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Characterization and *in vitro* studies on anticancer activity of exopolymer of *Bacillus thuringiensis* S13

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The objective of the present work was to isolate and characterize the exopolymer producer from a marine environment. The exopolymer producing strain (S13) was identified as *Bacillus thuringiensis* S13. Characterization of exopolymer shows the presence of brominated compound responsible for cytotoxicity on lung cancer cell line (A549) on XTT assay. An *in vitro* study of bacterial exopolymer shows the presence of cytotoxic effects on cell lines. Further, active compound in exopolymer responsible for cytotoxicity has to be characterized. The exopolymer produced by *B. thuringiensis* S13, showed potent cytotoxic effects, and could be used as therapy in cancer after further studies.

Key words: *Bacillus thuringiensis* S13, exopolymer, IR, GCMS analysis and lung cancer cell line.

INTRODUCTION

Ocean inhabits millions of unexplored organisms, which secretes valuable unexplored compounds. Up to 2008, only about ten thousand compounds have been evolved from marine organisms. The assorted environments in marine, force the bacteria to secrete some compounds to survive (Jensen and Fenical, 1994). In the marine ecosystem, bacteria secrete many bioactive exopolymers compared with another local, since marine has numerous and distinct capabilities to endure (Annarita et al., 2010). Majority of bacterial species produced secondary metabolites as exopolymer containing sugar and non-sugar components like amino sugars, protein (Sutherland, 1977) and fatty acids (Hayashida-Soiza et al., 2008). The adaptation of bacteria to diverse habitat can determine the production of unique secondary metabolites as exocellular. Sea provides an abundant source of nutrients and other parameters; the bacteria on the sea surface aggregates to form biofilm (Ng and Hu, 1989; Donlan and Costerton, 2002). Some bacteria do not produce any compounds because they make contact with other bacterial species or extracellular products from other bacteria. Several investigation exhibits number of biologically active compounds from marine bacteria (Barsby et al., 2001) showing antimicrobial activities,

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antiviral, immunosuppressive, enzyme inhibitor metabolites, receptor antagonistic, antitumor activities and anticoagulant properties (Reichenbach, 2001). The chemical nature of bacterial exopolymer is often highly complex. It was found that most of the marine isolates exhibit antagonistic activity. A term cancer is used to define uncontrolled cell growth. The cells divide rapidly, forming malignant tumour, and invading the adjacent cells. Cancer affects various parts of the body. Each cancer is different, and the chance of surviving depends on the cancer and site of cancerous growth. Cancer can affect any human being regardless of age. About 13% of human death is due to cancer throughout the world. Lung cancer causes more people to die than other cancers worldwide, accounting 1.3 million people to die annually (Khuri et al., 2001). At present, the clinical responses to patients of effectively targeted therapies for lung cancer are still inadequate (Danesi et al., 2003). Hence, the development of new effective anti-cancer drugs for lung cancer is the most important need of the day.

Polysaccharides derived from a microorganism have specific broad ranged properties such as antitumor, antioxidant activity. Bacterial exopolymeric substances have a wide range of applications (Adriana et al., 2005; Chen et al., 2008) that makes them interesting from the biotechnological point of view.

