

## Gas Chromatography - Mass Spectrometry Analysis and Antibacterial Activity of Bluish-Green Pigment from *Pseudomonas* sp. JJTBVK (KF836502)

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### ABSTRACT

The present study was conducted for the isolation of potential bacteria from the desert soil, their molecular identification and prediction of restriction sites of the potential isolate using the bioinformatics tools. Production of the metabolites was done by inoculating in nutrient broth of pH 8.6. Metabolite was bluish-green in color; it was extracted and dried by using methanol and used for partial characterization by using GC-MS spectroscopy. Antibacterial activity was performed with the clinical human pathogenic isolates. The bacterium was identified as *Pseudomonas* sp. JJTBVK on the basis of 16S rRNA sequencing. The sequence was analyzed for the restriction cleavage sites, which showed that the sequence had various restriction sites for different enzymes. Antibacterial activity (MIC) of methanol extract of the bacterial culture broth showed antibacterial activity (MIC), which was 29, 30, 30 and 29 mm for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Salmonella typhi*, respectively. GC-MS analysis of the methanol extract showed the presence of naphth [2,3-B] azet-2 (1H) -one, 1-phenyl-, which was the characteristic compound showing the antibacterial activity.

**Key words:** Characterisation, *Pseudomonas*, Extracellular, Pigment, Antibacterial activity

### INTRODUCTION

Microorganisms can withstand the adverse conditions and grow in the environment by secreting various colored components as metabolites. Some microorganisms can also be identified on the basis of the type and nature of the secretion of the metabolic compounds. *Pseudomonas* is a Gram negative bacterium, present in the soil, water, human skin flora and also opportunistic plant pathogen. The synthesis of bluish-green, yellow-green, fluorescent, water-

soluble extracellular pigments is a characteristic property of some *Pseudomonas* spp. under certain growth conditions. These species are members of the same intra-generic genetic homology group, e.g., *Pseudomonas aeruginosa*, *P. putida*, *P. fluorescens* and phytopathogens of the *P. syringae* type (Stanier et al. 1966; Palleroni et al. 1973; Palleroni and Doudoroff 1974; Meyer 1978). *Pseudomonas* is known to produce pigments resolved to survive under oxidative stress imposed by exhibiting antagonism, environmental variation and deposition of hazardous materials by human

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activities. This type of beneficial bacteria form colonies on the plant surfaces and secrete potential antibiotics and hydrogen cyanide that are lethal to plant pathogens; it also promotes plant growth by suppressing the pathogens in the root zone and upper parts and by involving in nutrient assimilation. It also can be even used to control environmental hazards in the biodegradation and bioremediation, and can be used to control the phytopathogens (Jayaswal et al. 1990). Water soluble and diffusible pigments producing ability of the *Pseudomonas* species are the primary characteristic, which have been used for their identification. There are reports on the production of specific bluish-green colored pigment in the culture medium of *P. aeruginosa*, which consisted of blue pyocyanin and also the production of un-specific yellow colored pigment, namely fluorescein (Howarth and Dedman 1964). *Pseudomonas aeruginosa* produced various phenazine pigments identified as pyocyanine, phenazine-1-carboxylic acid, 1-hydroxy phenazine and phenazine-1-carboxamide (Mavrodi et al. 2001). Defined conditions for the reliable production of phenazines have greatly aided by biosynthetic studies (MacDonald 1967).

Phenazines (Phz) are N-containing heterocyclic pigments synthesized by *Brevibacterium*, *Burkholderia*, *Pseudomonas* and *Streptomyces*. Currently, over 50 naturally occurring Phz compounds have been described and mixtures of as many as ten different Phz derivatives can occur simultaneously in one organism. The growth conditions determine the number and type of Phz synthesized by an individual bacterial strain (Dwivedi and Johri 2003). Production of the pigments from the bacteria have been studied by inducing the stress condition by varying the media components or by the incubation conditions such as pH and temperature or by varying the type or composition of carbon and nitrogen sources in the medium for the higher productivity (Goswami and Bhowal 2014).

