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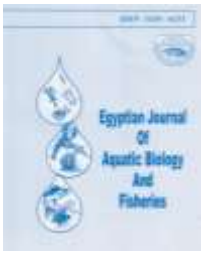

Antimicrobial and anti-tumor activities of exopolysaccharides produced by the biofilm of marine *Halomonas saccharevitans* AB2 isolated from Suez Gulf, Egypt

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ABSTRACT

Thirteen different attached marine bacteria were isolated from the surface of algae, rocksin biofilm forming device in Red Sea. They were screened for their potentiality in the exopolysaccharides (EPS) production. The most potent marine isolate 2D was identified by 16S rDNA analysis as *Halomonas saccharevitans* AB2 with 99% similarity. Optimum culture conditions which ease the synthesis of EPSsfrom *Halomonas saccharevitans* AB2 were evaluated. It grew well at 25˚C, pH 9 and on potato sea water extract with concentration 100%, lactose and malt extract as the best carbon and nitrogen sources. Application of Plackett-Burman experimental design achieved the maximum yield of EPSs (138 gl^{-1}) with 1.4 fold increase when compared to growth under the basal conditions. The maximum yield of EPSs (153gl^{-1}) with 1.6 fold increase was obtained upon applying Box-Behnken design. The EPSs showed antimicrobial activity by inhibition of different pathogenic bacteria and fungi with the maximum absolute unit (AU= 14.1 and 25.1) against *Vibrio fluvialis* and *Aspergillus niger* ATCC 16404, respectively. Moreover, these EPSs exhibited activities as antitumor agent against 8 cell lines with maximum inhibition (87%) at 50 µg with IC50 =20.3 µgml^{-1}against human lung carcinoma (A-549). Potential antimicrobial and antitumor activities property of EPSs produced by *H. saccharevitans* AB2 may lead to the development of novel medical drugs.

INTRODUCTION

A huge and extensive source of natural compounds can be retrieved from the marine environment (Bhatnagarand Kim, 2010). Marine microorganisms exhibit unique metabolic and physiological capabilities conferring them the ability to survive in extreme conditions and consequently produce novel metabolites that cannot be found elsewhere (Carvalho and Fernandes, 2010; Satpute *et al.*, 2010). Bacteria offer a great diversity of EPSs which could play an important role in the food, pharmaceutical and biomedical industries as well as in the bioremediation field, particularly in the metal recovery from industrial wastes (Ullrich, 2009). Various marine bacteria, such as *Bacillus, Halomonas, Planococcus, Enterobacter, Alteromonas, Pseudoalteromonas, Vibrio, Rhodococcus,* etc., have been extensively studied for the marine EPS isolation and characterization (Parkar *et al.*, 2017). Many

researchers have optimized the productionconditions for EPS in submerged cultureby *Fomesfomentarius* (Chen *et al.,* 2008), *Tremellafuciformis* (Cho *et al.,* 2006), *Pholiotasquarrosa* (Wang *et al.,* 2004), *Agrocybecylindracea* (Kim *et al.,* 2005), *Collybiamaculate* (Lim *et al.,* 2004), *Cordycepsjiangxiensis* (Xiao *et al.,* 2004), *Cordycepsmilitaris* (Kim *et al.,* 2003), *Aureobasidiumpullulans* (Moubasher and Wahsh 2014), and *Tremellamesenterica* (De Baets*et al.,* 2002). Statistical experimental designs such as Plackett–Burman (BP) design and response surface methodology (RSM) are successfully employed to screen and optimize the process parameters in bioprocess field (Sharmila *et al.*,2013). *Halomonas* is the type genus of the family Halomonadaceae and contains more than 70 species (Euzeby, 2011). Strains belonging to the genus *Halomonas*is are very heterogeneous and includes quite diverse species in terms of their physiology, ecology and nutrition. Different species of *Halomonas* that produce EPSs: *H. almeriensis* (Martı´nez-Checa *et al.,* 2005), *H. anticariensis* (Martı´nez-Ca´novas*et al.,* 2004a), *H. eurihalina* (Quesada *et al*., 1990; Dobson and Franzmann1996), *H. maura* (Bouchotroch *et al.,* 2001), *H. nitroreducens* (Gonza´lez-Domenech *et al.,* 2008a), *H. cerina* (Gonza´lez-Domenech *et al*., 2008b), *H. salina* (Valderrama *et al.,* 1991; Dobson and Franzmann, 1996) and *H. ventosae* (Martı´nez-Ca´novas *et al.,* 2004b) were discovered.Marine microbial polysaccharides are characterized by unique properties making them a good source of bioactive agents that can be used in many fields as anti-tumor, antiviral, antioxidant, anticoagulant, food and feed (Elsakhaw *et al*., 2017). The EPSs produced by these halophilic bacteria have different chemical compositions and functional properties from those already marketed and used by industry,their high sulphate content being of special interest. Sulphated polysaccharides are known to inhibit the growth of some viruses and tumors (Arena *et al.,* 2009). Another study (Ruiz-Ruiz *et al.,* 2011),screened the anti-tumoral activity of a panel of sulphated EPSs excreted by a group of newly discovered halophilic bacteria and found that strains N12T and B-100 produce a heteropolysaccharide that exerts a potent inhibitory effect on the proliferation of some tumor cell lines. Furthermore, the EPS produced by strain B-100, when oversulphated (B100S), exercises anti-tumoral activity on T-cell lines derived from acute lymphoblastic leukaemia via the intrinsic apoptotic pathway. The newly discovered B100S is therefore the first bacterial EPS that has been demonstrated to exert a potent and selective pro-apoptotic effect on T leukaemia cells.