MATERIALS AND METHODS

Screening and identification

Soil samples were collected from Mandapam, coastal area (790 8' E, 9° 17' N) of south India at a depth of 5 m. The bacterial strains were screened in Zobell agar plates (Himedia India) followed by serial dilution method. The plates were incubated at 25°C for a period of seven days. Morphology of bacterial colonies were observed and selected based on their mucoid morphology (Ng and Hu, 1989). After isolation, the strain (S13) was characterized phenotypically and biochemically, which was subcultured and maintained in 20% glycerol at 4°C as stock (Jean-Marc et al., 1990). For 16S rDNA gene sequencing, the bacterial colonies were picked and suspended in 0.5 µl of sterilized saline and centrifuged at 10,000 rpm for 10 min. After removal of the supernatant, the pellet was suspended in 0.5 µl of InstaGene Matrix (Bio-Rad, USA) and incubated at 56°C for 30 min, then heated at 100°C for 10 min. After heating, supernatant can be used for PCR. Template DNA (1 µl) was treated with 20 µl of PCR reaction solution, and 27F/1492R primers were used for amplification. Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR clean up kit (Millipore). The 16S rDNA was amplified by polymerase chain reaction (PCR) using the primers 518F CCAgCAgCCgCggTAATACg and 800R TACCAgggTATCTAATCC universal primers. The purified PCR products were sequenced by using 2 primers as described earlier. Sequencing was performed using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on automated DNA sequencing system. DNA sequence was obtained using DNA sequencer and the PCR products were sequenced using the same PCR primers and other internal primers to confirm the sequence (Dereeper et al., 2008).

The sequence of 16s rDNA gene was compared with the sequence data through BLAST searching tool in NCBI. As a result of BLAST search, the sequences were aligned to their closest sequence with MUSCLE (v3.7) and refined using Gblocks 0.91b (Castresana, 2000). The phylogenetic tree was constructed by SH-like method implemented in the PhyML program.

Extraction and characterization exopolymeric substance

The isolate S13 was grown in Zobell marine broth for 7 days at 32°C. The exopolymer was extracted, treated with two volumes of 95% cold ethanol to the cell free supernatant and stored at 4°C for 24 h. The precipitate was collected by centrifugation and washed with distilled water. The precipitate was redissolved in distilled water and dialyzed (molecular weight (mol. wt.) cut off 8000 dalton) against distilled water for 2 days to remove the salts from exopolymer. The weight of the exopolymer was measured after drying at 45°C (Bragadeeswaran et al., 2011).

Characterization of bacterial exopolymeric substance

The total sugar content was measured using phenol sulphuric acid assay with glucose as standard (Bruckner, 1955). The total protein content was measured by Lowry's method with bovine serum albumin as standard (Lowry et al., 1951). FT-IR spectra were recorded for exopolymer with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} region (Lijour et al., 1994). GCMS analysis was performed in JEOL GCMATE II GCMS (Mancuso et al., 2004). Helium was the carrier gas. Peaks were identified by comparing with known standards.

Optimization

The optimum carbon source for the growth and exopolymer production was achieved by fermentation in 100 ml of basal salt media. The different concentration (0.5, 1, 1.5, 2 and 2.5%) of carbon sources (glucose, sucrose, lactose and galactose) (Mata et al., 2008) were tested by inoculation with 2 ml of 24 h culture. The basal salt medium was supplied with 1% of different nitrogen source (peptone, yeast extract, ammonium chloride, and ammonium nitrate) to determining the effect of nitrogen source (Sung-Hwan et al., 2000). The isolate, which exhibited maximum growth and yield of exopolymer for carbon and nitrogen sources respectively, was selected for optimization of carbon: Nitrogen ratio. The basal salt medium with carbon source and nitrogen source which showed maximum yield were selected and different concentration of nitrogen was supplied (0.05 to 0.1%) for the respective isolate (Read and Costerton, 1987). The optimum pH for exopolymer synthesis was determined in basal salt media with different pH (5, 5.5, 6, 6.5, and 7) and, exopolymer production was achieved in production media (casein hydrolysate media) at 37°C in a shaker. The growth rate was measured at 540 nm.