There are several health risks associated with methicillin-resistant *Staphylococcus aureus* (MRSA) infections, including its potential to produce invasive infections, particularly in vulnerable patients and its resistance to multiple antibiotics (Cardozo et al. 2013). Phenazines are redox-active small molecules produced by the bacteria and have been studied extensively for their roles as toxins. However, how the phenazines benefits the producing organisms is

still not clear. These phenazines represent a large class of natural antibiotics that are produced by diverse bacteria and exhibit unique redox properties and broad-spectrum antibiotic activity (Marvodi et al. 2006). *Pseudomonas* can survive under anoxic conditions in the presence of glucose and oxidized phenazines, including pyocyanin, 1-Hydroxyphenazine and phenazine-1-carboxylic acid. Many microorganisms produce phenazines, brightly colored, heterocyclic small molecules that reversibly gain and lose electrons. *Pseudomonas* are perhaps the best studied phenazine producers, known as agents of human infection and for their potential as biological control agents that protect agricultural crops (Marvodi et al. 2013).

The green technology comprises to produce less toxic products from natural materials for today's production lines. Some synthetic dye manufacturing has been prohibited due to the carcinogenicity of the precursor or product and also due to the issue of the administration of their industrial wastes in the ecosystem (Venil et al. 2013). Phenazine compounds are biologically active metabolites that function in microbial competitiveness (Mazzola et al. 1992). These bacterial pigments can also be used as food colorants instead of the synthetic dyes, which are not degradable easily with the nature. Natural pigments are easily degradable in the nature; they possess antimicrobial and anticancer activity, contain pro-vitamin A and have some desirable properties such as stability to light, heat and pH (Mekhael and Yousif 2009; Muthuselvam et al. 2009). Various microbial pigments such as carotenoids, prodigiosin, astaxanthin and violacein have found application in medical areas due to their activities as immunosuppressive, anticancer, anti-aging and antioxidative agents (Perez-Thomas et al. 2003; Williamson et al. 2007; Raj et al. 2009; Goswami and Bhowal 2014).

Pyocyanin has a variety of pharmacological effects on eukaryotic and prokaryotic cells (Vukomanovic et al. 1997) and also antibiotic activity against bacteria, fungi and protozoa (Karpagam et al. 2013). The majority of antimicrobial agents are of microbial origin. The resistance of the pathogenic microbes to the commonly used antibiotics is increasing as a result of the wide-spread and long-term use of these common antibiotics. Therefore, development of a strategy to produce effective antibiotics is an urgent need. Interspecies interactions can be the possible utility in the search of effective

antibiotics. When different microbial species interact and communicate, as it happens in the nature, this may lead to novel pharmaceutical compounds. The aim of the present study was to search for bacterial metabolite having multiple functions such as natural color and which also acts as antimicrobial compound, which could be applied in the food and textile or under-garment industries.

## MATERIAL AND METHODS

### Sample collection

The soil sample was collected from the desert soils of Jhunjhunu, Rajasthan, India during September 2013 at a depth of 10-25 cm using sterile spatula and stored in a sterile polyethylene bag.

### Isolation and Identification of Bacteria

The soil sample was serially diluted and the dilutions were inoculated on the nutrient agar. One of the isolates showed the bluish-green colored pigment, which was selected and sub-cultured for the cultural, morphological, biochemical and physiological characteristics identification (Cappuccino and Sherman 1999; Schaad et al. 2001); the results were compared with Bergey's Manual of Determinative Bacteriology (Buchanan et al. 1974) and further confirmed based on molecular identification.

### Molecular characterization

The DNA was isolated by a commercial kit (Amnion Pvt. Ltd, India). The universal bacterial primers (Forward:- 5' - CWG RCC TAN CAC ATG SAA GTC - 3'; Reverse:- 5' - GRC GGW GTG TAC NAG GC - 3') were used for the amplification of 16S rRNA gene. The PCR was carried out in a 50 µL. PCR mixtures included gDNA (~50 ng), Forward Primer (100 ng), Reverse Primer (100 ng), 10 mM dNTPs mix (2 µL), 10X *AMTaq* Pol. Buffer (5 µL) and *AMTaq* Polymerase enzyme (3 U). Cycling conditions were as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.30 min, and a final extension of 5 min at 72°C. The 16S rRNA sequence was determined using an ABI3730XL capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan). The homology of the 16S rRNA partial gene sequence was analyzed using BLAST search (National Center for Biotechnology Information

(NCBI), USA). The phylogenetic tree was constructed by using tree view software (version 1.6.6). The restriction sites in 16S rRNA gene sequence of the potential isolate was predicted using Bioinformatics tool, NEB cutter (Version 2.0).