Thus, the present work aimed to study the EPS production potential of biofilm forming marine bacteria. The optimization and chemical investigation of the produced EPS by the potent isolate were evaluated. Also, the antibacterial and anticancer activities of the produced EPS wereinvestigated.

MATERIALS AND METHODS

Biofilm formationand isolation of the attached marine bacteria

The formation of biofilm was studied in sea water which was collected in plastic containers from a coastal area of Suez Gulf following the method of Lehtola *et al.* (2002).

Biofilm development was studied using polyvinylchloride (PVC) pipes which were 35 cm inlength and 4 cm in width. The glass slides (25 mm in width, 75 mm in length and 1mm in thickness) were used for the biofilm formation. The glass slides were precleaned with 1N HCl and treated with sodium hypochlorite solution of 10 mgl^{-1} for 24 h and rinsed with sterile distilled water before the experimental setup.

Then the slides were placed in PVC chamber at 21^{o}C and covered by aluminum foil. Sea water was pumped at flow velocity of 10 to 20 drops/minute in the PVC chambers. Ten slides were placed in the PVC chamber at regular distance for the formation of biofilm (Hong *et al*., 2002). The sea water passed through the PVC chamber with inserted glass slide for 2 months for bacterial adherence (Lehtola *et al*., 2002), Figure 1 shows the biofilm formation device.

Fig. 1: Biofilm formation device

The surfaces of the glass slides were carefully removed from the device, scrapped and suspended in 10ml of filter sterilized aged sea water using a cell scrapper and the scrapped materials were serially diluted. After a series of dilutions, 100 µl of diluents was spread on YMG marine agar medium plates (glucose 10 g, yeast extract 3 g, malt extract 3 g, peptone 5 g, distilled water 500 ml, aged sea water 500 ml). The plates were incubated at 25^{o}C for one week and bacterial colonies showing different morphological characteristics were transferred onto fresh YMG agar medium plates (Indraneel *et al*., 1999).

Isolation of attached bacteria to surface of rocks and algae was carriedout according toWilson *et al*. (2010).Specimens were washed 3 times in filtered seawater to remove loosely attached bacteria from the surface. Swabs were then taken from the surfaces of all specimens using sterile cotton swabs and streaked onto marine agar plates, then were incubated at 25^{o}C for 18 h. Bacterial colonies with distinct morphologies were picked off the plates, restreaked onto fresh media and incubated for a further 18 h at 25^{o}C. This restreaking process was continued until pure morphotype colonies were obtained.

Isolates were stored as glycerol stocks (25% glycerol, 75% marine broth). When required, glycerol stocks were inoculated in marine broth and grown on an orbital shaker at 25^{o}C.

