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Original Article

Halophilic Exopolysaccharide Isolated from Swamithope Salt Pans Induce Cell Death through Apoptosis in A549, MCF-7, HT-29 Cancer Cells

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ABSTRACT

Bacteria that are adapted to live in high salt concentrations were isolated from crystalline salt works of Swamithope, India. Selected strains were observed for the production of extra polymeric substances. 16S rRNA sequence of the selected strain revealed that the isolate belongs to the genus *Virgibacillus*. After routine analysis of microbial and biochemical methods, the polysaccharide was partially purified and identified as surfactant. The compound shows good emulsifying property with various oils. GC MS results revealed the presence of diphenyl-methane, methyl-D-glucose, enolide, cobalt, dicyano-methylene, phenoxaphosphine, benzene and ethanol derivatives in the compound. Pharmacological screening of antibacterial and anticancer assays showed inhibition effect towards various bacterial pathogens and cancer cells. Cell proliferation assay shows growth inhibition effect towards A549, MCF-7 & HT-27.As per our findings it is clear that certain exopolysaccharides possess multiple applications which can be useful for industrial applications and to develop new drugs.

Key words: *Virgibacillus*, 16S rRNA, A549, MCF-7, HT-27, GC MS, Salt Pans

INTRODUCTION

Exopolysaccharides are polysaccharide compounds that consist of monomeric subunits adjoining to form macromolecular structures. Many bacteria are known to synthesis polysaccharides for their livelihood and serve protection against pathogens which have been recently observed to have important role in various industries. Many such polymeric substances were isolated and used as surfactants and emulsifiers that are indispensable in daily life [1]. Hence are classified under biosurfactants. Biosurfactants are biologically active compounds that influence on surface interfaces [2]. Rather they make differences in their structure and composition most of the exopolysaccharides carry functional organic groups [3] such as acetyl, succinyl or pyruvate and some inorganic constituents such as sulfate as well. Moderate halophilic microorganisms are phylogenetically very diverse, including a great variety of microorganisms. They grow either in an absence or up to 20% (w/v) NaCl [4]. *Virgibacillus* is a new genus comprising of mesophilic and moderately halophilic bacteria. They grow at NaCl concentration between 5-10% (w/v). This genus was introduced in 1998 to accommodate *Bacillus pantothenticus* [5]. They reduce the surface tension and interfacial tension between individual molecules at the surface and interface respectively.

MATERIALS AND METHODS

Isolation and characterization of Virgibacillus sp.: Crystalline soil samples mixed with water were aseptically collected from salt works of Swamithope in Kanyakumari district, Tamil Nadu, India (8.12^0N 77.49^0E) using sterile polythene bags. The samples were transported immediately to the laboratory and stored at 4^0C for culture studies. 1gm of the sample was diluted in 100ml conical flask using distilled water. 0.1ml of water sample was serial diluted up to 10^{-8} dilutions in order to reduce the microbial load. 100µl from each dilution was spread plated on Zobell agar plates supplemented with NaCl. The plates were allowed to incubate at 37^0C for 4-7 days. After incubation, the colonies appeared were morphologically and biochemically identified. Individual colonies having varied visual features were picked for

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further studies. 16S rRNA sequence was performed to confirm the strain identity.

Optimization of strain and EPS production: The strain *Virgibacillus* sp. was optimized for varied parameters such as temperature (35 – 55^{0}C), pH (5 – 9), NaCl (7 – 15%), amino acid (mg) and carbon sources (mg) in the growth medium. Similarly the optimum condition for EPS production was also estimated. The growth of the organism was periodically checked by taking optical density at 600 nm.

EPS production and extraction: The broth culture (100ml) was centrifuged at 12, 000 rpm for 10 minutes in a cooling centrifuge. The supernatant was collected and mixed with equal volume of ice cold methanol and incubated for 24 hours at 4°C. The refrigerated solution was then centrifuged at 2500 rpm for 20 minutes. After that the pellets and supernatants were separately collected in sterile containers and subjected to proximate chemical estimation. The obtained pellet was resuspended in distilled water along with equal volume of ice cold methanol and the mixture was kept into refrigerator for overnight. Then it was centrifuged at 2500 rpm for 20 minutes. The final pellet obtained was dried at 60°C and weighed**.**

16S rRNA sequence: DNA was isolated from the Bacterial culture provided, The DNA was used in PCR to amplify the 16S region using 16S Forward and 16S Reverse primers described in the literature. The ~1500 bp amplicon was gel eluted and subjected to sequencing. The sequencing results were assembled and compared with NCBI database.

