

ISOLATION AND IDENTIFICATION OF ANTIBIOTIC PRODUCING MICROORGANISMS FROM WHEAT RHIZOSPHERIC SOIL

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Abstract—Multidrug resistance of bacteria is the major global healthcare problem in the post-golden era of antibiotics. Initially, the multidrug resistance (MDR) of the bacteria was associated with hospitalized patients, specifically immunosuppressed and ICU patients. But, now a days MDR has become a common problem in most patients and it is difficult to identify and cure. The molecular mechanism of multidrug resistance is very complex and it is mostly associated with the presence of resistant plasmid or transposon, or due to the action of multidrug efflux pump activated by increased expression of genes or enzymatic inactivation of drug, etc. To overcome these scenarios there is a need to search for novel antibiotics. In the present investigation, the antibiotic-producing microorganisms were isolated from wheat rhizospheric soil by using the crowded plate method and screened further against pathogens by the Giant plate technique. Among the five soil samples screened, only one soil sample from the wheat rhizospheric region has shown the zone of inhibition. The organism was then purified and checked for antagonistic activity by cross streak method using pathogens such as *Escherichia coli*, *Pseudomonas*, *Salmonella typhi*, *Klebsiella*, and *Staphylococcus aureus*. The molecular identification of the isolate was done by using 16s rRNA sequence analysis. The outcome demonstrated that the isolated organism belonged to the genus *Bacillus spizizenii* NRRL B-23049(T), which was its 100% closest neighbor. The detected organism's nucleotide sequence was submitted to GenBank under accession number PP911443. Thus, in the present investigation, we tried to find a new antimicrobial compound-producing microorganism from wheat rhizospheric soil.

INTRODUCTION

The modern age started with the accidental discovery of the first miraculous drug Penicillin by Sir Alexander Fleming in 1928 proved significantly beneficial for use by the military during World War II (Alanis, 2005). During the period 1950s -1970s, a remarkable discovery of Novel antibiotics was made and named the golden era of antibiotics. Antibiotics are the secondary metabolites secreted by microorganisms that are harmful to other bacteria at low concentrations. The discovery of antibiotics saves millions of lives (Aminov, 2010). But now a days, the emergence of multidrug resistance of pathogenic microbes (superbugs) has raised serious threats to human health and becoming a rapidly growing concern throughout the world. It has been reported that antibiotic resistance leads to about 700,000 people's deaths per year (O'Neill, 2016). To overcome these scenarios the search for novel

antibiotics is always in high demand. Many of the potent antibiotics that are widely used as antibiotics are obtained from microorganisms such as actinomycetes, bacteria, fungi, etc. (Abbas *et al.*, 2014). Microorganisms are omnipresent and among the reservoir soil is the most dynamic source of microorganism-producing antibiotics. The natural soil harbours over 10^{10} bacterial cells per gram of soil and the species diversity goes up to the order of about 10^4 (Roesch *et al.*, 2007). The species diversity of microorganisms depends on the nutrient richness and physicochemical properties of the soil. Rhizospheric soil harbors a large number of microorganisms compared to the other areas of soil as root exudates influence the growth of microorganisms. Soil morphology, depth and, root frequency influences microbial communities (Dong *et al.*, 2019; Steenwerth *et al.*, 2008). Every year more than 500 different antibiotics have been discovered and among them, more than 80% of antibiotics have

been isolated from soil microorganisms. Both the Gram-positive and Gram-negative bacteria as well as fungi have the potential to produce the antibiotic compound. Among the antibiotic-producer, genera of actinomycetes remain at the top position (Magarvey et al., 2004; Abuzeid et al., 2022). The next potent antibiotic producers belonging to the genus *Bacillus* have endospore formation ability and production of antibiotics like bacitracin, pumulin, and gramicidin which are inhibitory to the growth of other organisms (Singh et al., 2018). *Bacillus* species are widely spread in nature and found in every environment with favourably competence with the other organisms within the environment (Kuta et al., 2009). It has been reported that the *Bacillus* genus produces more than 45 antimicrobial compounds having effective antibacterial activity (Sonenshein et al., 2000; Stein et al., 2005)