In vitro anticancer activity

Briefly, the cancer cell lines (A549 lung cancer) were procured from National Centre for Cell Science, Pune. The cell lines were grown and maintained in Minimal essential medium (MEM, GIBCO) supplemented with 4.5 g/L glucose, 2 mM L-glutamine and 5% fetal bovine serum at 37°C in 5% CO_2 incubator. From the T-25 flask, the trypsinized cells were seeded in each well of 96 well flat-bottomed plates and incubated in 5% CO_2 at 37°C. After 24 h of incubation, the supernatant was discarded and, the cells were

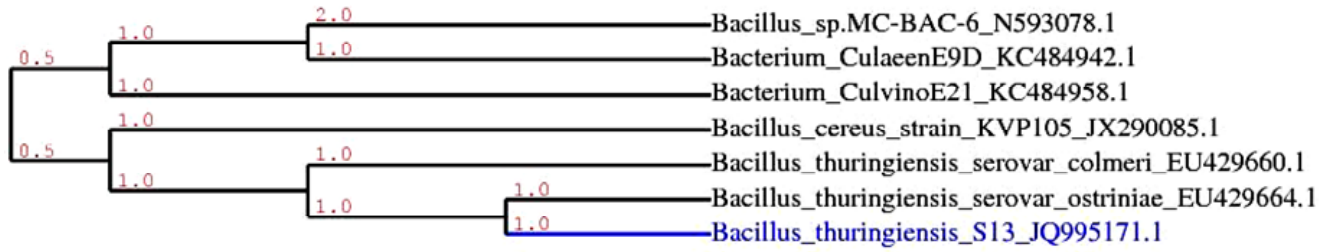

Figure 1. Phylogenetic tree of isolate S13 to their closest NCBI (BLASTn) strains based on the 16S rDNA gene sequences.

pretreated with growth medium. Subsequently, it was mixed with different concentrations (12.5, 25, 50, 100 and 200 µg/ml) of exopolymer and cisplatin (an anticancer drug as standard) separately in triplicates to achieve a final volume of 100 µl and then cultured for 48 h. The exopolymer and cisplatin were prepared separately as 1.0 mg/ml concentration stock solutions in dimethyl sulfoxide (DMSO). Culture medium and solvent were used as blank. Each well then received 50 µl of fresh XTT (0.9 mg/ml in RPMI along with XTT activator reagent) followed by incubation for 2 h at 37°C. At the end of incubation, 96 micro well plates were shaken for 15 s (Roehm et al., 1991; Stevens and Olsen, 1993).

RESULTS

The exopolymer producing strain was isolated from marine sediment; it forms circular convex mucoid colonies in Zobell agar medium. The bacteria was characterized as Gram positive; rod shaped showing positive results to VP, catalase, oxidase, protein and starch hydrolysis reactions, negative for indole, MR, citrate utilization test. The isolate produce endospore and showed the presence of crystals. The 16S rRNA sequence revealed the isolate was *Bacillus thuringiensis.* The sequence was submitted in Genbank NCBI, and the accession number assigned as JQ995171.1. Figure 1 shows the phylogenetic relationship of *B. thuringiensis* S13 with its closely related sequence of blast results. From the results, it was confirmed that, it belonged to the Phylum *Firmicutes* and family *Bacillaceae.* Calorimetric study showed 62.01% of sugar and 3.27% of protein in exopolymer. FT IR spectrum (Figure 2.) revealed characteristic functional groups of exopolymer; C-Br stretching noticed at 605.61 and 651.89 cm^{-1}. Four or more CH_2 groups occur in an open chain at 752.19 cm^{-1}. Further, aryl fluoride C-F stretching peak was noticed at 1195.78 cm^{-1}. A broad stretching of CH_3, CH_2-CH_3 at 1334.65 to1454.23 cm^{-1}. Stretching peak at 1668 and 1334 cm^{-1} indicates the presence of COOH group. C-H stretching was noticed in 2885.31 to 2974.03 cm^{-1}, O-H stretching was observed in the range of 3195.83 to 3313.48 cm^{-1}.