Screening for EPSs production by the marine bacterial isolates

Isolates were grown on YMG agar medium (gl^{-1}), The pre-inoculum was prepared in YMG broth by incubating at 25^{o}C for 24 h and 200 µl of this culture broth was inoculated into 50 ml of YMG broth and incubated at 25^{o}C for 5 days at 120 rpm. The contents were centrifuged (10,000xg for 20 min) and the culture supernatant was mixed with three volumes of isopropanol or ethanol slowly along the side wall of the conical flask and allowed to stand at 4^{o}C for 20 min for precipitation

of EPSs. The weight of the precipitated EPS was measured after drying at 40^{o}C for three days (Hong *et al.*, 2002**).**

Molecular characterization of the promising isolate AB2

The genomic DNA of AB2 was isolated according to Sambrok *et al.* (1989). The 16S rDNA gene was amplified by the PCR using the primers 16S 357 F; ACT CCT ACG GGA GGC AGC AG and 16S 907R; CCG TCAATT CAT TTG AGT TT. The PCR reaction mixture contained 200 µM of each dNTP, 0.5 µM primers, 10mM Tris-HCl pH 8.3, 1.5 mM magnesium chloride, 50 mM potassium chloride, 2.5 units Tag polymerase and 1 µl of template DNA. Amplicons were obtained with a PCR cycling program of 94^{o}C for 1 min followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec, and polymerization at 72^{o}C for 2 min. At the end of thermocycling, the PCR reaction was incubated at 72^{o}C for 7 min. As described by Ausubel *et al.* (1999), amplicons were visualized by electrophoretic separation on 1% agarose gels stained with ethidium bromide. PCR fragments were purified from amplification reactions with QlAquick PCR purification reagents (QlAGEN) according to the kit manual. DNA sequence was determined using the ABIPrism^{TM}DNA automated sequencer and dyeterminator cycle sequencing kit with AmpliTaqDNApolymerase (Applied BioSystems). These primers were separately used for sequencing of the amplified16S rDNA fragments. The PCR sequencing program used has three steps: denaturation at 96^{o}C for 15 sec, annealing at 60^{o}C for 15 sec and extension at 72^{o}C for240 sec. Blast program (www.ncbi.nlm.nih.gov/blast) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREEVIEW program (Page, 1996).

Optimization of EPSs production using Plackett-Burmanexperimentaldesign

In order to determine the major factors affecting polysaccharides production, different variables were evaluated using Plackett-Burman statistical design which is a 2 factorial design that identifies the critical variables required for elevated polysaccharides production and is very useful for screening the most important factors with respect to their main effects (El-Naggar, 2015). For each variable a high (+) and low (-) levels were tested. Eight different trials were performed in duplicates. (Table 1) represents the high and low concentration of the different Independent variables affecting the production of EPSs. The rows represent the different trials (row no. 9 represents the basal control). The main effect of each variable was determined with the following equation: Exi = (Mi+ – Mi-) / N, where Exi is the variable main effect, and Mi+, Mi- are the radius of the dry weight per gram in the trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2. Statistical t-values for two samples assuming unequal varianceswere calculated using Microsoft Excel to determine the variable significance.

Table 1: Plackett -Burman design for 7 independent variables

Trial	Factors							Response	
	Tryptone (T) (g/l)	**Maltose (M) (g/l)**	**pH**	**Inoculum Size (10^5 CFU/ml) (IS) (ml)**	**Incubation period (IP) (d)**	**Culture Volume (CV) (%)**	**Potato water extract (PWE) (ml)**	**Biomass dry weight (g/l)**	**EPS dry weight (g/l)**
1	- [5]	-[15]	-[8]	+[1.5]	+[10]	+[100]	-[100]	7	96
2	+[15]	- [15]	−[8]	-[0.5]	-[5]	+[100]	+[400]	8	114
3	−[5]	+[25]	-[8]	−[0.5]	+[10]	-[50]	+[400]	11	94
4	+[15]	+[25]	−[8]	+[1.5]	−[5]	-[50]	-[100]	10	118
5	−[5]	_[15]	+[9]	+[1.5]	-[5]	−[50]	+[400]	6	92
6	+[15]	−[15]	+[9]	−[0.5]	+ [10]	-[50]	−[100]	9	96
7	−[5]	+[25]	+[9]	_[0.5]	−[5]	+ [100]	_[100]	3	118
8	+[15]	+[25]	+[9]	+[1.5]	+[10]	+ [100]	+[400]	11	116
9	0[10]	0[20]	0[8.5]	0[1]	0[7]	0[75]	0[200]	10	90
Main effect	0.13	0.1	-0.015	0.02	-0.085	0.1	-0.0075		
t-value	2.015048	2.015048	1.9431803	1.9431803	1.9431803	2.015048	1.9431803		