GC MS: GC-MS analysis of partially purified biosurfactants were analysed individually using Agilent GC-MS 5975 Inert XL MSD (United States) gas chromatography equipped with J and W 122 – 5532G DB-5 ms 30 × 0.25 mm× 0.25 µm and mass detector (EM with replaceable horn) was operated in EMV mode. Helium was used as carrier gas with the flowrate of 1.0 ml min-1. The injection port temperature was operated at 250°C. The column oven temperature was held at 80°C for 2 min then programmed at 10°C min-1 to 250°C, which was held for 0 min, and then at 5°C min-1 to 280°C which was held for 9 min. Electron impact spectra in positive ionization mode were acquired between m/z 40 and 450.

Antimicrobial screening of EPS: The EPS extracted from *Virgibacillus sp.* was tested against common pathogens (*Vibrio cholera, Vibrio harveyi, Salmonella typhi, Pseudomonas aeruginosa and Klebsiella pneumonia*) using agar well diffusion method. Agar plates were prepared and final pH was adjusted to 7.0. The selected organisms were spread plated on solid agar media with appropriate inoculums (≈104 CFU). Using a sterile cork border approximately 7mm size wells were punched and maintained which were subsequently filled with 50µl of EPS extract. The plates were incubated for 24 - 48 h at 37^{0}C. After incubation, the zone of inhibition was measured and calculated.

Cell culture: The breast cancer cell line MCF-7, lung cancer cell line A549 and colon cancer cell line HT-29 were obtained from NCCS (National Centre for Cell Science), Pune and cultured in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°c in 5 % CO2 in a humidified atmosphere in a CO2 incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (Himedia)) for 2 minutes and passaged to T flasks in complete aseptic conditions. Extracts were added to grown cells at a final concentration of 6.25 µg, 12.5µg, 25µg, 50 µg and 100µg/ml from a stock of 1mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation.

Cell proliferation assay: MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (Himedia) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

The cells was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT - 5mg/ml dissolved in PBS). It was then incubated at 37^{0}C for 3 hours. MTT was removed by washing with 1x PBS and 200µlof DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a microplate reader (ELISASCAN, ERBA).

RESULTS AND DISCUSSION

Five different colonies were isolated from agar plates based on their morphology and colour variation. All the isolates were obtained from varied locations of solar salt works of Swamithope. Growth at different salt concentration proved all the isolates were moderate halophiles that failed to grow at increased NaCl concentration of 15%. From the five isolates, a single colony was picked for biochemical analysis and its 16S rRNA sequence revealed the strain belongs to the genus *Virgibacilllus* (Fig 1). In order to obtain clearance, a phylogenetic tree was constructed which showed the place of *Virgibacillus* with relation to other Halomonas strain (Fig 2). Species related to *Virgibacillus* strains were joined using evolutionary distances revealed close similarity. The sample BACTERIA – 1307 shows 99% identity at 96% coverage with the sequence from *Virgibacillus sp.* Recent research led by Donio [3] has found halophilic bacterium BS4 from crystalline soils of Thamaraikulam region. The colonies of *Virgibacillus* are thread like. Each individual colony form 4µm in size with dim white colouration. Biochemical analysis of the selected strain showed Catalase, Gelatine hydrolysis, Sugar, Glucose, Sucrose, Arabinose and Dextrose positive whereas Oxidase, TSI, Indole, Methyl red, Voges proska, Citrate, Urease and Lactose negative. The selected organism is gram negative and showed motility nature.