### Production of Pigment

The culture was inoculated into nutrient broth (pH 8.6) in the conical flask and incubated at 37°C for 48-72 h. The bacterial cells were separated from the liquid medium by using a refrigerated centrifuge at 12,074 g for 10-15 min. The bluish-green colored supernatant was used to extract the metabolic compounds through the methanol extraction method. Further centrifugation and filtration (Whatmann filter paper) was done to remove the insoluble components and precipitated proteins in the medium. Concentration of the pigment was done by using Buchi Roto evaporator and then it was used for further processing.

### Gas chromatography-mass spectrometry analysis

The methanol extract of *Pseudomonas* sp JJT BVK was analyzed by gas chromatography-mass spectrometry (GC-MS) using a THERMO GC - TRACE ULTRA VER: 5.0, Thermo MS DSQ II with a TR 5 - MS Capillary Standard Non - Polar Column (30 m, film 0.25 µm, ID 0.25 mm). The temperature was 80 to 250°C at 8°C/min. The carrier gas was Helium with a flow rate of 1.0 mL/min. The chemical constituent was identified using NIST08.LIB library spectra provided by the software on a GC / MS system.

### Antibacterial Activity

The methanol extract of *Pseudomonas* sp JJT BVK was used in different concentrations (25, 50, 75 and 100 mg/mL) in comparison with the standard drug to study the *in vitro* antibacterial activity of human pathogenic bacteria to find out the minimum inhibitory concentration by agar well diffusion method. The human pathogenic clinical isolates were *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Bacillus subtilis*. A loopful of each pathogenic bacterial culture was inoculated into the different test tubes containing 5.0 mL sterile Luria Bertani (LB) broth and incubated at 37°C for 18 h. Then transferred 100 µL of the inoculums of the each test pathogen on to different nutrient agar plates

and spread plate method was done with a sterile swab. A well of 5.0 mm diameter was made in the plates equidistantly, using a sterile cork borer. Filter sterilized (0.25 µm pore size) methanol extract of *Pseudomonas* sp JJTBVK with four different concentrations (25, 50, 100 and 200 mg/mL) compared with chloromphenicol (25 mg/mL) were tested. A 50 µL of each concentration of methanol extract and standard antibiotic was transferred into their respective labeled wells and the plates were incubated at 37°C for 24 h; sterile water was used as a control. Each bacterial strain was tested against the methanol extract with three replications were made during the study. After the incubation, the inhibition zone (minimal inhibitory concentration) around the well was recorded and expressed in millimeter (mm).