The majority of antimicrobial compounds used today have been obtained from the soil actinomycete and most of them remain uncultivable. Hence, there is a need to search for novel and more efficient antimicrobial compounds other than soil actinomycete. In this study, we investigated the evaluation of an antimicrobial compound produced by a bacterium isolated from the wheat rhizospheric soil samples collected from the locality of Saswad region. The molecular identity of the organism is investigated by 16S rRNA sequence analysis. The results of these analyses have shown that the isolated organism belonged to the closest neighbor of *Bacillus spizizenii* NRRL B-23049(T). The extracted secondary metabolites of the identified organism show effective antimicrobial activity against the tested human pathogens. Thus, in the present investigation, we tried to find a new antimicrobial agent-producing microorganism for controlling the multidrug resistance of pathogens.

MATERIALS AND METHOD

Collection of soil sample

Soil samples were collected from the wheat rhizosphere at a farm in the Saswad region. The site was dug 5-15 cm, and approximately 10 g of the rhizosphere soil was randomly collected in a sterile polythene bag. The debris from the soil sample was removed before collection.

Primary Screening

A crowded plate technique was employed to screen antibiotic-producing microorganisms. The soil

samples were air-dried. 1 g of soil sample was added to 100 ml of sterile distilled water. Serial dilution was done up to 10^{-6} and 100 μ l of each was placed on a sterile nutrient agar plate. The plates were incubated at 37 °C for 24 to 48 hours. The colonies that showed antagonism were picked up and streaked on nutrient agar plates separately to obtain pure isolated colonies (Fig.1 and 2).

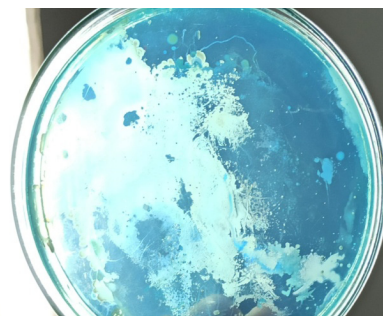


Fig. 1. Crowded plate technique showing the antibiotic producing organism.

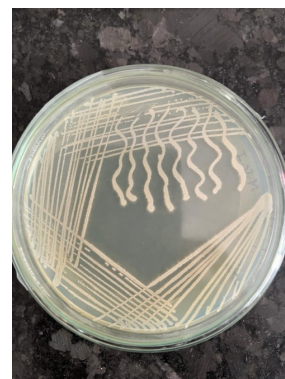


Fig. 2. Pure culture of isolated strain

Cross streak method

The isolated strains were streaked as a parallel line on nutrient agar plates and incubated at 37 °C for 24-48 hours. After observing growth, *Escherichia coli*, *Pseudomonas*, *Salmonella typhi*, *Klebsiella*, and *Staphylococcus aureus* were streaked at right angles to the original streak of isolate and incubated at 37 °C.

Characterization of the Isolate

Morphological characteristics, such as colony characteristics, of the isolated strain were determined by inoculating the isolated strain on nutrient agar. Gram staining was carried out to determine the cellular morphology and Gram stain phenotype. Endospore staining was carried out to check the isolate's ability to form endospores.

Biochemical characterization of the isolate was

carried out according to the method described in Bergy's manual of systematic bacteriology. The isolate was identified by performing tests such as oxidase test, catalase test, gelatin hydrolysis test, starch hydrolysis test, nitrate reduction test, urease test, growth at 45°C, and growth with 7% NaCl.

16s rRNA sequence analysis

The isolated strain was identified via sequencing the 16s rRNA gene. genomic DNA was isolated by the standard phenol/chloroform extraction method (Sambrook 1989). Followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequence. Essentially, sequencing was carried out from both ends using additional internal primers so that each position was read at least twice. Assembly was carried out using Lasergene package followed by identification using the EzBioCloud database (Yoon *et al.*, 2017).

