

Effect of pH and Temperature on Secondary Metabolite Isolated from Soil Bacteria

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Received: 15 Dec 2017/Revised: 17 Jan 2018/Accepted: 27 Feb 2018

ABSTRACT- Secondary metabolites used to treat infections caused by microbial pathogens. It can cause illness to humans and animals. This study was carried out to screen for potential antimicrobial producing microbes from soil samples collected from different area of Garhwal region in Uttarakhand, India. Dermatophytes are a major group of closely pathogenic fungi that infect skin, hair and nails in humans and animals. In the present study, a trial was done to find out a new antimicrobial agent producing bacteria from soil samples. Antifungal activity of each bacterial isolate against dermatophytic fungus was performed with dual culture and agar well diffusion methods using SDA medium. All the isolated bacterial colonies were observed for primary screening for their anti-dermatophytic activity against the pathogenic species of dermatophytes *Trichophyton* (MTCC-272), *Epidermophyton*, (MTCC-465), *Microsporum* (MTCC-964), *Candida albicans*, and *Aspergillus niger* were screened by well diffusion methods. Among the total 75 bacterial isolates, only 15 of them were capable of synthesizing antimicrobial metabolites in primary screening. Out of these fifteen isolated bacterial sp. only four Bacterial colonies were found to most potent that was obtained from the agricultural region of Srinagar Garhwal, in Uttarakhand found to exhibit the highest antagonistic and anti-dermatophytic activity against most of the used pathogenic dermatophytes in the study. The Physicochemical and biochemical characters of the isolated bacterial species were matched with *Bacillus* and *Pseudomonas* sp. Then antifungal activity was measured in different pH and temperature. Thus, the isolated strain was given the suggested name PA-4(a), PA-2(a), PA-2 (PK-1), and PA-1(E). This study indicated that the microorganisms isolated from agriculture land of Garhwal region in Uttarakhand (India) soil could be an interesting source of antimicrobial bioactive compound.

Key-words- Antibiotic, Antagonistic activity, Dermatophytes, Secondary metabolites, Soil Bacteria

INTRODUCTION

Antimicrobial agents are the most promising field worldwide with the need of continuous search of new ones. The incidence of antibiotic resistance towards current synthetic drugs has been rapidly increasing. Natural organic compounds produced by microorganisms are an important screening target for a variety of bioactive substances. These active compounds can be further explored as new drugs or antibiotics [1] the need for new antibiotics has been highlighted recently with the increasing pace of emergence of drug resistant pathogens (MRSA, XDR-TB, etc).[2] Current drug development methods have been slow to produce effective new antibiotics as they have primarily focused on modifying existing classes of antibiotics or using genomics to the identify new drug targets. San Lang has reported bacterial strains of *B. subtilis* isolated from soil that produced an antifungal agent active against *Fusarium oxysporum* [3].

Dermatophytic fungi usually do not invade living tissues, but colonize the outer layer of the skin and their products such as acid proteinases, elastase, keratinases, and other proteinases act as virulence factors. Antibacterial and antifungal drug development today takes place in either the academic or the pharmaceutical company environment. The increase in antibiotics resistance is prevailing due to inappropriate use of antibiotics by general health practitioners and leads to the effectiveness of front line antibiotics worldwide. This situation has become an alarming condition to drug manufacturers and public health practitioners. Therefore, this study is an attempt to investigate indigenous soil resources with potential of antimicrobial production that could be used to produce new product with better efficacies.[4-6]

MATERIALS AND METHODS

Collection of Soil Sample- Soil samples were collected in various locations from maletha in Srinagar Garhwal region of Uttarakhand during the research work. Samples were collected in the month of February 2012 to November 2012. Several diverse habitats included rhizosphere of plant, agricultural soil, herbal garden, and usar land in different areas were selected for the isolation of bacteria having good antimicrobial activity. The samples were taken up to the depth of 20cm of the soil

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Quick Response Code	Website: www.ijlssr.com
	 DOI: 10.21276/ijlssr.2018.4.2.19

surface. The samples were placed in polythene bags, closed tightly and stored in cool and dry place.