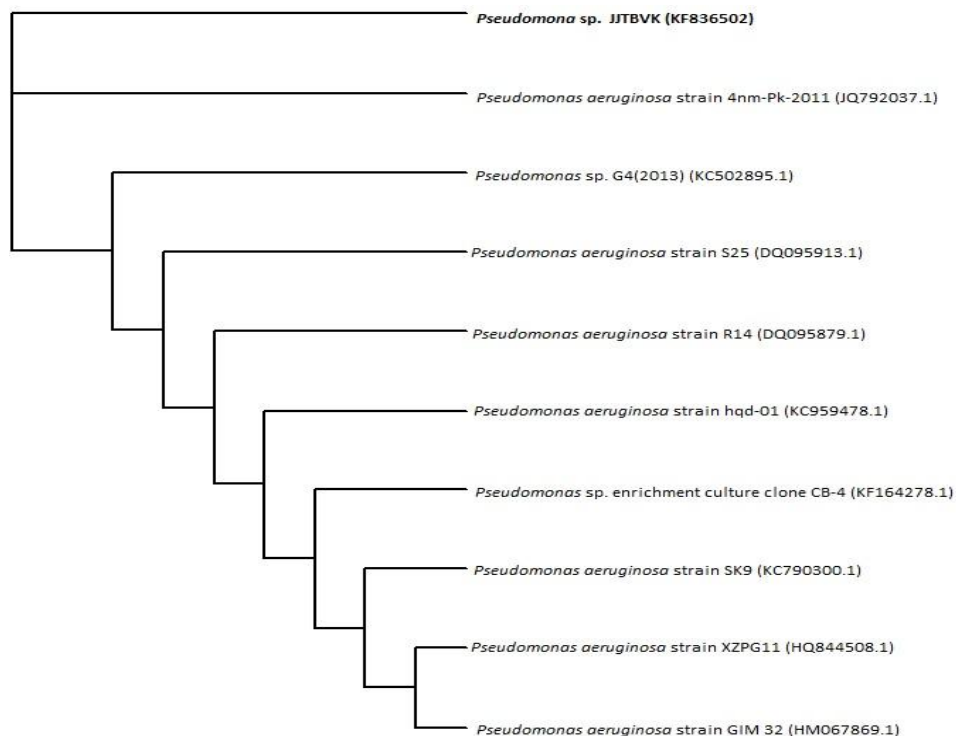
## RESULTS

The isolated bacterium was used for the cultural and morphological study. The bacterial colony was circular, raised and smooth margin. It was a

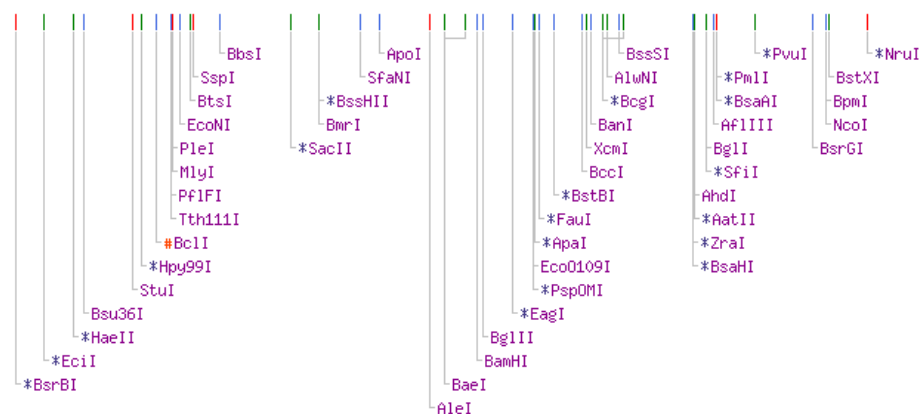
Gram negative bacterium, rod in shape, motile and aerobic, which gave positive test for citrate utilization, starch hydrolysis, H<sub>2</sub>S production, lipid hydrolysis, gelatine liquefaction, oxidase and catalase test and negative for methyl red, vogues procure and indole production. Based on these, the organism was identified as *Pseudomonas* sp. Then further confirmation was made by using molecular characterization.

### Molecular characterization

On the basis of 16S rRNA partial gene sequence, the bacterium was identified as *Pseudomonas* sp. through the analysis of the sequence using BLAST search. It was designated as *Pseudomonas* sp. JJTBVK. The constructed phylogenetic tree showed the phylogenetic relationship with the *P. aeruginosa* with 99% similarity (Fig. 1). The accession, given to the bacterial sequence is KF836502. The gene sequence of *Pseudomonas* sp JJTBVK was analyzed for restriction enzyme cleavage sites, which showed the different cleaving sites for different restriction enzymes (Fig. 2).



**Figure 1** - Dendrogram of 16S rRNA gene sequence in *Pseudomonas* sp. JJTBVK.



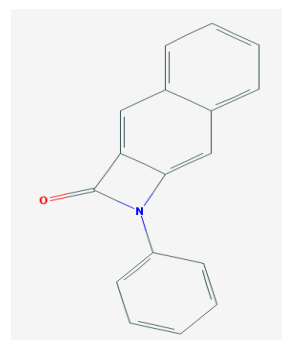
**Figure 2** - Restriction enzyme cleaving sites in 16S rRNA gene sequence of *Pseudomonas* sp. JJTBVK.