Gas chromatography–mass spectrometry (GC-MS) analysis showed the presence of saturated fatty acid and brominated compounds (Table 1). All sugars support the growth and exopolymer production. As far as the carbon source is concerned, the growth and production rate of *B. thuringiensis* S13 is directly proportional to the concentration of sugars. For all sugars, the growth and production rate dramatically increased for sugar concentration. However, the highest yield (58.5 mg/l) of exopolymer was observed when glucose was supplied as a carbon source (Figure 3). Of the various nitrogen source tested, peptone produced the highest yield of exopolymer (peptone 49.5 mg/l, yeast extract 47.2 mg/l, ammonium chloride 41.5 mg/l, and ammonium nitrate 40 mg/l). The optimum carbon and nitrogen concentration for exopolymer production was noticed when 0.6% peptone was supplemented with 2.5% of glucose (121 mg/l) (Table 2). At different pH, the cultures showed a sigmoid growth (Figure 4). The effect of pH (6.0, 6.5, 7.0, 7.5 and 8.0) on growth and production was assessed and summarized in Table 3. The isolate showed sigmoidal growth and production at variable pH. However, the significant amount of exopolymer production was observed at pH 7.0 (292 mg/l). At low or high pH, the exopolymer production was stunned.

XTT assay showed that the bacterial exopolymer has an anti-proliferative activity on A549 lung cancer cells. After the addition of different concentration of exopolymer and cisplatin (control) to the cancer cells separately, the growth rate was measured by its optical density value at 490 nm followed by the addition of XTT. It was interesting to note that the exopolymer inhibits 50% of cancer cell growth at a concentration (100 µg/ml) (Table 4). From the results, the half maximal inhibitory concentration (IC50) of the bacterial exopolymer was estimated at 133.27 µg/mL, and IC 50 value for cisplatin at 23.7 µg/mL. Metabolically active cancer cells potentially reduced and convert the yellow tetrazolium salt (XTT) into orange formasan dye (Figure 5a) by the enzyme mitochondrial oxidoreductases. This conversion occurs only in the live cells. However, the bacterial exopolymer treated cells fail to reduce (Figure 5b) indicating that the bacterial exopolymer can inhibits the cell proliferation.

DISCUSSION

The promising strain was selected based on the development of mucoid morphology because it was one of the fundamental screenings for isolation of exopolymer producing bacteria. The presence of mucoid morphology is one of the key factors to screen the exopolymer-produ-