Box-Behnken design was applied to determine the suitable levels of the most significant three variables selected from Plackett-Burman experimental design. To perform this task, performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model were carried out. The three significant variables elucidated through Plackett-Burman experimental design were malt extract (X_1), Incubation period (X_2) and culture volume (X_3). The low, middle and high levels of each variable were designated as -1, 0 and +1, respectively. A design matrix for the 15 trials, along with the natural values for the three factors was constructed (Table 2). The trials were carried out in triplicate and mean values of production of EPSs was estimated. To predict the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variables and the response. The equation for the three factors was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Table 2: Optimization of the culture conditions using Box-Behnkendesign

Trial	Factors							Response	
	Malt extract (ME) (g/l)	**Incubation period (IP) (d)**	**Culture volume (CV) (%)**	**Lactose (L) (g/l)**	**pH**	**Inoculum siz (10^5 CFU/ml (IS) (ml)**	**Potato water extract (PWE) (ml)**	**Biomass dry weight (g/l)**	**EPS dry weight (g/l)**
1	0[15]	+[7]	-[50]	+[25]	+[9]	+[1.5]	-[100]	50	140
2	+[20]	+[7]	0[75]	+[25]	+[9]	+[1.5]	-[100]	90	180
3	0[15]	0[5]	0[75]	+[25]	+[9]	+[1.5]	-[100]	90	160
4	+[20]	-[3]	0[75]	+[25]	+[9]	+[1.5]	-[100]	60	164
5	-[10]	-[3]	0[75]	+[25]	+[9]	+[1.5]	-[100]	40	168
6	+[20]	0[5]	+[100]	+[25]	+[9]	+[1.5]	-[100]	10	180
7	0[15]	-[3]	+[100]	+[25]	+[9]	+[1.5]	-[100]	40	168
8	0[15]	0[5]	0[75]	+[25]	+[9]	+[1.5]	-[100]	30	160
9	+[20]	0[5]	-[50]	+[25]	+[9]	+[1.5]	-[100]	40	140
10	-[10]	0[5]	+[100]	+[25]	+[9]	+[1.5]	-[100]	40	180
11	-[10]	0[5]	-[50]	+[25]	+[9]	+[1.5]	-[100]	30	140
12	-[10]	+[7]	0[75]	+[25]	+[9]	+[1.5]	-[100]	30	160
13	0[15]	-[3]	-[50]	+[25]	+[9]	+[1.5]	-[100]	60	150
14	0[15]	0[5]	0[75]	+[25]	+[9]	+[1.5]	-[100]	40	160
15	0[15]	+[7]	+[100]	+[25]	+[9]	+[1.5]	-[100]	30	90

Statistical analysis of the data

The data of EPSs production was subjected to multiple linear regressions using Microsoft Excel to estimate t-value, P-value and confidence level.The significance level (P-value) is determined using the Students test. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. If this probability is sufficiently small, the idea that the effect was caused by varying the level of the variable under test is accepted. Confidence level is an expression of the P-value in percent. Optimal values were estimated using the *solver* function of Microsoft Excel tools. The simultaneous effects of the four most significant independent factors on each response were visualized using three-dimensional graphs generated by STATISTICA 10.0 software.

Model verification

The optimal conditions realized from the optimization experiments were verified experimentally. The predicted near optimum and far from optimum levels of the independent variables were examined and compared to the basal condition settings.

Chemical characterization

Chemical characterization of the purified EPS included UV, Fourier transform-infrared (FTIR) spectrum analysis and X-ray diffraction (XRD). Chemical characterization included UV was carried out using Heλiosα. Hydrolysis of the sample was done by the method of Kang and Veeder(1982).

Pellets for infrared analysis were prepared by grinding a mixture of 2 mg EPS with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The FTIR spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} region.