#### Extraction of bacterial pigment

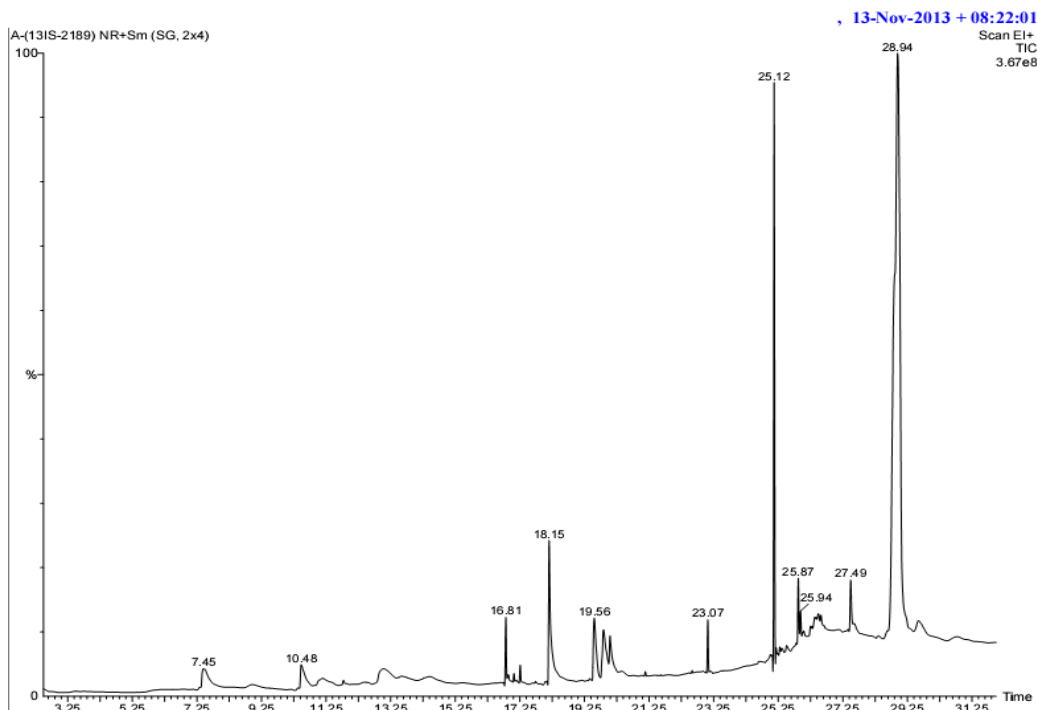
After the incubation, the broth was centrifuged to separate the supernatant and the metabolite was extracted using methanol, which was green in color.

#### Gas chromatography-mass spectrometry analysis

GC-MS Chromatogram of the methanolic extracted pigment showed different peaks (Fig. 3); the highest peak was observed and identified as Naphth [2,3-B] Azet-2 (1H) -One, 1-Phenyl- (Fig. 4). The IUPAC name of this compound is 1-Phenyl naphtho [2,3-b] azet-2 (1H) -one and the chemical formula is  $C_{17}H_{11}NO$ .



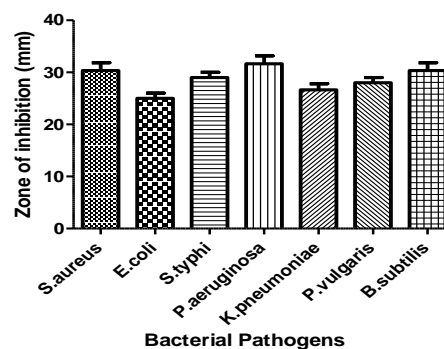
**Figure 4** - Structure of Naphth [2,3-B] Azet-2 (1H) -One, 1-Phenyl-



**Figure 3** - GC-MS Chromatogram shows the different peaks for the extracted compound.

### Antibacterial Activity

Antibacterial activity of the methanolic extract of *Pseudomonas* sp. KF836502 showed significant differences in their activities depending on the microorganisms tested (Table 1). The metabolite showed the maximum zone of inhibition against *P. aeruginosa* (32 mm), followed by *S. aureus* (30 mm), *B. subtilis* (30 mm) and *Salmonella typhi* (29 mm) than other clinical isolates when compared with the standard antibiotic chloromphenicol (Fig. 5).