Main focus on the isolation of bacteria producing good antimicrobial substances from the selected region where so many area having good cultivation and no any serious type of epidemics and endemics assuming that present of soil bacteria having good antimicrobial activity. The bacterial isolates showing antifungal activity will be further tested for activity by spot inoculation method or Agar well method.

Fungal culture Used- The cultures i.e. *Trichophyton* sp., *Microsporium* sp. *Candida*, *P. citrinum*., *A. niger* were obtained from the standard culture collection centers and maintained in the Research Laboratory of Devsthal Institute of Training and Research, Dehradun, India. These cultures were maintained on nutrient agar slants at first being incubated at 37°C for 16-24 hours and then stored at 4°C as stock cultures for further study.

Isolation of Microorganisms from soil sample-

Soil samples were collected from different area as mentioned was treated with physical and chemical method before plating to eliminate common microbes. One gram of each soil samples was suspended in 10 ml of sterilized distilled water for serial dilution followed by crowded plate, pour plate, and spread plate technique. Further isolation of pure culture from the primary isolation was carried out by streak plate method. The soil samples were plated by serial dilution method on Nutrient agar (NA), Sabouraud's Dextrose agar (SDA), Potato dextrose agar (PDA), Yeast Glucose broth, Trypticase Soy agar/broth, and Carbohydrate fermentation medium. Plates were incubated at 27°C for 1-2 weeks for 48-72 hrs.

Identification of Antibiotic producing microorganisms-

Secondary metabolites producing microbes from soil samples were further identified by various morphological, biochemical and physiological characteristics were identified according to the scheme described in Sneath^[7] in Bergey's Manual of Systematic Bacteriology.

Preparation of the Bacterial strain inoculums-

The isolated bacterial strains from primary isolation were inoculated in nutrient broth media and Trypticase soy broth medium for 48 hours. The cultures were centrifuged at 7000 rpm for 10 min, the supernatant was collected and stored at -7°C until used. This supernatant was used to study antibacterial activity of isolated bacteria among pathogenic Bacterial species.

Determination of Antibacterial activity-

An aliquot of 0.5ml of the cell free supernatant of the isolated bacterial strain from primary isolation was added into the well of seeded test bacterial culture agar plate. The plates were incubated in upright condition at 37°C for bacteria and 27°C for fungal isolates for 24 to 48 hours till the supernatant diffused into the agar plate was further incubated for 72 hrs then observed for appearance of zone

of inhibition around the wells. The zone diameter was measured in mm.

Effect of environmental factors

Effect of Temperature on Antimicrobial activity-

Sucrose medium in 4 separate flasks were inoculated with the isolated strains. The tubes incubated at 27, 30, 37, and 40°C for 72 hours. At the end of the incubation broth was assessed for growth and antibiotic production.

Effect of pH on Antimicrobial activity-

The 50 ml of sucrose medium in different flask were adjusted at initial pH 6.0, 6.5, 7.0, 7.5, and 8.0. The tubes were sterilized at 121°C for 15 mm and after cooling were incubated with the test organisms. The tube were incubated at 37°C for 72 hours and assayed for antibiotic production and biomass yields.

RESULTS

In the present study, total fifteen isolates was found active against in primary screening. Out of these fifteen only four soil isolates was found to maximum inhibition against test microorganisms which on microscopic examination. These isolates were identified by various physical, chemical, and biochemical method, which belongs to genus *Bacillus* and *Pseudomonas*. The present study was undertaken to explore the antifungal potential of this bacterial isolates and to access the effect of physical factors on the production of antifungal substances. It was found that temperature and pH is also one of the most important factors for the growth of microorganisms and the production of secondary metabolites.