X-ray diffraction (XRD) was performed on X-ray powder diffractometer (Al-azhar university) instrument equipped with a PW3123/00 curved Ni-filtered CuKα (λ=1.54056°A). Radiation generated at 40 kV and 30 mA with liquid nitrogen cooled the solid-state germanium detector to study the physical properties of EPS using slow scan in different ranges of two-theta angles (2–80°). The specimen length and irradiated length were 10 mm with receiving slit size of 0.2- and 200-mm goniometer radius. Distance between focus and divergences slit was 100 mm. Dried EPS sample was mounted on a quartz substrate, and intensity peaks of diffracted X-rays were continuously recorded with scan step time 1 s at 25°C. The d-spacings appropriate to diffracted X-rays at that value of θ were calculated according to Bragg's law. Crystallinity index (CIxrd) was calculated from the area under crystalline peaks normalized corresponding to total scattering area (Ricou*et al.,* 2005).

Antimicrobial activity of EPS producedby *H.saccharevitans*AB2

Fifty millimeters of nutrient agar medium inoculated with indicator pathogens were poured into all plates. After solidifying, wells were punched out using 0.5 cm cork-porer, and each of their bottoms was then sealed with two drops of sterile water agar.One hundred microliters of filtrated EPS were transferred into each well after sterilizing by ultra-filtration using 0.22 µl sterilized filters. All plates were incubated at appropriate temperature for 24-48 h. After incubation period, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm, where dividing Y^2 over X^2 determines an absolute unit (AU) for the clear zone. The absolute unit of each EPS, which indicates a positive result in the antimicrobial action, was calculated according to the following equation (El-Masry*et al.,* 2002):

$AU= Y^2/X^2$

Antitumor activityof EPS produced by *H.saccharevitans* AB2

Subculture of cell lines

Cultures were viewed using an inverted microscope to assess the degree of confluence and the absence of bacterial and fungal contamination. Cell monolayer was washed with PBS using volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1 ml per 25 cm^2 of surface area. Flask was rotated to cover monolayer with trypsin/ EDTA then the flask was returned to the incubator and left for 2-10 min.The cells were examined using inverted microscope (CKX41; Olympus, Japan) to ensure that all cells were detached and floated. The cells were re-suspended in a small volume of fresh serum containing Dulbecco's Modified Eagle's Medium (DMEM). The required number of cells were transferred to a new labeled flask containing pre warmed growth medium and incubated at 37°C in humidified atmosphere of 5% CO_2 (Water jacketed double incubator, Shel Lab, Sheldon manufactured, Inc.®, USA (Mosmann, 1983**)**.

Evaluation of antitumor activity

The purified EPS was tested for any cytotoxic activity against eight tumor cell lines i.e., MCF-7 cells (human breast cancer cell line, HepG-2 cells (human Hepatocellular carcinoma), A549 cells (human lung carcinoma), HCT-116 cells (colon carcinoma), PC-3 cells (human prostate carcinoma), HELA cells (human Cervical carcinoma) and HEP-2cells (human Larynxcarcinoma) CACO2cells(human intestinal carcinoma). They were obtained from VACSERA Tissue Culture Unit. When the cells grown on 75 cm^2 tissue culture flasks reached confluence (usually 24 h), the cell suspension of tumor cell lines were prepared in complete growth medium (DMEM). The aliquots of (100 µl) of cell suspension were added to each well on a 96-well tissue culture plate. The blank wells contained medium in place of cell suspension. The cells were incubated for 24 h at 37°C in humidified atmosphere of 5% CO_2. After the formation of a complete monolayer cell sheet in each well of the plate, the medium was aspirated and replaced with DMEM with 2% fetal bovine serum. Then the EPS were dispensed into 96- tissue culture plate at 50 (µl/well). Another set of well was kept for including wells of cell controls in which (50 µl) of DMEM with 2% FBS being added instead of the plant extract as negative control. Serial three fold dilutions of the EPS were added into a 96-well sterile tissue culture plate using multichannel pipette (Eppendorff, Germany). The treated and untreated cells were covered with a plate sealer then allowed to grow and proliferate by further incubation the plate for 24 h at 37ºC in humidified atmosphere of 5% CO_2**.** At the end of incubation the plate was examined using the inverted microscope **(**Riyadh *et al.,* 2015).