The strain was noted to produce extrapolymeric substance in Zobell agar media after 4-7 days of incubation. To obtain higher EPS yield the strain was optimized using different pH, NaCl concentration, temperature, carbon and amino acid sources. The growth characteristics of the strain showed log phase after 3rd day and continued producing EPS till 20th day and started to decline on 25th day. The growth at every day was determined using the optical density at 600nm. The purpose of optimization was to find the strain growth in a suitable condition to produce maximum EPS. From the results obtained, it was concluded that the strain can grow well at 40^{0}C in an optimum pH of 7±2. Optimum growth and EPS production was obtained at NaCl concentration of 9 %. The different role of carbon and amino acid sources were studied in order to enrich the media for enhanced production of EPS. It was found that the presence of lactose (1 mg) and tyrosine (1mg) in the culture media promote EPS by 5%. EPS production was observed from the fourth day during log phase and continued throughout the exponential phase. A study conducted by Reddy and co-workers have found time dependent variation in EPS production which shows maximum yield during log phase. After three days of *Bacillus licheniformis* incubation, it produces a maximum EPS yield of 576mgl^{-1} which is comparatively higher than our study [6, 7]. We compared the strain growth and EPS production in media containing carbon and amino acid sources. Pandey reported that media containing sucrose reduced EPS production to >1g/l. Rather sucrose, glucose enhanced EPS production by 1-5% and the maximum yield was achieved at 4% glucose concentration [8]. Halomonas sps BS4 isolated from Thamaraikulam solar salt pans revealed optimum growth at 8% NaCl to produce surfactant compound. The optimum pH was concluded to be 6-8 [9]. Six distinct compounds were obtained at varied retention time (Rt) (Fig 4 and 5). The peak obtained in the Rt-12.713 indicates the presence of diphenlymethane (100%), Rt-18.315 indicate the presence of methyl-D-glucose and enolide (11.98%), Rt-20.576 indicate the presence of cobalt and dicyanomethylene (31.69%), Rt-21.516 indicate the presence of phenoxaphosphine (9.27%), Rt-22.747 was unknown compound (11.12%) and Rt-24.197 indicate the presence of benzene and ethanol (28.73%).

The partially purified surfactant isolated from moderate halophilic bacterium *Virgibacillus sp.* was checked for antibacterial activity against gram negative pathogens. Sample aliquots treated in spread plate cultures of *Vibrio cholera, Vibrio harveyi, Salmonella typhi, Pseudomonas aeruginosa and Klebsiella pneumonia* showed growth inhibition which were confirmed by the formation of inhibition zones around the wells. The plates were incubated at 37^{0}C for 24 h. It was noted that 25µg EPS resists *Pseudomonas aeruginosa* growth by forming 15 mm zone (Diameter); whereas *Vibryo cholera and Vibrio harveyi* showed 13 & 17 mm inhibition zones. The EPS showed moderately resistance against *Salmonella typhi* and *Klebsiella pneumonia* with 9 & 7 mm inhibition. The zone of inhibition thus formed can be increased by increase in sample concentration. The antibacterial activity of EPS isolated from *Bacillus sp* BS4 from neighbouring areas of Swamithope salt pan showed resistance against *Staphylococcus aureus* (15.35mm), *Klebsiella pneumonia* (15.60mm), *Salmonella typhi* (11.98mm) *and Streptococcus pyrogenes* (17.33mm) [9]. Our records of *Salmonella typhi* showed 12mm inhibition which is similar to the data obtained by [10]. Regarding *Klebsiella pneumonia* a 3mm variation in zone of inhibition was recorded.

The anti-proliferative effect of EPS produced by *Virgibacillus sp.* was studied against A549 (Lung carcinoma), HT-29 (Colon cancer cells) and MCF-7 (breast cancer) cell lines. The death of cancer

cells were assessed by cell proliferation assay (MTT) (3-(4,5 – Dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide. The selected cell lines were treated with aliquots of EPS at varied concentrations (6.25, 12.5, 25, 50 & 100 µg/ml) for 24 – 48 h. It was noted that EPS inhibited the proliferation of cell lines in a dose and time dependent manner (Fig. 6). The IC_{50} values for each cell lines were determined from the MTT data are 351 µg/ml (A549), 114 µg/ml (HT-29) and 159 µg/ml (MCF-7). Cells treated with EPS (100 µg/ml) showed moderately Cytotoxicity in A549 cell lines after 48 h. The % of viability was recorded to be 23.69 % (Table. 1). Similarly in HT-29 treatment, the % of viable cells was calculated to be 53.57 % (Fig. 7) (Table 2). In MCF-7 cell lines the EPS showed 47.40% viability when compared with A549 and HT-29 (Table. 3).