Screening of isolates against known fungal pathogens-

The bacterial isolates showing good antimicrobial activity in primary isolation as antagonistic activity in crowded plate techniques (as judged by the zone of inhibition around the colony) was further tested against *T. rubrum*, *Candida* sp., *Microsporium* sp., and *A. niger* assessed for antimicrobial activity by perpendicular streak method and Agar well diffusion method^[8]. Out of the 15 isolates in primary screening, only 4 isolated strains were measured for strong antimicrobial activity namely as PA-4(a), PA-2(a), PA-2 (PK-1) & PA-1(E).

Extraction of the Antifungal substances

Mass cultivation and Antimicrobial assay-

Mass cultivation of isolated bacterial sp. showed antimicrobial activity was carried out by using 250 ml Erlenmeyer flasks containing 100 ml of Trypticase soy broth were incubated on a rotary shaker (200 rpm) at 30°C for 2-3 days. After incubation the culture was extracted by using centrifugation at 2500 rpm for 15 min in centrifuge tube. Antibacterial activity of this fraction against test organisms was done by well diffusion method^[9] with 0.5 ml of bacterial extracts used in the assay. The plates were kept at 40°C for 2 hr. before they incubated at 27°C for 48 h. Antimicrobial activity was assessed by measuring zone of growth inhibition around the well.

Selection of most potent producer and its characterization- Among different promising isolates, four most potent bacterial isolate showed broadest inhibitory spectrum was characterized and identified according to the criteria as given in Bergey's Manual of

Determinative Bacteriology ^[10] this method used for studying morphological, cultural and biochemical characterization of the isolates was given ^[11] shown in Table 1.

Table 1: Morphological and cultural characteristics of the isolate

S. No.	Characteristics	PA-4(a)	PA-2(a)	PA-2 (PK-1)	PA-1(E)
1	Gram staining	Gram +ve	Gram +ve	Gram +ve	Gram -ve
2	Cell shape and size	Rod measuring 0.7–0.9x 2-4 micrometer in size. Rode occurring singly in pairs and in chains of 2-9 cells	Rod	Rod	Rod
3	Spore	Oval, central to sub-terminal in position	+ve	+ve	Non spore forming
4	Anaerobic growth	–	–	–	–
5	Catalase Test	+ve	+ve	+ve	+ve
6	Pigment Production	+ve	-ve	+ve	+ve
7	Acid from				
	Glucose	+	+	+	+
	Galactose	+	+	+	+
	Maltose	+	+	+	+
	Mannitol	+	+	+	+
8	IMVIC	+	+	+	+
	Growth at temperature (°C)				
9	30	+	+	+	
	37	+	+	+	
	42	+	+	+	
	45	–	+	+	
	50	–	–	–	

Effect of incubation Temperature on antibiotic production- The effect of different parameter of selected pH 6.0, 6.5, 7.0, 7.5, 8.0, & 8.5 and temperature 27, 30, 37, and 40°C for 72 hrs on antibiotic production were studied. It was found to be the best pH and temperature for antibiotic production in comparison to many other inorganic and organic sources tested. Maximum production was found, when the concentration of pH and temperature in the medium was pH 7.5, 8, 8.5 (Table 2 to Table 4), Fig. 1 & Fig. 2 and temp. 30°C, 37°C, and 40°C (Table 5 to Table 7 & Fig. 3 & Fig. 4).

In the present study, these four isolate PA-4(a) have maximum antimicrobial activity against all tested pathogens. At this temperature maximum growth was recorded at O.D. of 1.814 and antibiotic activity in the terms of zone of inhibition was measured. Less antibiotic production was recorded at temperature range of 27°C and pH 6, where O.D. was 1.128 and zone diameter of 6 mm was observed.

