requirement of novel antimicrobial compounds (Alanis, 2005; Sharma *et al.,* 2011). Natural sources are for the most part unexplored and could serve as a possible source for novel antimicrobial drug discovery (Shah *et al.,* 2017). For exploring antimicrobial compound producing microorganisms that are acidophilic and acid tolerant in nature, red soils hence provides a potential source. It was revealed by the anecdotes that red soil has some antibiotic like properties, so this may be a reason that, historically red soil have been used by people to treat skin infections and diaper rash, and still it is used as an easy on the pock*et al*ternative to pharmaceutical products in some communities (Falkinham *et al.,* 2009). Based on the above considerations, plan of this study is to explore red soil for its antimicrobial activity and to isolate different antibacterial compound producing strains and their characterization, for the search of new antibiotics to combat the antibiotic resistance crisis faced by the world.

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## MATERIALS AND METHODS

### *Collection of soil sample*

Red soil samples were collected from selected locations of Kashmir region of Northwestern Himalaya. After removal of approx 3cm of the soil surface which is mostly debris, soil samples up to the depth of 15cm to 20cm were collected in sterile polythene bags and transferred to the laboratory for further procedures. Red soil samples were air dried for 3 to 4 hours, crushed, and sieved prior to use for isolation of microbial strains (Saadoun *et al*., 1999).

### *Isolation of bacterial strains*

Microbial strains were isolated by the serial dilution method. One gram of dried soil was weighed and added to 9ml of double distilled water (dd $H_2O$) in a sterile test tube and shaken well using vortex mixer, this stock solution was then diluted serially up to the dilution of $10^{-5}$. Three types of media were used for the isolation of bacterial strains from the soil sample named as Nutrient agar (NA), Tryptone soya agar (TSA) and MacConkey agar (MA). Spread plate technique was used for the inoculation of sample and strain isolation. From each dilution, 100µl of the aliquot was taken with the help of micropipette and spreaded over the surface of different media, with the help of a glass spreader. Plates were incubated at 37ºC for 24 to 48 hrs.

### *Test microorganisms*

Different clinical strains of bacteria and fungi were used as test strains for the secondary screening of selected isolate for evaluation of antibiotic production. Test bacterial organisms were collected from the microbiology laboratories of Pakistan Institute of Medical Sciences (PIMS), Armed Forces Institute of Pathology (AFIP), Rawalpindi Institute of Cardiology (RIC) and Bilal Hospital Rawalpindi, while fungal strains were collected from National Agriculture and Research Center (NARC). Test bacterial strains included: *Escherichia coli, Staphylococcus aureus, Shigella flexneri, Klebsiella pneumonia, Enterobacter aerogenes, Lactobacillus acidophilus, Staphylococcus epidermidis, Salmonella typhi, Listeria monocytogenes and Bacillus subtilis. Test fungal strains included: Aspergillus niger, Aspergillus fumigatus, Candida albicans and Trichophyton tonsurans.*

### *Preliminary screening of isolated strains for antibiotic production*

Isolated strains expected to be the antibiotic producers were screened for their antimicrobial spectrum by Agar well diffusion method to assess their capability of producing antibiotic metabolites (Uzair *et al*., 2008). For antibacterial activity a lawn of test bacterial strain was spreaded over the surface of Muller hintoin agar over the surface of nutrient agar plates by using sterile swabs, and a hole was punched aseptically, with the help of borer, in the center of agar plate. A volume of 100µl of the bacterial, grown in broth, was taken with the help of micropipette and introduced in the well of agar plates. Inoculated agar plates were then incubated at 37ºC for 24 & 48 hrs, for bacterial after incubation zone of inhibition was observed.

### *Identification and characterization of bacterial isolate*

After preliminary screening, the suspected antibiotic producer isolate was identified and characterized by morphological, cultural and biochemical tests. For preliminary identification of bacterial strain gram staining was done. Morphological identification of the selected isolate was done by observing color, shape, size and appearance of the colonies of the bacterial isolate grown on agar plate. Odor of the colonies grown on the agar plate and pigment produced (if any) were also observed. While biochemical tests Standardized identification of the selected strain was performed by using API 20 NE kit.

### *Induction of antibiotic metabolite production*

Before extraction of the bioactive compounds from the bacterial strains, selected through secondary screening, the optimization of culture media was done by using different chemicals such as Copper sulphate (CuSo4), Sodium sulphate (Na2So4) and Glycerol. 25ml nutrient agar was prepared in 4 volumes in 100ml conical flasks, 2mM $CuSo_4$, 2mM$Na_2So_4$ and 30%Glycerol were added separately in each flask respectively and one flask was taken as control which contained nutrient agar only. These differently supplemented nutrient agar media were poured into labeled petri dishes, media was allowed to set and then bacterial isolates were inoculated into the culture media and plates were incubated at 37ºC for 24hrs. After incubation the difference in growth of the isolates was observed as compared to the control and the supplemented media which supported maximum growth of *Pseudomonas aeruginosa* S2 was selected for further tests.