Cells treated with EPS were further examined by microscopic observation to reveal the cell morphological changes associated with cell death. It was noted that cell lines treated with exopolysaccharide are irregular in their morphology that indicate blebbing of membrane and shrinkage of cells. Increase in incubation time causes cell rupture and death of cells. A recent study conducted by [7, 9] have identified Bacillus sp. BS4 to produce a surfactant compound under moderate condition which also showed anti cancer properties. Partially purified EPS sample was treated against mammary epithelial carcinoma cell lines (0.00025 µg) showed cell inhibition by 4.44%. With increased EPS concentration (25 µg) suppressed 47.42% cell proliferation [10].

CONCLUSION

The strain *Virgibacillus sp.* has been isolated first time at crystalline salt pans of Swamithope region which is known to produce an exopolysaccharide. In order to obtain maximum strain growth and EPS production, the strain was optimized at different concentrations of NaCl tolerance and compound to be found enhanced EPS production in culture broth. Routine biochemical and spectral screening revealed the exopolysaccharide is a surfactant compound named ‘surfactin’. The biological role of EPS showed good antibacterial and anti-cancer properties. Hence it is concluded that the compound have potential role in industrial application in Microbial Enhanced Oil Recovery (MEOR). Further study is needed to investigate the active ingredients present in the more purified form of EPS as a target based drug molecule in cancer drug development.

1 cgtagtatat gctgtagcgt gctcgtcgag cacgggaagt tggaagatcc tctccaagag

61 gtgacgcctg tggaacgagc ggcggacggg tgagtaacac gtgggcaacc tgcctgtaag

121 attgggataa ccccgggaaa ccggggctaa taccggataa tacttttcgt tgcataacga

181 gaagttgaaa ggcggctttt agctgtcact tacagatggg cccgcggcgc attagctagt

241 tggtaaggta acggcttacc aaggcgacga tgcgtagccg acctgagagg gtgatcggcc

301 acactgggac tgagacacgg cccagactcc tacgggaggc agcagtaggg aatcttccgc

361 aatggacgaa agtctgacgg agcaacgccg cgtgagtgat gaaggttttc ggatcgtaaa

421 actctgttgt cagggaagaa caagtgccgt tcaaataggg cggcaccttg acggtacctg

481 accagaaagc cccggctaac tacgtgccag cagccgcggt aatacgtagg gggcaagcgt

541 tgtccggaat tattgggcgt aaagcgcgcg caggcggtcc tttaagtctg atgtgaaagc

601 ccacggctta accgtggagg gccattggaa actggaggac ttgagtacag aagaggagag

661 tggaattcca cgtgtagcgg tgaaatgcgt agagatgtgg aggaacacca gtggcgaagg

721 cgactctctg gtctgtaact gacgctgagg

Fig 1. I The sequence of the 16S region for sample **BACTERIA – 1307** is as follows

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident
Virgibacillus sp. NCCP-182 gene for 16S rRNA, partial sequence	1306	1306	96%	0.0	99%
Uncultured Virgibacillus sp. isolate DGGE gel band ntu249 16S ribosomal RNA gene, partial sec	1306	1306	96%	0.0	99%
Virgibacillus marismortui strain I121 16S ribosomal RNA gene, partial sequence	1306	1306	96%	0.0	99%
Virgibacillus marismortui strain I15 16S ribosomal RNA gene, partial sequence	1306	1306	96%	0.0	99%
Bacillus sp. HPB29 16S ribosomal RNA gene, complete sequence	1303	1303	96%	0.0	99%
Virgibacillus salarius strain 01 16S ribosomal RNA gene, partial sequence	1301	1301	96%	0.0	99%
Virgibacillus sp. T2 16S ribosomal RNA gene, partial sequence	1301	1301	96%	0.0	99%
Virgibacillus sp. F2 16S ribosomal RNA gene, partial sequence	1301	1301	96%	0.0	99%
Virgibacillus salarius strain KSI 760 16S ribosomal RNA gene, partial sequence	1301	1301	96%	0.0	99%
Virgibacillus salarius strain MSP10.4 16S ribosomal RNA gene, partial sequence	1301	1301	96%	0.0	99%
Virgibacillus sp. A355 16S ribosomal RNA gene, partial sequence	1301	1301	96%	0.0	99%