Table 2: Effect of pH 7.5

S. No.	Isolated strain	Zone of Inhibition (mm) against Test Organisms			
		<i>T. rubrum</i>	<i>M. canis</i>	<i>A. niger</i>	<i>Candida sp.</i>
1	PA-4(a)	20	16	15	10
2	PA-2(a)	14	18	19	13
3	PA-2(PK-1)	12	13	15	10
4	PA-1(E)	10	15	16	10

Table 3: Effect of pH 8

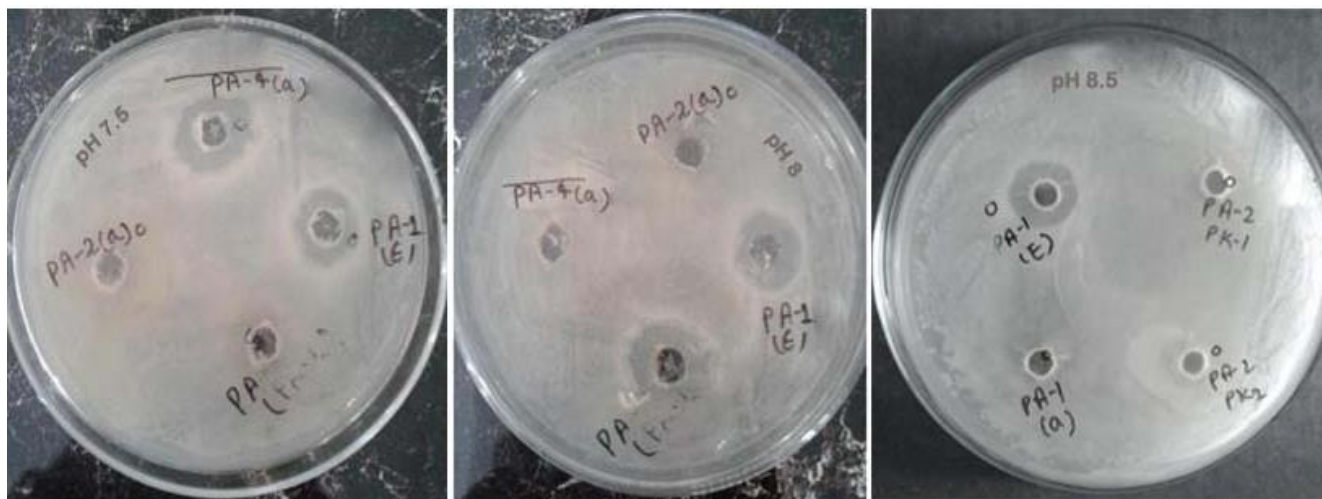
S. No.	Isolated strain	Zone of Inhibition (mm) against Test Organisms			
		<i>T. rubrum</i>	<i>M. canis</i>	<i>A. niger</i>	<i>Candida sp.</i>
1	PA-4(a)	10	12	15	19
2	PA-2(a)	14	-	-	-
3	PA-2(PK-1)	22	-	-	-
4	PA-1(E)	18	19	15	13

Table 4: Effect of pH 8.5

S. No.	Isolated strain	Zone of Inhibition (mm) against Test Organisms			
		<i>T. rubrum</i>	<i>M. canis</i>	<i>A. niger</i>	<i>Candida sp.</i>
1	PA-4(a)	12	09	28	19
2	PA-2(a)	10	-	18	-
3	PA-2(PK-1)	16	-	-	12
4	PA-1(E)	20	10	25	13



Fig. 1: Effect of pH on *Candida sp.*



A: pH 7.5

B: pH-8

C: pH-8.5

Fig. 2: Effect of pH on *Trycophyton rubrum*

Table 5: Effect of temperature 30°C

S. No	Isolated strain	Zone of Inhibition (mm) against Test Organisms			
		<i>T. rubrum</i>	<i>M. canis</i>	<i>A. niger</i>	<i>Candida sp.</i>
1	PA-4(a)	16	18	20	10
2	PA-2(a)	18	15	18	12
3	PA-2(PK-1)	19	20	-	14
4	PA-1(E)	12	15	22	11

Table 6: Effect of temperature 37°C

S. No.	Isolated strain	Zone of Inhibition (mm) against Test Organisms			
		<i>T. rubrum</i>	<i>M. canis</i>	<i>A. niger</i>	<i>Candida sp.</i>
1	PA-4(a)	14	12	-	19
2	PA-2(a)	12	11	10	14
3	PA-2(PK-1)	19	18	15	20
4	PA-1(E)	16	19	15	13