### *Solid state fermentation for the preparation of crude extract*

Extraction of the bioactive compound produced by *Pseudomonas aeruginosa* S2 was achieved by growing the strain using solid state fermentation method using nutrient agar supplemented with 30% glycerol., *Pseudomonas aeruginosa* S2 was inoculated and plates were incubated at 37ºC for 5 to 7 days for maximum growth. After 7 days incubation when maximum growth of the isolates was achieved, the culture media from all the petri dishes was transferred into 1000ml beakers and was chopped in to very small pieces. Later on the crushed media was shifted into the conical flasks and 10 grams of sodium chloride (NaCl) was added in each conical flask, 80% acetone was prepared, and added in a volume such that the crumbled media were submerged in it. The active metabolite was separated from the solid residues by filtration of the acetone and fermented culture mixture by

using Whatman No.1 filter paper. Antibiotics are produced extra cellularly so the supernatant filtrate was collected in a conical flask, an equal volume of ethyl acetate was added in it, and was vigorously shaken. The Sample was transferred into the separating funnels in three batches, and was allowed to stand for 15 to 30 minutes, until two layers were clearly visible. The organic layer or the solvent phase was evaporated at room temperature and finally the dried residues were dissolved in Dimethyl Sulfoxide (DMSO). The crude extracts obtained from the isolates were stored at 4ºC temperature, and then were used for further studies (Raja and Prabakaran, 2011).

***Antibacterial compound purification by column chromatography***

Antibacterial compounds were purified from crude extract by silica gel column chromatography. The crude ethyl acetate extract was again extracted with hexane, chloroform, ethyl acetate respectively. The ethyl acetate extract (4.95g) was subjected to column chromatography (CC) over silica gel column using hexane with gradient of ethyl acetate up to 100%. Purity of separated compounds was evaluated on TLC cards.

***Determination of antibacterial activity of the crude extract***

Antibacterial activity of the major compound purified by column chromatography was determined by the Kirby Bauer disc diffusion method. DMSO was used as a control and test organisms against which this activity was performed are described earlier. Nutrient agar media was used and lawn of bacterial test strains were inoculated into the agar plates by using sterile swabs. Sterile filter paper disc of about 6mm diameter which contained 10µL of purified compound was placed on the agar plate, with one disc containing DMSO as a control and plates were incubated at 37ºC for 24hrs. After incubation the zone of clearance were measured in mm and recorded.

## RESULTS

***Isolation of bacterial and fungal strains***

From different localities ten different red soil samples (table 1) were collected and evaluated for the isolation of bacterial strains with antibiotic producing potential. A total of 21 morphologically different bacterial strains were isolated. The bacterial isolates were given code as S1, S2, S3, S4… S20 and S21.

***Screening of isolated strains for antibiotic production***

Bacterial strains that were isolated from the soil samples were then subjected to the preliminary screening for antimicrobial activity against clinical strains. Out of these 21 bacterial isolates only identified as *Pseudomonas aeruginosa* S2 produced zones of inhibition against test clinical strains.

***Optimization of culture conditions and solid state fermentation***

Glycerol (30%) supplemented nutrient agar plate showed maximum growth of S2 isolates and maximum pigment production. A total of 24.86grams of crude extract infused in DMSO was obtained and collected in sterile vials and then was evaluated for the antibacterial activity and subjected to silica gel column chromatography for purification.

***Antibacterial activity of pure compounds obtained from Column chromatography***

A UV active compound purified from column chromatography (fig. 1) showed antibacterial activity against five Gram positive clinical strains and 5 Gram negative clinical strains. Calculated zones of inhibition against these strains are demonstrated in table (2) and zone of inhibition against *Staphylococuus aureus* is shown in fig. 1

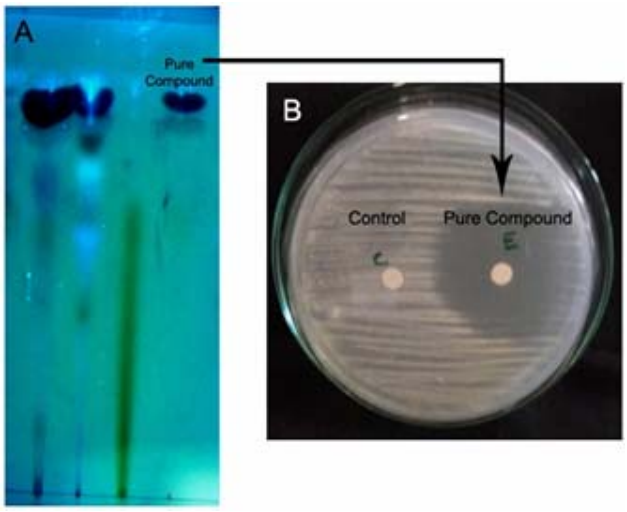


**Fig. 1**: A, Thin layer chromatography of elute obtained from crude extract loaded on silica gel column showing pure UV active compound. B, Antibacterial activity of pure UV active compound against *Staphylococcua aureus.*

