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**Original Research Article**

# Preliminary Screening of Primary and Secondary Metabolite Production from Soil *Bacillus* by Spot Inoculation Technique

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**A B S T R A C T**

The present study deals with isolation and preliminary screening of industrially important strains of bacillus species for maximum production of proteolytic and anti-microbial activities. Total of 29 strains were isolated from Rhizosphere soil of chilli plant from kaza agricultural fields. According to Bergey's manual of systematic bacteriology, 04 out of 29 isolates were tentatively identified as genus bacillus. Out of 29 isolates four strains were more effective against the indicator test bacterial and fungal pathogens. Were in case of proteolytic activity, all four isolates had shown positive results, among four, AS-10 (++), AS-9 (++) and AS-6 (++).were as AS-7 shows least proteolytic activity. These hyper-producing strains of bacillus can be exploited in industries for commercial product formation.

**Keywords**

Bacillus, Rhizosphere, anti-bacterial, proteolytic hyper-production, metabolite

## Introduction

Microorganisms are good sources for the production of biologically active secondary metabolites (Monaghan RL *et al.*, 1990). Bacteria belonging to the genus *Bacillus* are among the most widely distributed microorganisms have produced antibiotics in the soluble protein structure and that these antibiotics have been found to be cheaper and more effective, these microorganisms are preferable for commercial production (Priest FG *et al.*, 1989). Also, Strains of *Bacillus* are known producers of bioactive cyclic lipopeptides (Konz D *et al.*, 1999). *Bacillus* has been investigated for their ability to produce so called bacteriocin like inhibitory substance BLIS. It has been reported that strains of *B. thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium and B. cereus* produce BLIS (Motta AS *et al.*, 2007). On the other hand, *Bacillus subtilis* showed a production of a large number of antibiotics, which are classified as non-ribosomal (Stein T *et al.*, 2005). In another study, wild type strains of genus *Bacillus* were screened for their antimicrobial activity. Two strains exhibited antimicrobial activity against *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aspergillus niger* and were identified as *Bacillus polymyxa* and *Bacillus*. An antibiotic compound called Permetin A was purified from the culture filtrate of *Bacillus circulans (*Zuber P *et al.*, 1993*).* It showed in *vitro* activity against Gram negative, Gram-positive and some anaerobic bacteria. Butirosin produced by *Bacillus circulans* is among the clinically important 2-deoxy

streptamine containing amino glycoside antibiotics. Although, *Bacillus circulans* recorded its antimicrobial activity against Gram positive, it exhibit poor antimicrobial activity Gram negative bacteria. Not only bacterial secondary metabolites have antimicrobial activity against the growth of microorganisms, but also some bacterial enzymes involved in such important phenomena eg. Proteolytic activity. Notable among these are the large number and variety of antibiotics that bacillus produce which today are indispensable for the treatment of variety of infections. They are produced as beta lactums metabolites and have no physiological role in growth phase (Srinivasan MC *et al.,* 1991). The aim of this study was to screen and search for hyper-producer strains of bacillus for production of antimicrobial and proteolytic secondary metabolites.

## Materials and Methods

### Isolation and identification of bacteria

Bacterial samples were collected from soil Rhizosphere of chilli plant from the agricultural fields of kaza, Andhra pradesh India. Samples were obtained 5 to 7cm depth from the surface of the soil and kept in sterile plastic bags, then were transferred directly to the laboratory and air dried. 10 g of each soil sample was added to 40 ml sterilized distilled water in 250 ml conical flask, shaked well for 10 min and the soil suspension was allowed to stand for 1 min until suspension supernatant appears clear, then 10 ml of supernatant were added to 90 ml sterilized distilled water in 250 ml conical flask. Then 10 fold serial dilutions were prepared in sterilized distilled water up to 10-6, and 0.1 ml from each dilution were spread on the surface of nutrient agar plate, plates were incubated at 35ºC for 24 h. All the obtained colonies were isolated, purified and streaked on nutrient agar slopes, maintained at 4 ºC, sub cultured at regular intervals in nutrient agar. The most prominent bacterial strains isolated from Rhizosphere soil of the chilli plant were tentatively identified on the basis of cultural, morphological and biochemical characteristics as per their genera laid down in the Bergey's manual of systemic bacteriology (Mew TW *et al.,* 1976, Cross T *et al.,* 1989). Bacterial isolates were maintained on nutrient agar media at $4^{o}$C and was sub-cultured periodically on the same media at $37^{o}$C.