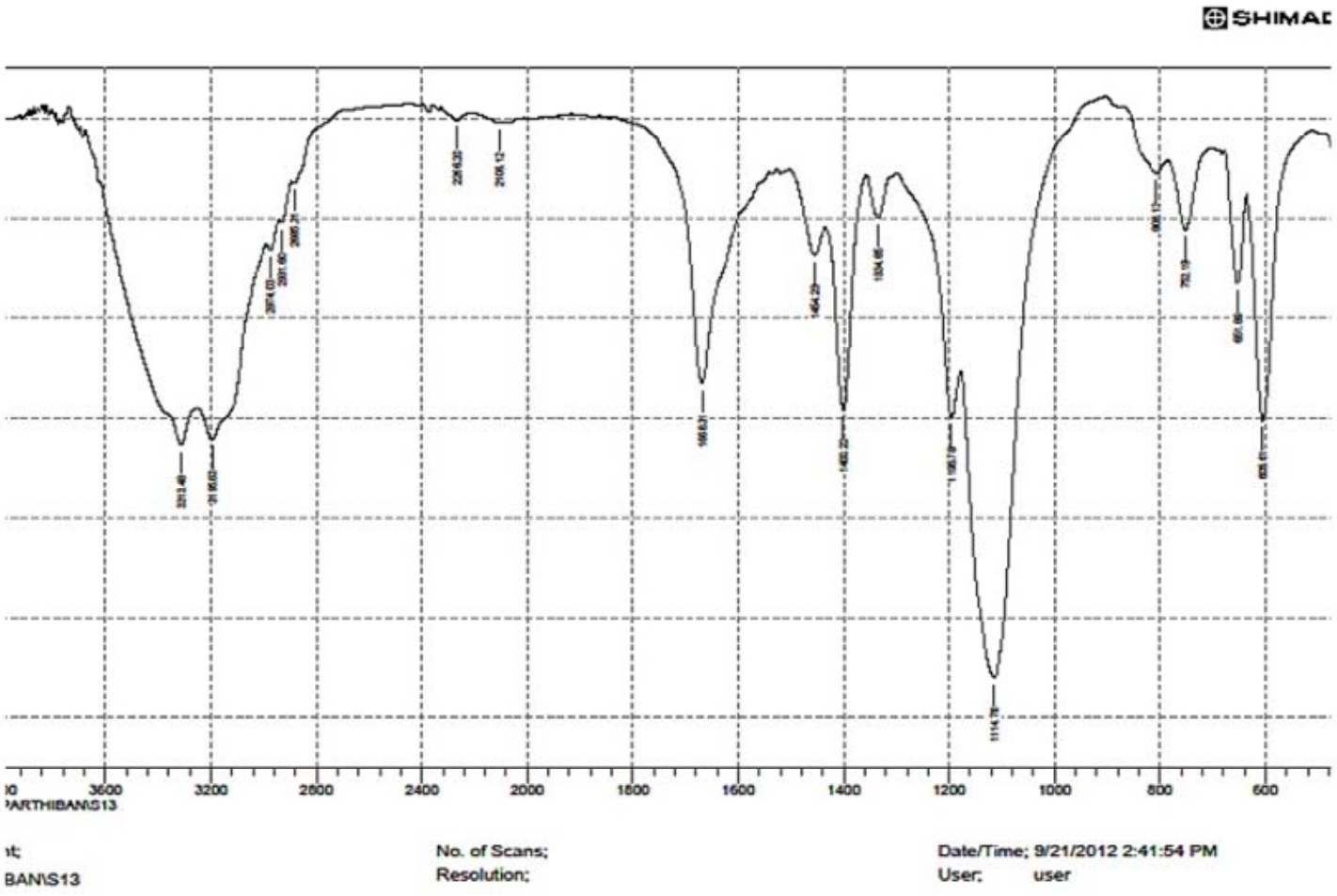

Figure 2. FTIR spectrum of exopolymer. **Note:** Stretching at 752.19 cm^{-1} indicates the presence of CH_2 groups and 1668 and 1334 cm^{-1} indicates the presence of COOH group. C-H stretching at 2885.31 to 2974.03 cm^{-1} and O-H stretching was observed in the range of 3195.83 to 3313.48 cm^{-1}.

Table 1. GCMS analysis of non carbohydrate fraction of bacterial exopolymer.

Rt	Name of compound	Mass	Molecular formula
12.58	Pentanoicacid,5-hrdroxy,2,4-di-t-butylphenyl esters	306.4397	$C_{19}H_{30}O_3$
17.65	1,1,3,1"-Terphenyl,3,3,5,5-tetrabromo-5-{3,5-dibromophenyl}	780	C24H12Br6
23.27	Docosanoic acid,1,2,3-propanetriyl ester	1059.81	C69-H134-O6

cing bacteria (Inmaculada et al., 2010). The exopolymer producing strain was identified by standard morphological, physiological and biochemical methods, compared with Bergey's manual of determinative bacteriology (Holt et al., 1994). Al-Nahas et al. (2011) also reported that the exopolymer production increased, when glucose was supplied as a carbon source in *Pseudoalteromonas* sp. Sutherland (1977) and Cerning et al. (1994) also found that excess of sugar in the culture medium stimulates the production of exopolymer by lactic acid bacteria. In accordance with this, it is a recognized fact that a similar association exists in the concentration of sugars and the exopolymer production. Kim et al. (2008) in *Weissella hellenica* reported a similar result. The exopolymer synthesis started during the late exponential growth phase and reached the maximum after 3 to 5 days. According to Gancel et al. (1994), the subsequent decrease in the production was probably due to various effects such as physiological stress, enzymatic degradation. Cerning et al. (1994) and West et al. (1998) found similar observation