**Figure 5** - Antibacterial activity of the methanol extract of *Pseudomonas* sp. JJTBVK.

**Table 1** - Antibacterial activity of the methanol extract of *Pseudomonas* sp. JJTBVK.

Species	Methanolic Extract (Crude)				Standard (Chloromphenicol)
	25 mg/mL	50 mg/mL	75 mg/mL	100 mg/mL	
<i>Staphylococcus aureus</i>	7±1	12±2	20±1	30±1.5	29±1
<i>Escherichia coli</i>	6±0.6	8±1	16±2	25±1	26±0.5
<i>Salmonella typhi</i>	7±1	10±1.5	18±2.5	29±1	27±1.5
<i>Pseudomonas aeruginosa</i>	9±1.5	14±2.5	22±1.5	32±1.5	29±2
<i>Klebsiella pneumoniae</i>	6±0.6	9±1.5	15±1	27±1.2	28±1
<i>Pseudomonas vulgaris</i>	8±2	9±1.2	16±2	28±1	28±0.5
<i>Bacillus subtilis</i>	9±1.5	13±1.5	20±1	30±1	29±1

## DISCUSSION

Several bacterial cultures were isolated from the agricultural soil and screened for characteristic extracellular pigment producing ability. One of these was characterized as *Pseudomonas* sp. JJTBVK (KF836502), which produced bluish-green colored pigment by inducing the stress condition in the media by varying the pH. There are reports on inducing the stress by varying other conditions such as carbon source for characteristic and good rate of pigment productivity (Muthuselvam et al. 2009). An important factor is the concentration of the iron, which must be controlled to ensure the secretion of fluorescent pigments by *Pseudomonas* (Totter and Moseley 1953). Muthuselvam et al. (2009) reported maximum pigment productivity was at 40°C, pH 7.0, lactose as a carbon source and ammonium chloride as nitrogen source by the *P. aeruginosa*. Luti and Yonis (2013) worked on the interactions with elicitor cells such as dead and live, elicited *P. aeruginosa* to produce more phenazine albeit at different levels and reported increased in the production of phenazine in elicited cultures. Based on the type of microbial elicitors such as *E. coli*, *Bacillus subtilis* and *S. cerevisiae*, the maximum

production of phenazine was achieved in the culture elicited with heat killed cells of *S. cerevisiae*.

Naphth [2,3-B] Azet-2 (1H) -one, 1-Phenyl has beta lactum ring in its structure and belongs to the phenazine derivatives, which is responsible for antibacterial activity. The functional groups are largely responsible for differences in the physical and chemical properties of the individual phenazines, and hence their biological activity (Marvodi et al. 2006). The phenazine compounds are phenolic in nature, which include pyocyanin and pyorubrin pigments and exhibit a broad spectrum antibiotic property for both fungi and bacteria (Smirnov and Kiprianova 1990). The antagonistic effects of almost all of phenazine derivatives are usually attributed to one general characteristic redox activity (Price-Whelan et al. 2006), and these compounds have been recognized as a major factor in controlling many pathogens (Saha et al. 2008). More than 6000 phenazine-containing compounds that have been described over the past century and around 100 are of natural origin (Smirnov and Kiprianova 1990; Laursen and Neilsan 2004).

Natural phenazines are produced almost exclusively by eubacteria; they often are excreted

to very high levels in milligrams to grams per litre during *in vitro* bacterial growth and they typically are pigmented with absorption spectra that include two distinct maxima in the UV range and at least one in the visible range that varies according to the nature and position of substituents on the heterocyclic ring (Britton 1983). Some of the fluorescent *Pseudomonads* found in the rhizosphere region of the soil may act as plant growth promoting rhizobacteria and suppress the soil borne plant pathogenic fungi by secreting the antifungal metabolites such as pyoluteorin, pyrrolnitrin, phenazines and 2,4-di acetyl phloroglucinol (Dwivedi and Johri 2003). Luti and Yonis (2013) carried out the agar diffusion test for the antimicrobial activities of the phenazine on the elicitor microorganisms; they observed that phenazine was noticeably effective against *S. cerevisiae* and in a lesser extent against *B. subtilis* and *E. coli*. Some microbial pigments are sensitive to heat, light, acidity, air and water activity and unstable and highly degradable (Joshi et al. 2003); hence, this isolated phenazine compound needs to be studied for the applicability.

## CONCLUSION

In the present study the molecular characterization and cleavage sites for the restriction enzymes were studied for *Pseudomonas* sp. JJTBVK. The culture produced Naphth [2,3-B] Azet-2 (1H) -One, 1-Phenyl in alkaline pH conditions (under the stress conditions). These compounds have potential to inhibit the growth of the human pathogenic bacteria and used as a coloring agent in food and textile industries.

## ACKNOWLEDGEMENT

The authors are thankful to JJT University, Rajasthan and North-West University, South Africa for the support.

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Received: March 16, 2015;  
Accepted: May 03, 2015.