Determination of viable cells

The number of survival cells was determined by staining with crystal violet. Briefly, after the end of incubation period, the plate containing the treated and untreated cells were inverted or aspirated to remove the medium. Then, the wells were washed by 100 µl of PBS and then the cells were fixed with 10% formalin for 15 min at room temperature the cells were then stained with 100 µl of crystal violet for 20 min the excess of stain was removed and the plates were rinsed with deionized water, and then dried. The cellular morphology was observed by using inverted microscope with digital camera to capture the images representing the morphological changes in the treated cells compared with control cells. To obtain quantitative data the dye was extracted from the cells by adding glacial acetic acid (33%) to each well and mixed the contents of each well before reading the absorbance on the ELISA

reader (SunRise TECAN, Inc.®, USA) at 490 nm. The absorbance is proportional to the number of survival cells in the culture plate (Saintigny*et al.*, 2011).

Data analysis

The percentage cell viability was calculated using the Microsoft Excel ®. Percentage cell viability was calculated as follows:

% Cell viability = Mean Abs control – Mean Abs of extract × 100

Mean Abs control, where: Abs absorbance at 490 nm.

RESULTS AND DISCUSSION

In the marine environment, EPSs play important roles in the production of aggregates, adhesion and colonization of surfaces, biofilm formation, sequestering of nutrients, and thus provide protection and ecosystem stability (Nichols *et al.*, 2005).The present study concerned with the isolation of biofilm attached marine bacteria using biofilm formation device. Isolation of attached bacteria using the same manner was performed in different studies (Allison and Sutherland, 1987,Indraneel*et al.*, 1999; Kumar *et al*., 2011).

Thirteen morphologically distinct marine bacteria were isolated from surface of algae, rocks in addition to biofilm formation device. The electron micrographs in Figure 2 show the attached bacteria on the glass slide.

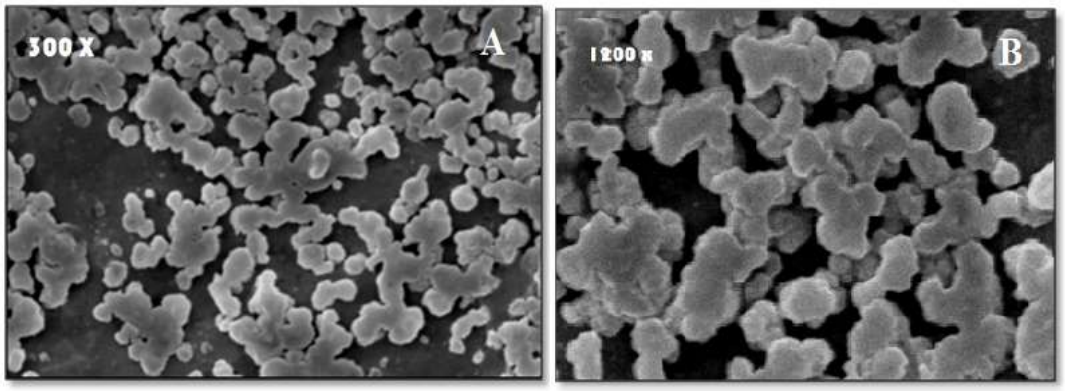

Fig. 2: Scanning electron micrographs showing biofilm of the attached bacteria on glass slide

They were tested for production of extracellular polysaccharides. All isolates exhibited different range of EPS production. Among the tested isolates, the most promising producer AB2 yielded 95 $gl^{-1}$was selected for further studies.

Molecular characterization of the selected EPS producing bacteria

The extracted 16SrRNA gene (approximately 1500 base pair) was amplified using the universal primers 16S 27F and 16S 1492R.The amplified DNA was partially sequenced using the sequencer (ABI 3730xl). The sequencing data obtained utilizing this strategy was 459 base pair. This sequence was compared with those which gave the highest homology using Blast search computer based program. The resulting data indicated that the isolate AB2 under study was identified as *Halomonassaccharevitans*AB2.

The phylogenetic relationships of this experimental isolate and the closely related relatives were analyzed as shown in (Fig. 3). The electron micrograph shows the cell shape of *H. saccharevitans*AB2.