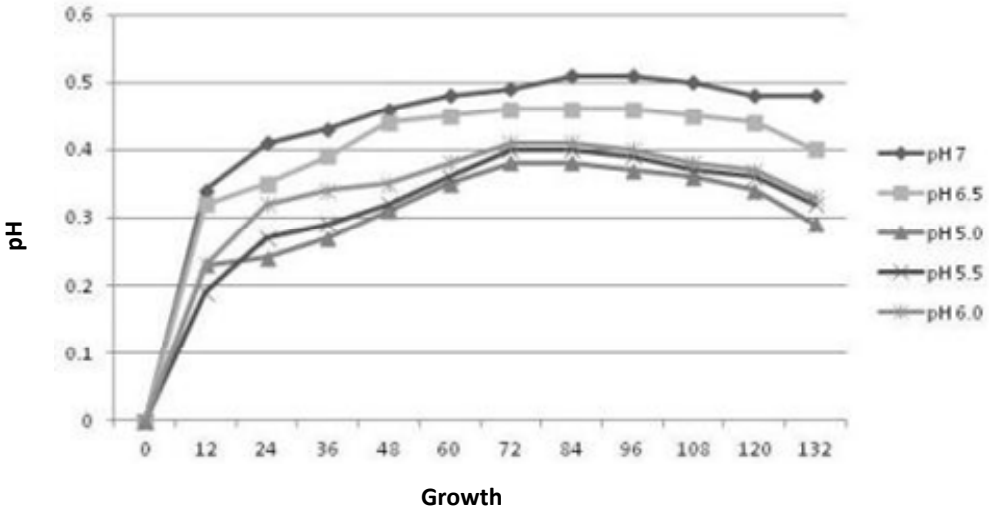

Figure 3. Effect of pH on growth of *Bacillus thuringiensis* S13. **Note:** The isolate exhibits sigmoidal growth and exopolymer production at pH 7 on compared with other pH condition.

Table 2. Effect of carbon nitrogen concentration on exopolymer production.

C:N Concentration	Peptone (%)	Exopolymer (mg/l)
	0.5	98.33 ±0.28
Glucose 2.5%	0.6	121.1 ±0.28
	0.7	84.33 ±0.28
+	0.8	65.33 ±0.11
	0.9	59.16 ±0.05
	1	41.33 ±0.28

The isolate produce highest amount of exopolymer on supplying 0.5% of peptone with 2.5% glucose. *Values are the means ± standard deviations of triplicate measurements. Effect of C:N on production of exopolymer.

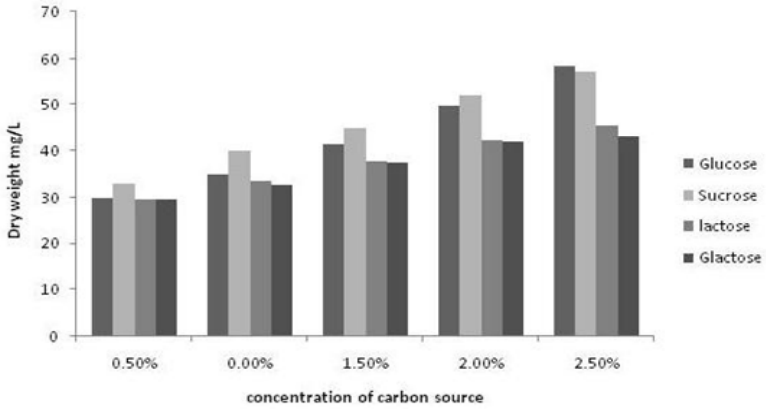

Figure 4. Effect of carbon source on production of exopolymer. **Note:** The isolate S13 produce highest amount of exopolymer when glucose was supplied as carbon source. The exopolymer production increased with the concentration of sugars.

Table 3. Effect of pH on exopolymer production.