Table 7: Effect of temperature 40°C

S. No	Isolated strain	Zone of Inhibition (mm) against Test Organisms			
		<i>T. rubrum</i>	<i>M. canis</i>	<i>A. niger</i>	<i>Candida sp.</i>
1	PA-4(a)	18	12	-	18
2	PA-2(a)	15	11	18	-
3	PA-2(PK-1)	-	-	14	12
4	PA-1(E)	13	19	15	14

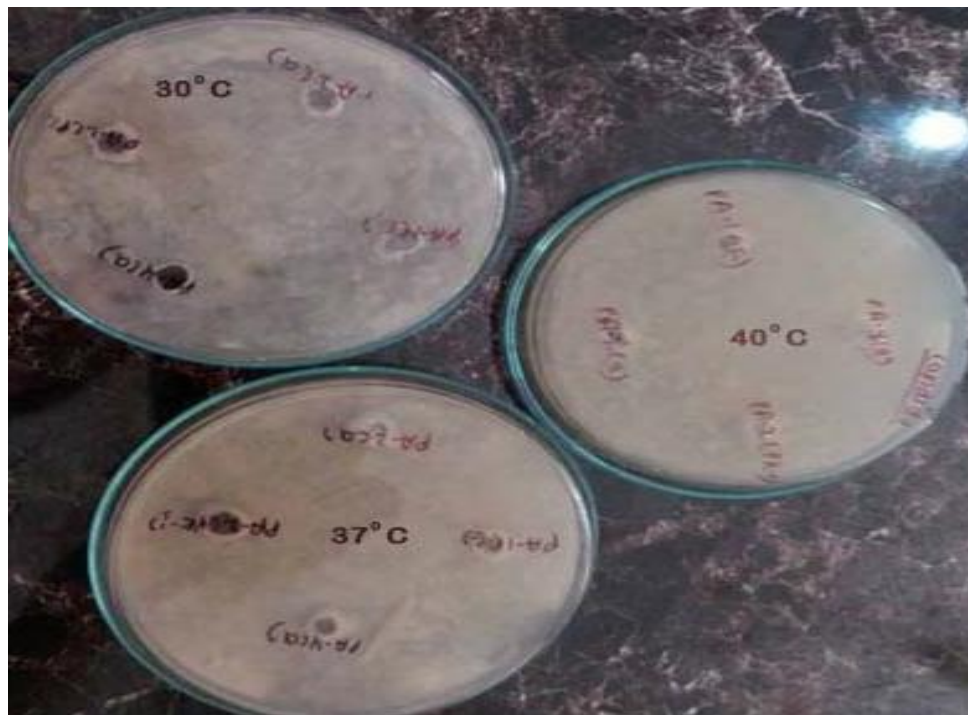


Fig. 3: Effect of Temperature on *Candida* sp.