Extraction of the antibiotic compound

The 96 hr old pure culture broth of the isolated strain was centrifuged to extract the antimicrobial agent. The obtained culture filtrate was mixed with an equal volume of organic solvents such as ethyl acetate, vigorously shaken, and allowed to separate. The lower phase (organic phase) was separated from the exhausted supernatant (upper phase). The solvent phase was concentrated by drying the samples at 37 °C until no solvent was left. Then the extract was tested for antimicrobial activity (Singh *et al.*, 2018; Rajan *et al.*, 2014).

Antimicrobial activity of the extracted compound

The extracted compound of RAK01 was then tested for its antimicrobial activity against the selected pathogen from the result of the cross-streak method. The Agar well diffusion method is used to carry out antimicrobial activity. 100 μ l pure cultures of selected microorganisms (equivalent density 10⁶ CFU/ml) was spread uniformly on a Sterile Mueller-Hinton agar plate. The wells were punctured by using stainless steel cork borer (6mm). Different concentrations of the extracted compound were then added to the well. The plates were incubated overnight at 37 \pm 2 °C. The inhibition zone was measured using antibiotic zone scale -C

(RIDACOM) (Valgas *et al.*, 2007).

RESULTS AND DISCUSSION

Screening and isolation of antibiotic producing organisms

The antibiotic producing organisms were screened by the crowded plate technique. The five different samples from the wheat Rhizospheric region were screened for antibiotic producers, and only one soil sample showed colonies with the zone of inhibition. The culture was then further purified and used for further study. The isolated organism was designated as isolate RAK01.

Identification of isolates

Morphological and biochemical characterization

The colony morphology was found to be white-colored with the entire margin on nutrient agar. The microscopic observation showed that the isolated organism stain gram-positive rod has endospore formation ability and is motile. According to Bergy's manual of systematic bacteriology, the biochemical characterization of isolated organisms was carried out. The isolate RAK01 showed positive for starch hydrolysis, gelatin hydrolysis, oxidase, catalase, reduction, growth at 45°C, and growth with 7% NaCl, and negative for urease. (Williams *et al.*, 1989) (Table 1; Fig.3)

Table 1. Biochemical characteristics of the isolate RAK01

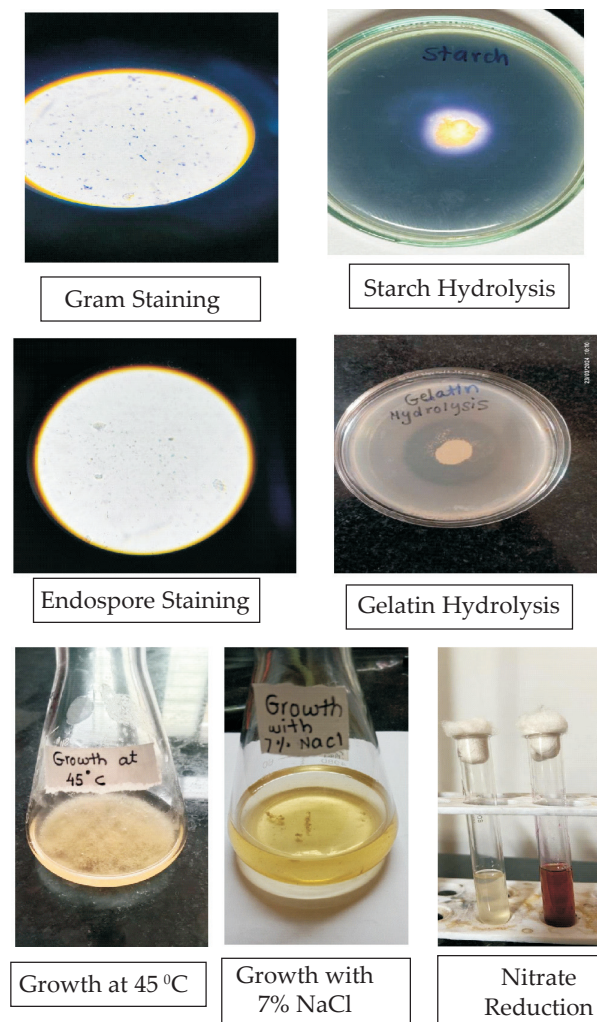
Biochemical test	Result
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Oxidase	Positive
Catalase	Positive
Nitrate reduction	Positive
Urease production	Negative