## DISCUSSION

Nowadays due to the emergence of multi drug resistant bacterial pathogens, crucial challenges for efficient treatment of diseases have appeared (Abo-Shadi *et al.,* 2010). The development of effective antibiotics would determine the future of modern medicine (Handelsman 2005; Laxminarayan *et al.,* 2016). Soil is an ultimate reservoir of biologically active micro flora, which provides nearly all of the clinical antimicrobial drugs used today (Demain and Fang, 2000; Foster and Woodruff, 2010). Even though, soils are considered as an excellent source for the isolation of antibiotic producing microbes, at present, exploration of formerly ignored ecosystems is the emphasized (Cragg *et al.,* 1997; Ouhdouch *et al.,* 2001; Prabavathy *et al.,* 2006). The unexplored Kashmir

**Table 1**: Samples collected from Kashmir region of the Himalayas and total bacterial count.

| Sample Codes | Sampling Area | Bacterial counts (CFU/ml) |
|---|---|---|
| H2 | Hajira | $8.1\times10^4$ |
| H3 | Muzafarabad | $2.1\times10^5$ |
| H4 | Narh | $4.9\times10^5$ |
| H5 | Abbotabad | $3.8\times10^2$ |
| H6 | Kohala | $2.5\times10^5$ |
| H7 | Thorar | $5\times10^2$ |
| H8 | Hattian bala | $3.8\times10^3$ |
| H9 | Sudhanoti | $7.4\times10^5$ |
| H10 | Khuiratta | $6.2\times10^4$ |

**Table 2**: Antibacterial activity of the pure compounds against clinical bacterial strains

| Test strain | Zone of inhibition | Sensitivity pattern |
|---|---|---|
| *Escherichia coli* | 3mm | _ |
| *Staphylococcus aureus* | 28 mm | ++ |
| *Shigella flexneri* | 0 | _ |
| *Klebsiella pneumoniae* | 1mm | _ |
| *Enterobacter aerogenes* | 38 mm | +++ |
| *Lactobacillus acidophilus* | 2mm | _ |
| *Staphylococcus epidermidis* | 14mm | + |
| *Salmonella typhi* | 0 | _ |
| *Listeria monocytogenes* | 2mm | _ |
| *Bacillus subtilis* | 0 | _ |

Resistant (-): For total diameter smaller than 10 mm, Sensitive (+): For total diameter between 11-20 mm
Very sensitive (++): For total diameter between 22-30 mm, Extremely sensitive (+++): For total diameter larger than 31mm

Himalayas are of great significance because of its richness in biodiversity, it can be explored for isolation and characterization of native microbes for biologically active secondary metabolites (Shah *et al.,* 2017). Hence aim of the present study was to isolate and identify antibiotic producing bacterial strains from the red soils of Himalayan region of Pakistan. Total 21 bacterial were isolated from the selected red soil samples which were then subjected to the preliminary screening for their antibiotic production ability. From the isolated bacterial strains, only one strain was found to inhibit bacterial growth. The selected strain was characterized on the basis of its colonial and cellular morphology and biochemical tests the antibiotic producing strain was identified as *Pseudomonas aerugionsa* S2. A diffusible blue green pigment production by the colonies was observed this type of colonial morphology was also reported by Isnansetyo and Kamei, (2009) and In this study when the antibacterial activity of the isolate was assessed against test strains, it was observed that Gram positive *Staphylococcus aureus* was extremely sensitive to the isolated strain with a zone of inhibition of 42mm in diameter whereas *Enterobacter aerogenes and Staphylococcus epidermidis* were also sensitive to the isolated strain and produced zone of clearance of 26mm and 22mm respectively. It was estimated from the present study that the isolate has more bactericidal effect against Gram positive pathogens. Antibacterial activity of the pure compound isolated from the crude extract of *Pseudomonas aeruginosa* S2 through column chromatography showed enhanced antibacterial activity against 10 different clinical isolates, among which seven strains showed non-sensitivity against it. Maximum activity was shown by *Enterobacter aerogenes* with 38mm inhibition zone, *S. aureus* and *S. epidermidis* also showed sensitivity to the crude extract of isolate. In a similar study by Rina *et al.,* (2015) it was reported that *Staphlococcus aureus* was inhibited by the supernatant of *P. aeruginosa* with a zone of 25mm in diameter. While here in this study the zone of inhibition against *S. aureus* was measured as 28mm, so a slight difference in the diameter was observed. Sensitivity of *S. epidermidis* to the isolate identified as *P. aeruginosa* was also reported by (Zhiqiang *et al.,* 2009).

## CONCLUSION

This study shows that the bacterial isolates from the soil sample has the potential to act as a source of new antimicrobial compounds, against pathogenic microbes of human origin. Here, we found that the Himalayan region is rich in biodiversity and has been sufficiently acceptable due to its vast microbial diversity. The extra cellular substance produced by *Pseudomonas aeruginosa S2* may become effective as antibiotics if exploited and purified as those of the commercially available antibiotics.

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