### In vitro screening of isolates for

### Antibacterial activity

Isolates were tested for their antibacterial activity against bacterial pathogens namely *Escherichia coli*, *Klebsiella* sp, *Pseudomonas* sp, *Salmonella typhi*, *S paratyphi* and *vibrio cholera*.The antibacterial activity was carried out by spot inoculating method. The bacterial isolates isolated from the rhizosphere soil were spotted on the pre-poured nutrient agar plates already having a lawn of indicator test bacteria on it. Plates were incubated at $37^{o}$C for 24 h and observed for clear zone formation around the spot. Antibacterial activity was expressed in terms of diameter (mm) of clear zone produced around the spot at $37^{o}$C for 24 h.

### Screening for antifungal activity

All the isolates were tested for their antifungal activity against the fungal strains namely *Aspergillus s., Fusarium sp, and Pencillium sp.* The antifungal activity was carried by agar cup plate method. For this, 24hr old culture of bacterial strains was centrifuged at 10000 rpm at $4^{o}$C for 10 min and supernatant was collected. Supernatant

(50 µl) was added to each well cut on a pre-poured nutrient agar Petri plate with the help of a sterile cork borer with 6 mm diameter, already having a bit of indicator test fungi on it. Plates were incubated at 25 ± 2°C for 72 h and observed for the formation of a clear zone around the well. The antifungal activity has been expressed in terms of diameter (mm) of clear zone formed around the well (Perez C *et al.*, 1993).

## Proteolytic activity

All the bacterial strains were screened out for the production of proteolytic activities by the method of (Flaming HP *et al.,* 1975) with slight modifications. 16hr old cell culture strains were spotted on the pre solidified skim milk agar plate with the help of sterile inoculation loop. Plates were incubated at 37°C for 24 h and were observed for the formation of clear zone around the spot. Proteolytic activity was expressed in terms of its effectiveness (++/+) of clear zone formed around the spot after incubation at 37°C for 24 h.

## Results and Discussion

Out of 29 isolates, isolated from soil Rhizosphere of chilli plant from the agricultural fields of kaza, Andhra pradesh India. Four strains shows highest antimicrobial activity against various tested organisms (Fig 1, Table 1). The bacterial isolate showed antimicrobial activity against not only various Gram-positive, but also Gram negative bacteria. Furthermore, a fungus belongs to genus, *Pencillium*, *Aspergillus and Fusarium* also inhibited (table 2). Thus, it showed a very broad spectrum of inhibition ranging from prokaryotes to some eukaryotes.

When coming to its proteolytic activity, all four isolates shows a prominent proteolytic activity on milk casein agar. (Fig 3 table 3) The bacterial strains was selected and initially identified as gram positive, rod shaped bacilli. In order to further investigate its morphology and biochemical properties, we observed that it is encapsulated motile that produce oval terminal spores. The vegetative forms occur singly and have rounded ends. On nutrient agar the organism gives a very mucoid adherent growth in 18 h at 37° C. In nutrient broth the growth is initiated much more slowly and a mucoid pellicle is formed. Acid without gas is produced from glucose, fructose, maltose, sucrose, galactose, raffionse and xylose.

The Vogues Proskuaer test is negative. Gelatin and starch are hydrolyzed. Nitrites are produced from nitrates, as reported by (Smith *et al.,* 1946). The comparison of our bacterial isolate results with Bergey's manual of systematic Bacteriology results shown in (Table 4).

All the four bacillus strains isolated from the Rhizosphere of chilli were screened out for the production of proteolytic activity. All the four strains were found to be positive for the production of proteolytic. The four strains viz AS6, AS7, AS9 and AS10 showed maximum production of proteolytic activity in milk casein agar in terms of clear zone. According to Bergey's manual of systematic Bacteriology, The bacterial isolates was suggested to be Bacillus spp.

