INVESTIGATION OF ANTICANCER EFFECT OF NOVEL LIPOPEPTIDE BIOSURFACTANT, ISOLATED FROM *BACILLUS PUMILUS AVS5*, AN ENDOPHYTIC INHABITANT OF *ALOE VERA*

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Abstract: Endophytic microorganisms that exist as symbionts of traditional medicinal plants are rich sources of secondary metabolites with potential medicinal and other biotechnological applications. Chemically diverse compounds were found to induce cytoxicity, anti-inflammatory and antibacterial activities. Lipopeptides, the microbial amphiphiles have recently emerged as potential new-generation anticancer agents, owing to low toxicity, high efficacy and easy biodegradability. In the current study, a novel lipopeptide was isolated and characterized from *Bacillus pumilus AVS5*, an endophyte of *Aloe vera*. The lipopeptide showed a dose dependant killing of K562, a human leukemic cancer cell line and the treatment induced extensive DNA damage. Therefore, the molecule may have potential application as an anticancer lead.

Keywords: Endophytes, lipopeptides, biosurfactants, anticancer agents.

Introduction: Endophytic microorganisms that inhabit the living plants are relatively unstudied and impending sources of novel natural products for utilization in medicine, agriculture, and industry. The earth harbors quite a lot number of plant species and it is remarkable that each individual plant is host to untold number of endophytes. Only a few of these plants have ever been completely studied relative to their endophytic biology [1]. *Aloe vera,* a traditional medicinal herb widely used in many countries is one of such kind. Consequently, the opportunity to find new bioactive natural products from their endophytic microorganisms is great. Bacillus strains are widely distributed in nature and their bioprospection led to the discovery of diverse class of secondary metabolites including lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides and isocoumarins. Among these compounds, lipopeptides (LPs) of the surfactin, iturin, and fengycin families have been found to exhibit a wide range of bioactivities including anticancer, antiviral, antimicrobial, immunosuppressive, antimycoplasmic and exceptional surfactant properties [2-6]

Lipopeptides are amphiphilic molecules made up of short linear chains structures of amino acids, linked to a fatty acid via ester or amide bonds. They are produced by little number of bacterial species and have important biological functions. Besides surfactant properties, they often show significant anticancerous or antitumor properties therefore have attracted huge interest from industry. They play important role in apoptosis induction process of tumor cells and mode of action involves membrane leakage through pore formation [7], ion channel formation [8], cationic carrier action [8] and detergent like effects [9] which finally lead to cytotoxicity.

The diversified structures along with unique functional groups of lipopeptides make them lucrative and the search for novel lipopeptides seem to be promising in anticancer research. The present study points out the screening, isolation, characterization and cytotoxic effect of lipopeptide biosurfactant extracted from *Bacillus pumilus AVS 5*, which exists as an endophyte of *Aloe vera* plant.

Materials and Methods:

Sampling and Isolation of bacteria: Healthy leaves of *Aloe vera* plant were cut and stored in sterile plastic bags. The plant parts were carefully washed in running water to remove external soil and debris. In a method adapted from Fisher *et al* (1993), the plant material was surface sterilized by immersion in 70% ethanol for 5 min, 10% Sodium hypochlorite for 15 min, followed by washing with sterile distilled water [10]. The material was once again subjected to 70% ethanol for 5 min and two washes with sterile distilled water. To ensure that surface sterilization was successful, sterile distilled water employed in final wash was inoculated into nutrient broth followed by incubation at 30 ^{0}C for three days and checking for any growth of plant surface-associated contaminating microorganisms. Surface sterilized plant material was cut into small pieces and subjected to homogenization under sterile environment. Homogenized serially diluted plant sample was then spread plated onto starch casein agar (Composition : Starch – 10 gL^{-1}, Cassamino acids – 0.3 gL^{-1}, $CaCO_3$ – 0.02 gL^{-1}, $FeSO_4.7H_2O$ – 0.01 gL^{-1}, KNO_3 – 2.0 gL^{-1}, $MgSO_4.7H_2O$ – 0.05 gL^{-1}, NaCl – 2.0 gL^{-1} Agar – 18 gL^{-1}) followed by incubation at 30 ^{0}C for three days. After three days, distinct colonies were identified and purified on the same medium for 5 to 6 generations and stored in glycerol stocks at – 80 ^{0}C.

Biosurfactant Screening: Different biosurfactant screening methods were done for the identification of potential biosurfactant producer. The methods

adopted were (a) drop-collapse test by the addition of mineral oil in 96 -well microtitre plates[11]; (b) Oil spreading technique by the addition of engine oil [12]; (c) Emulsification activity by adding kerosene and equal volume of cell free supernatant.