Molecular identification

The molecular identification of the isolate RAK01 was carried out by performing 16s rRNA sequence analysis. A sequence read of ~700 bp sequence was obtained. The outcome of the identified sequence demonstrated that the isolate RAK01 showed 100% similarities with the gene cluster sequence of *Bacillus spizizenii*. The outcome of the molecular identification showed that the identified organism has the taxonomic designation *Bacillus spizizenii* NRRL B-23049(T) (Table 2).

Table 2. Result of 16S rRNA sequencing

PRN	Strain No.	Closest neighbour*		%Similarity
		Taxonomic Designation	Accession no.	
A_MAR_24_107	RAK01	<i>Bacillus spizizenii</i> NRRL B-23049(T)	CP002905	100.00

**Fig. 3.** Biochemical characterization result

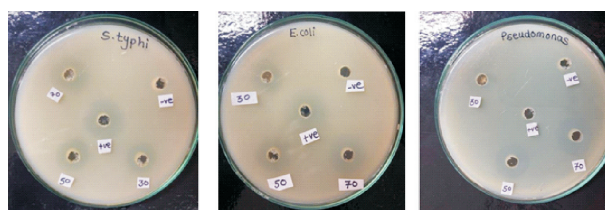
Antimicrobial activity

The extracted compound from the RAK01 strain shows effective antimicrobial activity against Gram-negative pathogenic strain. Hence, it may be

Table 3. Antimicrobial activity of extracted compound

Name of pathogen	Volume of Extracted antibiotic			Positive control (Ampicillin 50 µl)	Negative control (µl)
	30 µl	50 µl	70 µl		
<i>E. coli</i>	14 mm	18 mm	19 mm	20 mm	No inhibition
<i>S. typhi</i>	15 mm	17 mm	18 mm	20 mm	No inhibition
<i>Pseudomonas</i>	14 mm	16 mm	18 mm	-	No inhibition

narrow-spectrum antibiotics. Greater inhibition was observed at higher volumes of the extracted antimicrobial compound whereas lesser growth inhibition was observed with 20 µl of the compound. From the result, it was found that the inhibition by the extracted compound to be dose dependent (Table 3, Fig. 4).

**Fig. 4.** Antimicrobial activity of extracted compound.

CONCLUSION

Today the multi-drug resistance of bacteria has become a major global concern. To overcome this scenario there is a need to search for novel antibiotics. Microorganisms are the basis for the search for antibiotics. Rhizospheric soil is the most effective natural resource for locating antibiotic-producing microbes. Numerous methods have been employed to isolate microorganisms that produce antibiotics. Among these, the crowded plate technique is frequently used to isolate antibiotic producers which are further followed by different procedures such as the cross-streak method to study the antagonistic activity of isolated microorganisms.

In the present investigation, we investigated the evaluation of the antibiotic potential of *Bacillus spizizenii* isolated from wheat rhizospheric soil collected from Saswad, India by performing 16s molecular sequence analysis. The ethyl acetate was used as a solvent for the extraction of antimicrobial substances. The extracted compound shows

significant antimicrobial activity against the tested pathogen. The zone of inhibition was increased with increasing the volume of an extracted compound. From the observation the antimicrobial activity of the extracted compound was found to be dose-dependent.

Conflict of Interest – None

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