Fig 2. J The Top 10 matches for the sequence derived from **BACTERIA – 1307** is provided above.

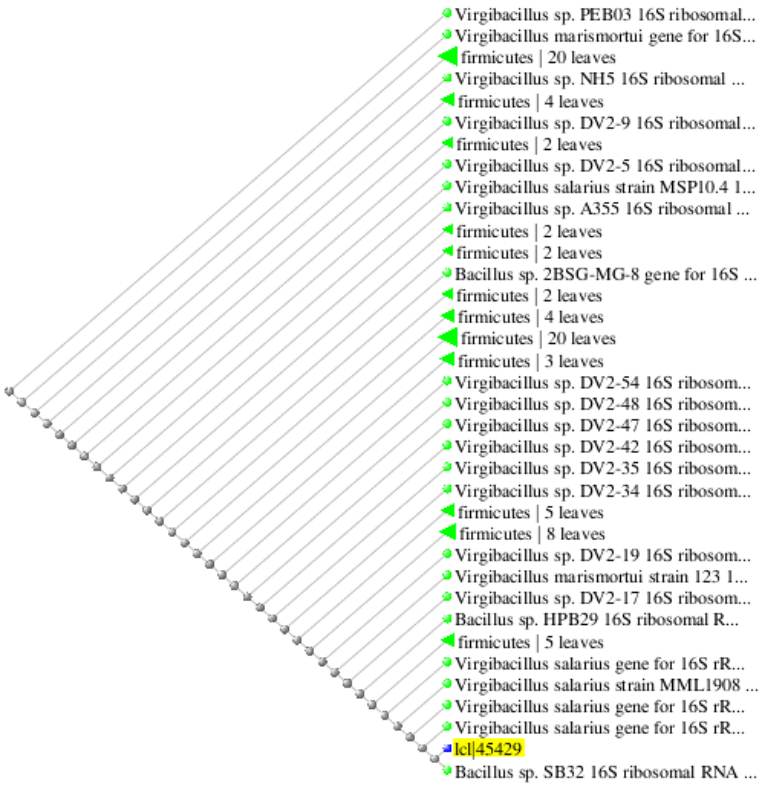
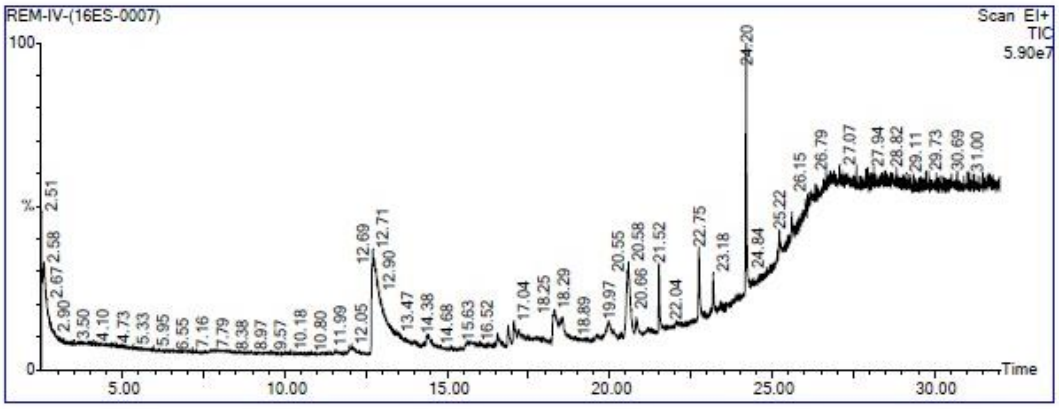


Fig 3. Phylogenetic relationship between *Virgibacillus* and other related species are presented here. The tree was constructed based on their 16S rRNA gene sequences.