Bacillus thuringiensis S13	
pH of the media	**Dry weight of EPS (mg/l)**
pH 5	040
pH 5.5	096
pH 6	134
pH 6.5	198
pH 7	292

The optimum pH for the highest yield of exopolymer is 7.

Table 4. Dose response of bacterial exopolymer on A549 (Lung cancer).

Concentration (ug/ml)	OD of cisplatin (STD) treated cells at 490 nm	% Cell survival	% Cell inhibition	OD of exopolymer treated cells at 490 nm	% Cell survival	% Cell inhibition
12.5	1.04±0.00	75.46±0.05	24.53±0.05	0.68±0.00	99.76±0.05	0.23±0.05
25	0.68±0.00	48.56±0.05	51.43±0.05	0.57±0.00	82.63±0.05	17.36±0.05
50	0.42±0.00	28.63±0.05	71.36±0.05	0.43±0.00	61.23±0.11	38.76±0.11
100	0.26±0.00	16.56±0.05	83.43±0.05	0.36±0.00	50.33±0.57	49.66±0.57
200	0.15±0.00	8.86±0.05	91.13±0.05	0.28±0.00	37.56±0.05	62.43±0.05

The half-inhibitory concentration of bacterial exopolymer against cancer cell is 50 (ug/ml), where as for control 25 (ug/ml). *Values are the means ± standard deviations of triplicate measurements. % = percentage.

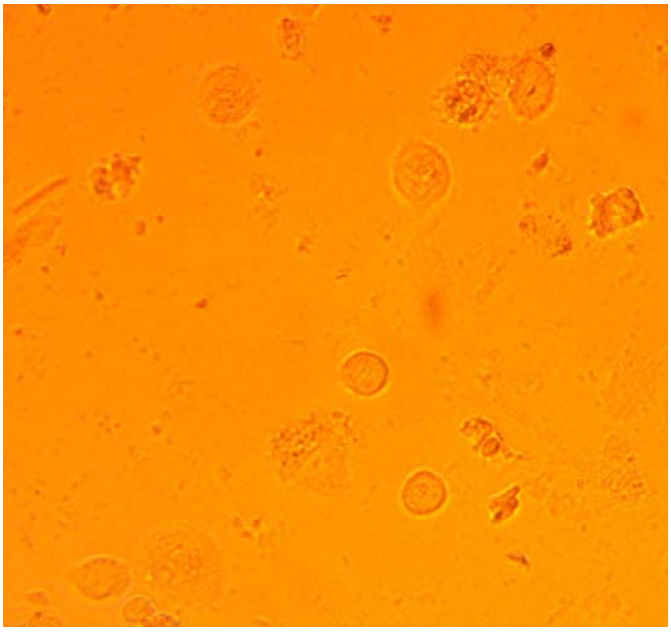

Figure 5a. A549 cell line treated with exopolymer.

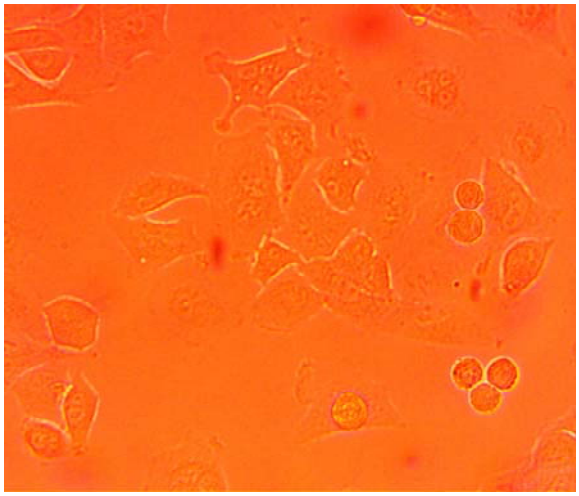

Figure 5b. A549 cell line. **Note:** The color change from yellow to orange (Figure 5b) indicates the reduction of yellow tetrazolium salt (XTT) into orange formazan dye by the metabolically active mitochondrial enzymes, meanwhile, the exopolymer treated cell lines fails to convert (Figure 5a) indicates that the cell become lyses.