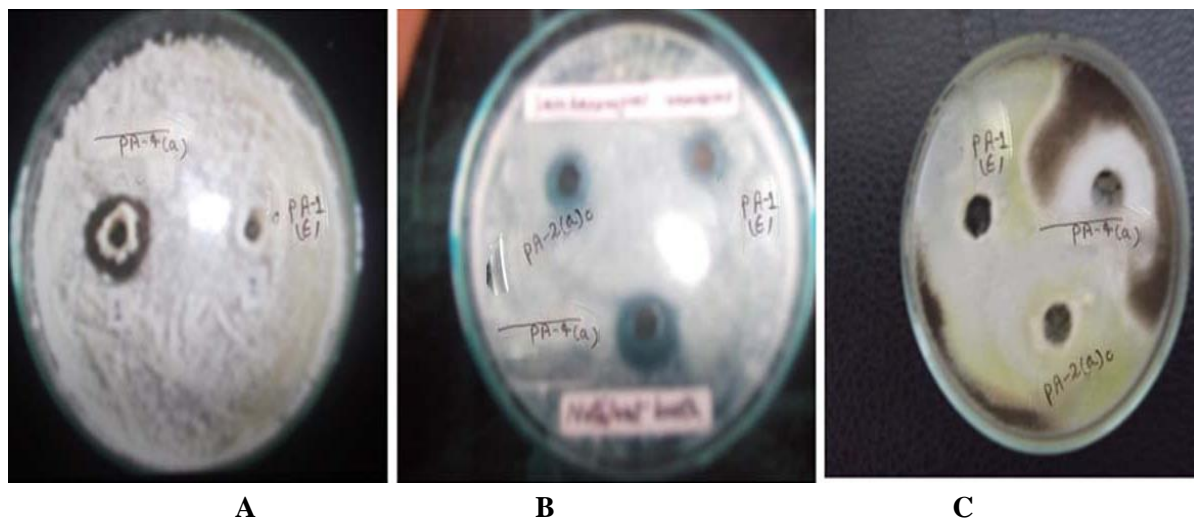


Fig. 4 A: Effect of Temperature at 30°C on *Microsporium* sp.
B: Effect of Temperature at 30°C on *Trychophyton* sp.
C: Effect of temperature at 30°C on *A. niger*

DISCUSSION

In comparison to bacterial and viral infections, fungal infections are not common yet fungal disease create sufficient burden on health care system. In the present study 37°C was found to be the optimum temperature for the synthesis of secondary metabolite. Optimum pH was measured 7.0 to 8.5 and maximum zone was obtained 28 mm. At all other temperature and pH less growth and antibiotic was observed. By the help of morphological, cultural and biochemical activity these spp. was fall into the genus *Bacillus* sp. and *Pseudomonas* sp. [12].

Inhibition of different fungi by isolates- Culture supernatant of isolated species grown in trypticase soy broth was tested for antifungal activity against 7 pathogenic and opportunistic pathogenic fungi viz.,

C. albicans, *Penicillium* sp., *A. niger*, *A. flavus*, *A. fumigatus*, *M. canis* and *Trichophyton rubrum*. Out of these isolates show antagonistic activity against only four fungi namely *T. rubrum*, *M. canis*, *C. albicans*, *Penicillium* sp, *A. niger*. *Bacillomycine D*, an aurantin group of antibiotic found to be produced by *Bacillus subtilis* AU 195 that was antagonistic to *A. fumigates* [13].

Effect of temperature on Antimicrobial activity- The optimum temperature for growth of and the production of antifungal substances was found to be 37°C and maximum zone diameter of 20 mm against *C. albicans*. Almost all isolated strain was active at 37°C against *T. rubrum*, *M. canis*, *C. albicans*, *Penicillium* sp, *A. niger*. In a recent study it was reported that in case of *Bacillus subtilis*, maximum synthesis of an antifungal substance occurred at 37°C [14].

Effect of pH on Antimicrobial activity- The optimum pH for the synthesis of antifungal substance was 7.5, 8 and 8.5. But all isolated strain was active against all four fungal pathogen showed inhibitory effect at pH 7.5. It was reported that in the case of *B. aurantinus* the maximum synthesis of antibiotic aurantin in occurred when the organism was grown at pH 7.5^[15].

CONCLUSIONS

Antagonistic activity was tested against eight fungal pathogens, of these only four were inhibited with a maximum inhibition of zone at 28 mm. Optimum temperature and pH for the growth of the producer organisms and the synthesis of antibiotic was at 7.5, 8, 8.5 & 30°C, 37°C, and 40°C. In this study, we concluded that the soil has maximum microorganisms and antimicrobial potential.

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How to cite this article:

Bamola N, Gupta RK. Effect of pH and Temperature on Secondary Metabolite Isolated from Soil Bacteria. *Int. J. Life Sci. Scienti. Res.*, 2018; 4(2): 1729-1735. DOI:10.21276/ijlssr.2018.4.2.19

Source of Financial Support: Nil, **Conflict of interest:** Nil