#	RT	Scan	Height	Area	Area %	Norm %
1	12.713	2042	18,437,916	5,276,027.0	51.871	100.00
2	18.315	3162	4,732,300	631,904.1	6.213	11.98
3	20.576	3614	13,186,950	1,671,886.5	16.437	31.69
4	21.516	3802	12,489,665	489,346.4	4.811	9.27
5	22.747	4048	13,024,900	586,525.2	5.766	11.12
6	24.197	4338	44,764,808	1,515,802.8	14.902	28.73

Fig 4. GC MS results showed six distinct peaks at varied retention time (RT) illustrate the presence of different compounds to be present in the exopolysaccharide.

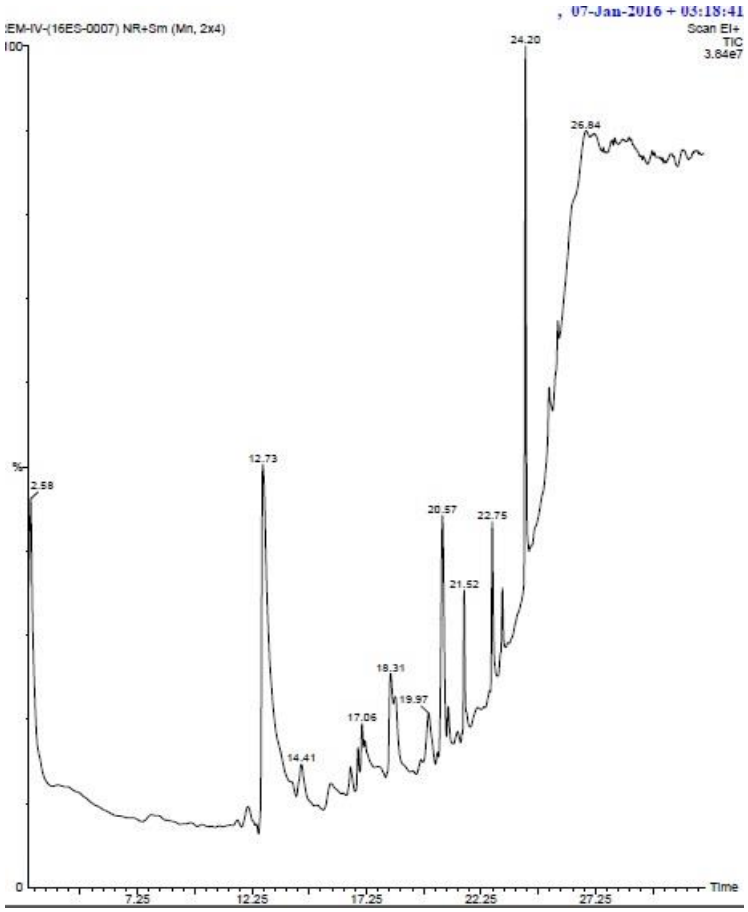


Fig 5. The height of each peaks represent the percentage of specific compounds present in the sample.