Phenotypic and Genotypic identification of strain: The strain was identified based on morphology and biochemical confirmations as well as based on the characteristics described in Bergey's Manual of Systematic Bacteriology [13]. The 16S rRNA gene of the bacterial isolates was amplified with a set of universal primers - 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') were used. After amplification, 12 µl of each reaction mixture were separated on 1.5% (w/v) agarose gel to confirm the size and purity of PCR products. The PCR product was outsourced to Bioserve India Ltd for sequencing purpose. BLAST analysis of the obtained 16s rRNA sequence was done in Eztaxon server. The phylogenetic tree was constructed by MEGA5 software and evolutionary history was inferred using the UPGMA method [14].

Isolation and purification of biosurfactant: Bacterial cells were separated by centrifugation at 10000 rpm for 15 min. The supernatant was subjected to acid precipitation by the addition of 6N HCl to achieve a final pH of 2.0 and allowed precipitation to occur at 4˚C. The precipitate was pelleted at 10000 rpm for 20 min, re-dissolved in distilled water, adjusted to pH 7.0, freeze-dried, and weighed. The dried surfactant was extracted with methanol and dried with the aid of a rotary evaporator under vacuum. To purify further the lipopeptide compound, the concentrated extract was subjected to silica gel (60–120 mesh) column chromatography with step wise elution using chloroform/methanol gradient. The active fraction was confirmed by the emulsification activity, and the purity was checked by thin layer chromatography.

Characterization using TLC and FT-IR Techniques: The purified compound was dissolved in distilled water and spotted on TLC sheets and run with $CHCl_3$ /CH_3OH /H_2O (65:15:1) as mobile phase. The chromatogram was developed under short UV light and homogeneity was checked. Further, the plate was sprayed with 0.2% ninhydrin (in absolute alcohol) and heated to 110°C. One milligram of purified biosurfactant powder was ground with 100 mg of KBr and pressed with 7, 500 kg for 30 s to obtain translucent pellets. The infrared spectra were recorded on Mattson 1, 000 FT-England FTIR system within the range of 500-4000 cm -1 wave number. All measurements consisted of 500 scans, and KBr pellet was used as background reference.

Anticancerous activity:

Cytotoxicity assay: The MTT assay was performed on K562 cell lines - a human leukemic cell line in order to determine the lipopeptide cytotoxicity. It was dissolved in dimethyl sulfoxide solution (DMSO). Cells were seeded into 96-well plates at a density of $1x10^4$ cells/well (in triplicate). Various concentrations of lipopeptide were added to the medium after 24 hours. Cells treated with equal concentration of DMSO without lipopeptide were used as controls. After incubation for 6, 24, 48 and 72 h, MTT solution (5 mg/ml) was added to the medium. The formazan crystals that formed were dissolved by DMSO, and absorption was measured at 490 nm with an automatic ELISA reader. The cell inhibition rate was calculated and IC50 (50% inhibiting concentration) was determined. Experiments were repeated three times.

DNA ladering assay for apoptosis analysis: For DNA laddering assay, K562 cells were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 M EDTA, and 0.5% SDS for 10 min. RNase A and proteinase K were added and incubated overnight at 50˚C. The lysates were extracted with phenol and chloroform and centrifuged at 12,000 rpm for 5 min. DNA was precipitated in ethanol. After treatment with 1 M Tris-HCL (pH 8.0), 0.5 M EDTA, 20 µg/ml RNase, DNA was electrophoresed in 1.5% agarose gel and photographed.

Results and Discussion:

Identification of effective biosurfactant producer: Among the various isolates, the effective biosurfactant producing bacteria was confirmed as Bacillus sp. by morphological, biochemical and 16s rRNA sequence analysis. As per results given in Table 1, the phenotypic characteristic features of Bacillus Sp. AVS5 are gram positive, rod shaped, non motile and able to ferment sugars such as glucose, sucrose and galactose. Phylogenetic and evolutionary analysis of the 16s rRNA sequence revealed that the Bacillus sp. *AVS5* have similarities of 99.79%, 99.58% and 99.58% with *Bacillus pumilus ATCC 7061*, *Bacillus aerophilus 28K* and *Bacillus stratosphericus 41KF2a* respectively.