in *Lacobacillus casei* and *Sphingomonas pancimobilis* that yielded high quantity of exopolymer when glucose is use as a carbon source. The isolate produced a maximum amount of exopolymer at pH 7. This result is consistent with Vincent et al. (1994) who studied in *Alvinella pompejana,* which produces the highest amount of exopoly-

mer at pH 7.0 to 7.3. Fourier transform infrared (FTIR) spectrum was relevant to the peaks already has been reported by Lungmann et al. (2007); Braissant et al. (2007); Vijayabaskar et al. (2011); Sathiyanarayanan et al. (2013).

GC-MS analysis showed the presence of non sugar components in bacterial exopolymer (Sutherland et al., 1977). The brominated compound 1,1,3,1"-Terphenyl,3,3,5,5-tetrabromo-5-(3,5-dibromophenyl) is found in bacterial exopolymeric substance (Figure 5). This was considered as an important characteristic feature of exopolymer. Lowell (1966) and Isnanetyo et al. (2003) also reported that the brominated secondary metabolites from the marine organisms exhibit a potent cytotoxic property. Lowell (1966) observed a low molecular weight brominated compound named 2,3,4,tribromo,5(5 hydroxy-2',2',dibromophenyl)pyrrole in *Thalassia* sp. 3,3,35,5'-tetra bromo-2-2 dihenyldiol was isolated from *Pseudoalteromonas phenolica* by Isnanetyo et al. (2003) which has strong antibacterial activity against methicillin resistant *Staphylococci aureus*.

In order to survive under stressful conditions, some of the marine bacteria synthesize modified proteins, fatty acids and sterols, bounded on their cell wall or incorporated with their exocellular substances (Weber et al., 1996). Polyunsaturated fatty acid synthesis by the marine bacteria is one of the adventitious characters to survive in an extreme habitat. Yano et al. (1997) also observed the presence of polyunsaturated fatty acid (PUFA) such as Docosahexanoic acid and eicosapentanoic acid in the cell wall of marine bacteria *Vibrio marinus* and sometimes are likely to be included with the exopolymeric substances. These polyunsaturated fatty acids are essential in maintaining the fluidity condition under high pressure and adaptation to saline and extreme temperature. These polyunsaturated fatty acids also act as a defense compound having antimicrobial activity (Hayashida et al., 2008). Meanwhile, saturated fatty acids namely Docosanoic acid, 1,2,3-propanetriyl ester and Pentanoic acid, 5-hrdroxy,2,4-di-t-butylphenyl esters were found in the exopolymer. Russell (1989) also noted that there is the presence of saturated fatty acid in *Marinococcus halophilus* for maintaining membrane fluidity and nutrient transport. Docosanoic acid, 1,2,3-propanetriyl ester from plant origin named as Tribehenin, which acts as a good skin conditioning agent and emulsifying agent (Daffodil et al., 2012).

The cytotoxic effect of bacterial exopolymer was due to the presence of brominated compound. The enzyme mitochondrial oxidoreductases facilitate reduction of slightly yellow compound XTT to orange at the cell surface by transplasma membrane electron transport. The succinate dehydrogenase system of the mitochon-drial respiratory chain reduced the tetrazolium salt of XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium -5-carboxyanilide salt) to soluble formazan only in living cells. The mitochondrial membranes of live cells have active enzyme system, which are inactivated shortly after cell death. The cell lines treated with bacterial exopolymer destroy the respiratory chain and inactivate the enzyme and, therefore, fail to form a soluble orange formazan by reduction of the yellow tetrazolium salt.

This work shows the potential application of bacterial exopolymer. However, biotechnological potential of the biopolymer produced by marine bacteria remains largely unexploited. The present study will give an idea on the significance and insight into the potential of the bacterial exopolymer.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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