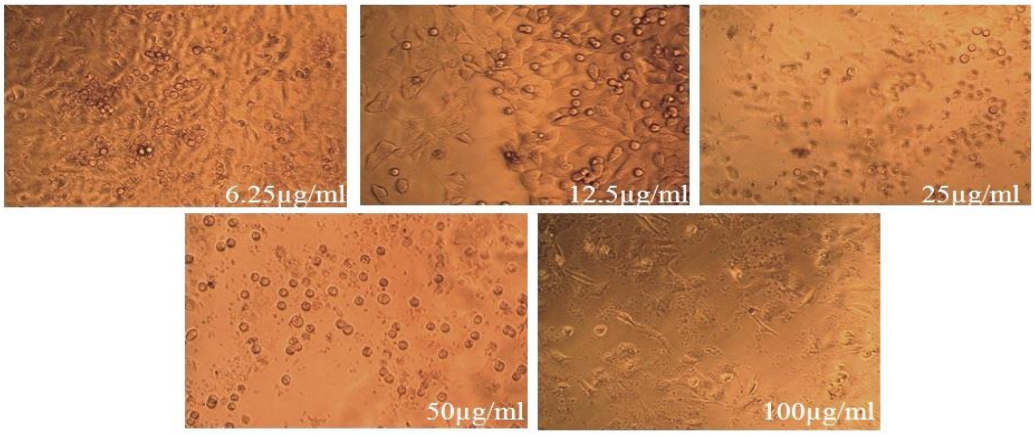


Fig 6: Effect of EPS on the proliferation and viability of A549 cells. A549 cell proliferation was assessed by MTT assay. Cells were treated with 6.25, 12.5, 25, 50 and 100 µg/ml of EPS extracted from *Virgibacillus sp.* for 48 hrs.

EPS (µg/ml)	**Average OD at 540nm**	**% viability A549**
Control	1.3573	
6.25	0.3122	44.89
12.5	0.2932	42.16
25	0.2121	40.56
50	0.2677	38.49
100	0.1648	23.69

Table 1. Cytotoxicity effect of EPS towards A549 cells.

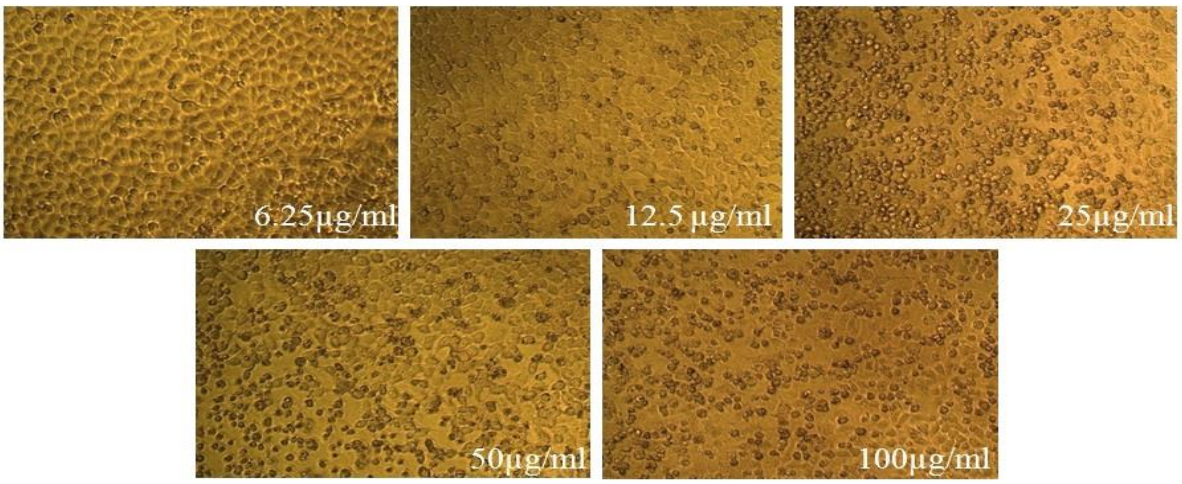


Fig 7: Effect of EPS on the proliferation and viability of HT-29 cells. HT-29 cell proliferation was assessed by MTT assay. Cells were treated with 6.25, 12.5, 25, 50 and 100 µg/ml of EPS extracted from *Virgibacillus sp.* for 48 hrs.

EPS sample (µg/ml)	**Average OD at 540nm**	**% viability HT-29**
Control	0.6934	
6.25	0.4975	96.88
12.5	0.4382	85.33
25	0.3707	72.19
50	0.3097	53.57
100	0.2751	53.57