Biosurfactant screening: Of the different biosurfactant screening tests such as drop collapse test, oil spreading test, and emulsification activity, the endophytic Bacillus sp. AVS5 was able to produce biosurfactants. *Bacillus sp.* has been reported as the major producer of biosurfactants including lipopeptides, glycolipids and surfactin etc. In the oil spreading test, a zone of about 2.4 cm was recorded against the engine oil. Morikawa et al. [15] showed that the extent of oil displacement is directly proportional to the concentration of the biosurfactant produced. Emulsifying capacity of the

same test sample against kerosene additionally proved the biosurfactant production potential.

Table 1 Phenotypic characteristic features of Bacillus sp. AVS5

S.No	Characteristic	Result	S.No	Characteristic	Result
	Growth characteristics	Positive	11	**Substrate Utilization**	Positive
	Gram Staining	rod	12		Positive
1	Cell shape	Non motile	13	Glucose	Positive
2	Motility	10 – 50 °C	14	Fructose	Positive
3	Growth Temperature	10 %	15	Galactose	Negative
4	NaCl tolerance		16	Mannose	Positive
5			17	Urea	Positive
	Biochemical			Xylose	
	Characterization	Positive	18	Sucrose	Negative
6	Amylase	Positive	19		Positive
7	Protease	Negative	20	Indole production	negative
8	Lipase	Positive		Voges-Proskauer	
9	Oxidase	Positive		Citrate utilization	
10	Catalase				

Purification and Structure Determination: When the plate was sprayed with 0.2% ninhydrin, the biosurfactant component was observed as a single spot on the TLC plate. The observation implied the presence of peptide in the sample. The present study TLC analysis revealed that, the Rf value of 0.68 was confirmed as lipopeptide [16]. As a result of C–H stretching vibrations and N–H stretching vibrations, a broad absorbance peak (centred around 3434 cm^{-1}) with wave numbers ranging from 3600 cm^{-1} to 3100 cm^{-1} was observed (Figure 1). This is typical of carbon-containing compounds with amino groups. Sharp absorbance peaks are observed at 2926 cm^{-1}, 1625 cm^{-1}, and 1048 cm^{-1} and are indicative of aliphatic chains (- CH_3 - and –CH_2–). These peaks reflect the presence of alkyl chains in the compound. A strong band was also observed at 1744 cm^{-1}, which is due to the presence of carbonyl group. The presence of C=O bonds causing C=O stretching vibrations leads to absorbance peaks in these regions. The FTIR spectrum implied the production of a lipopeptide biosurfactant.

Anticancer potential of Bacillus pumilus AVS5 lipopeptide: The interactions of surfactin-like lipopeptides with various cell lines have been studied by many researchers. Cell viability in K562 cells when various concentrations of biosurfactants treated was given in figure 2. The concentrations of 5 µg suppressed the