**Table.1** Bacillus species isolated from Rhizosphere of chilli Rhizosphere for the production of antibacterial activity at 37$^{o}$C

| Pathogens | Zone of inhibition(mm) | | | |
|---|---|---|---|---|
| | AS6 | AS7 | AS9 | AS10 |
| *E.coli* | 06 | 12 | 13 | 21 |
| *Klebsiella* oxytoca | 14 | 07 | 16 | 19 |
| *Pseudomonas aeruginosa* | 07 | 09 | 13 | 18 |
| *Salmonella typhi* | 05 | 13 | 15 | 20 |
| *Salmonella paratyphi* | 11 | 17 | 19 | 22 |
| *Vibrio cholerae* | 09 | 15 | 18 | 24 |
| *Proteus vulgaris* | 10 | 08 | 12 | 15 |

**Table.2** Screening of bacillus sp isolated from Rhizosphere of chilli for the production of antifungal activity at 28 ± 2$^{o}$C

| Pathogens | Zone of inhibition(mm) | | | |
|---|---|---|---|---|
| | AS6 | AS7 | AS9 | AS10 |
| *Pencillium spp* | - | +(05) | +(07) | +(13) |
| *Aspergillus spp* | - | +(06) | +(10) | +(12) |
| *Fusarium spp* | - | +(09) | +(11) | +(15) |

+ indicates activity, - indicates no activity.

**Table.3** Screening of *Bacillus* sp isolated from Rhizosphere of chilli for the production of proteolytic activity at 37 ± 2$^{o}$C

| Bacterial strain | Proteolytic activity |
|---|---|
| AS 6 | + |
| AS 7 | + |
| AS 9 | ++ |
| AS 10 | ++ |

++ indicates high activity, + indicates moderate activity & - indicates no Proteolytic activity

**Table.4** Identification of the strain *Bacillus* spp -N: Not found in Berge's manual

| Characteristics | Bergey's manual | Bacterial isolates | | | |
|---|---|---|---|---|---|
| | | AS-6 | AS-7 | AS-9 | AS-10 |
| Gram reaction. | + | + | + | + | + |
| Shape | Rod | Rod | Rod | Rod | Rod |
| Spore formation. | Spore former | Do | Do | Do | Do |
| Catalase. | Partial | + | + | - | + |
| Nitrate reduction. | + | - | + | + | + |
| H2S production. | - | - | - | - | - |
| Indole test. | N | - | - | - | - |
| Methyl red. | N | - | - | + | - |
| Vogues – Proskauer. | N | + | + | - | + |
| Amylase | + | + | + | + | + |
| Lipase | + | + | + | + | + |
| Pectinase | + | + | + | + | + |
| Gelatinase | + | + | + | + | + |
| Raffinose. | + | + | + | + | + |
| Starch. | + | + | + | + | + |
| L-rhammnose | + | + | + | + | + |
| . L-Arabinose. | + | - | - | - | - |
| D-glucose. | + | + | + | + | + |
| D-xylose. | + | + | + | + | + |
| D-Mannitol. | + | - | + | - | - |
| Maltose. | + | + | + | + | + |
| Sucrose. | + | + | + | + | + |
| Cellobiose. | + | + | + | + | + |
| L-arginine. | + | + | + | + | + |
| L-tyrosine. | - | + | + | + | + |
| L-alanine. | - | - | - | - | - |
| DL-tryptophan. | - | - | - | - | - |
| L-serine. | - | - | - | - | - |
| L-alanine. | - | - | - | - | - |
| DL-aspartic acid | - | - | - | - | - |

N: not identified

The results of this study show that many strains of the collection of *Bacillus from* natural isolates have strong antimicrobial activity against clinically important bacteria. According to the obtained results, in most strains this activity is the result of the production of diffusible antibiotic substances and in some cases surfactin family. On the other hand, the production of proteolysin is not that common among the strains from our collection, since we identified only 4 isolates that synthesizes a proteolysin like substance. Also, an interesting result of this work is a very strong antagonistic effect of *Bacillus* isolates

against Pencillium, Aspergillus & Fusarium spp. The available data concerning the control of important clinical pathogen are very limited, so further experimental work on this topic will be of great interest.

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