Table 2. Cytotoxicity effect of EPS towards HT-29 cells

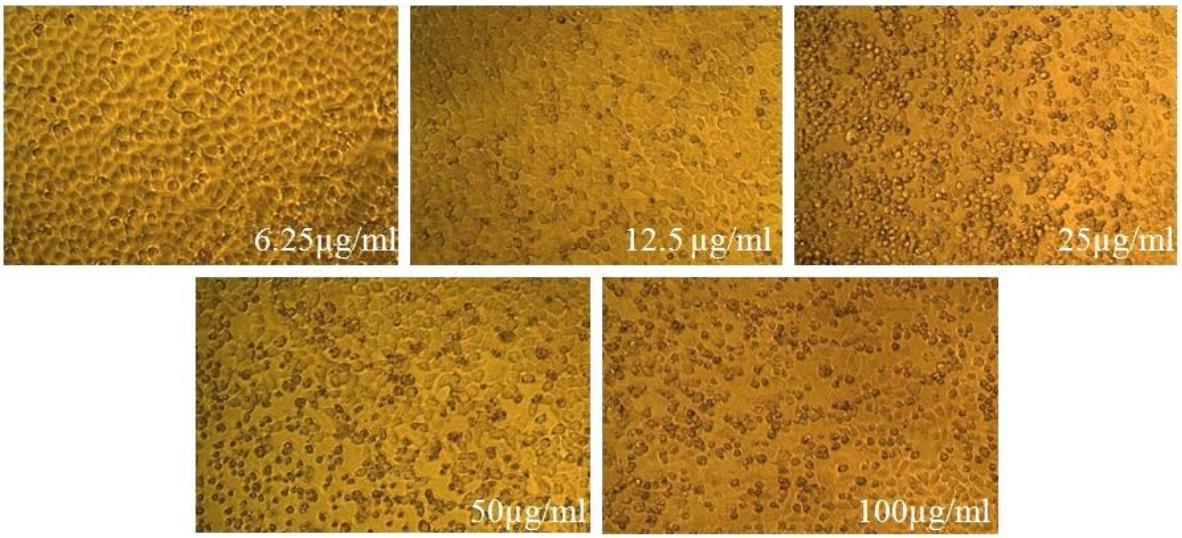


Fig 8: Effect of EPS on the proliferation and viability of MCF-7 cells. MCF-7 cell proliferation was assessed by MTT assay. Cells were treated with 6.25, 12.5, 25, 50 and 100 µg/ml of EPS extracted from *Virgibacillus sp*. for 48 hrs.

EPS sample (µg/ml)	**Average OD at 540nm**	**% viability MCF-7**
Control	0.5123	
6.25	0.2747	93.84
12.5	0.0324	76.00
25	0.9052	66.64
50	0.8585	63.20
100	0.6439	47.40

Table 3. Cytotoxicity effect of EPS towards MCF-7 cells.

REFERENCES

1. Reddy et al. Isolation and characterization of exopolysaccharides from seaweed associated bacteria Bacillus licheniformis. Carbohydrate Polymers 2011; 84: 1019 – 1026.
2. Ashok Pandey et al. Studies on structural and physical characteristics of a novel exopolysaccharide from Pseudozyma sp. NII 08165. International Journal of Biological Macromolecules 2013; 59: 84– 89
3. Donio et al. Halomonas sp. BS4, A biosurfactant producing halophilic bacterium isolated from solar salt works in India and their biomedical importance. Springer Plus 2013; 2:149.
4. Ventosa A et al. Biology of moderately halophilic aerobic bacteria. Microbiol. Mol Biol Rev 1998; 62: 504-544.
5. Ventosa A. Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76. Extremophiles. 2003; **7**: 221-228.
6. Heyndrickx M et al. *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and knight 1950). Emended description of *Virgibacillus pantothenticus.* International Journal of Systematic Bacteriology 1998; 48: 99-106.
7. Siegmund L. Biological amphiphiles (microbial biosurfactants). Current Opinion Colloid Interf Sci 2002; 7:12-20.
8. Yeh MS et al. Bioreactor design for enhanced carrier-assisted surfactin production with *Bacillus Subtilis*. Process Biochem 2006; 41: 1799–1805.
9. Joshi S et al. Biosurfactant production using molasses and whey under thermophilic conditions. Bioresour Technol 2008; 99:195–199.
10. Mandal SK et al. Isolation and characterization of exopolysaccharide secreted by a toxic dinoflagellate, Amphidinium carterae Hulburt 1957 and its probable role in harmful algal blooms (HABs). Microb Ecol 2011; 62:518-27.