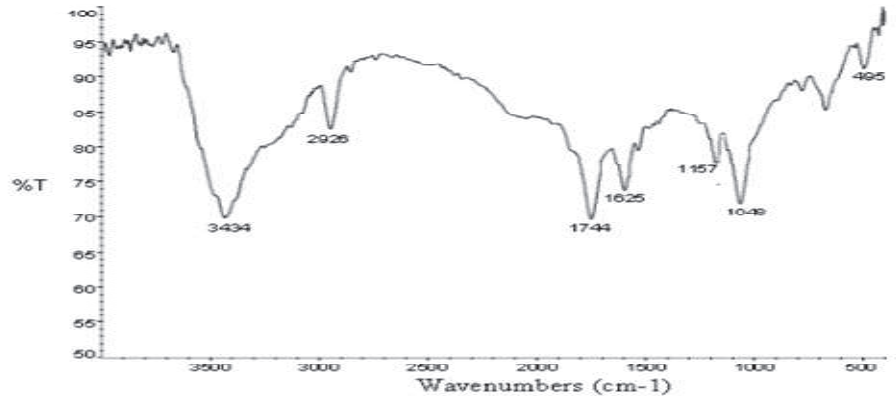


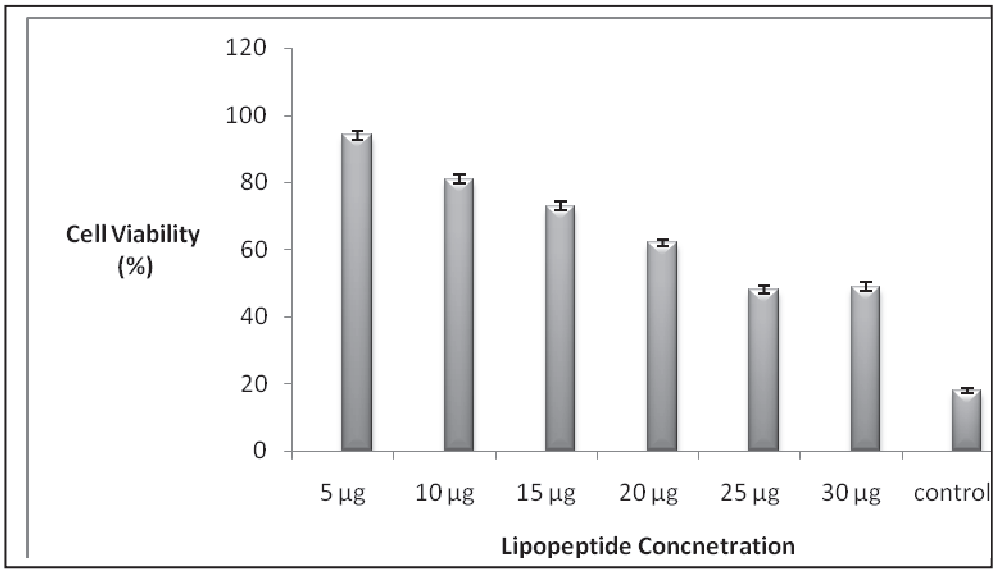
Fig 1: FTIR Spectrum of a lipopeptide produced by Bacillus pumilus AVS5.

cells of 5.78%; 10 µg at 18.94%; 15 µg at 26.65%; 20 µg at 38.84%; 25 and 30 µg at the maximum of 52.34 and 52.85 % respectively. Results indicated that that this lipopeptide exhibited cytotoxicity to the K562 cell

lines. Wang et. al., [17] reported a new cyclic lipopeptide that inhibited proliferation in K562 cells with an IC50 value approximately to 30 µg/mL. Surfactin is one of the most powerful biosurfactants and is known to have anti-inflammatory, antibiotic and anti-tumor functions [18].

Cao *et al* [19] demonstrated that surfactin induces apoptosis in human breast cancer MCF-7 cells through a ROS/JNK mediated mitochondrial/caspase pathway. An antitumor lipopeptide biosurfactant purified from Bacillus natto TK-1 was able to inhibit the proliferation of MCF-7 human breast-cancer cells [20].

Apoptosis is a genetically controlled mechanism essential for the maintenance of tissue homeostasis, proper development and elimination of unwanted cells. In cells undergoing apoptosis, a fraction of nuclear DNA is fragmented to the size equivalent of DNA in mono- or oligonucleosomes. When such DNA is analyzed by agarose gel electrophoresis it generates the characteristic 'ladder' pattern of discontinuous DNA fragments [21, 22]. Such a pattern of DNA degradation generally serves as a marker of the apoptotic mode of cell death. As shown in Fig 3, we observed that the DNA degradation clearly occurred in *Bacillus pumilus AVS*5 lipopeptide treated leukemic cells, indicating that the lipopeptide was capable to induce apoptosis.



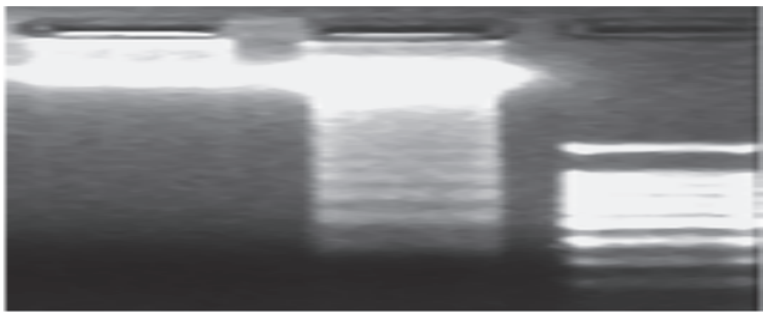


Fig 3: DNA ladder Assay. A – Cells treated with 0.025 µg of lipopeptide. B - Cells treated with 2.5 µg of lipopeptide and C – 1 Kilo Base pair Ladder

Conclusion: A major obstacle to cancer chemotherapy is the development of resistant cell populations following the relapse of an initially responsive malignancy. Such chemo-resistance has motivated investigators to develop new drugs that are more efficacious and have fewer side effects. In the present study, a novel lipopeptide, isolated from Bacillus pumilus AVS5, an endophytic icroorganism of *Aloe vera* was shown to exhibited marked cytotoxicity and effectively reduced the proliferation of K562 luekemic cells. Therefore, the particular molecule can be a potential candidate as an anticancer lead.

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