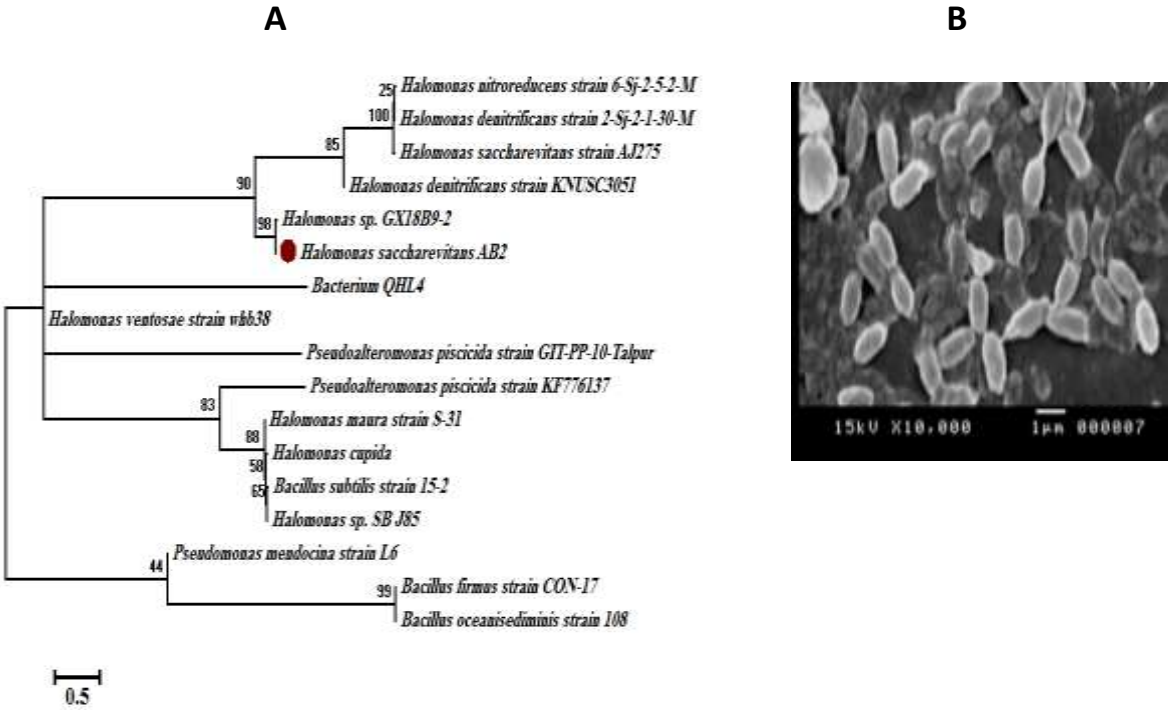

Fig. 3: Phylogenetic relationships among the representative experimental strain and the most closely related *Halomonas* species (A). The dendogram was generated using tree view program. Electron micrograph showing cells of *H. saccharevitans* AB2 (B).

Production of extracellular polysaccharides by halophilicarchaea and bacteria has been reported by several workers and the members of the genus *Halomonas* have been identified as the most potential producers (Llamas *et al*., 2012). Many microorganisms are present that are able to produce EPSs. Considering the *Halomonas* genus, the species *H. maura*, *H. eurihalina*, *H. ventosae*, *H. anticariensis* and *H. alkaliantarctica* isolated from hypersaline environments, were shown to be good EPS producing species (Bouchotroch *et al*., 2001; Quesada *et al*., 2004; Martínez-Cánovas *et al*., 2004; Poli *et al*., 2007).

It was reported that production and characterization of extracellular polysaccharides by the wide variety of halophilic microorganisms isolated from hypersaline environments are inadequate (Garcia *et al*., 2004; Quesada *et al*., 2004; Pal and Paul, 2008; Llamas *et al*., 2012).

Production of EPS by bacterial species in culture is greatly influenced by a number of factors, such as phases of growth, nutritional status and the environmental conditions. The nature and concentration of nutrients in particular, are necessary components for stimulation of growth and synthesis of EPS (Pal and Paul, 2008). Fermentation conditions play important role in determining the conformation, molecular mass, monosaccharide ratios and functional properties of the EPS (Finore*et al*., 2014).

A wide variety of carbon sources, including sucrose, glucose, lactose, maltose, mannitol, sorbitol, whey, starch, and even nonsugar sources like methanol and C9 to C16 n-alkanes, could affect EPS production as was reported in different studies (Antón, 1988).The present experiment aimed at optimization of the growth conditions for higher production of EPS using different carbon and nitrogen sources. Different carbon sources included glucose, fructose, sucrose, maltose, lactose, and soluble starch were amended separately in the medium and tested for their effect on EPS production. The preferable carbon source for growing *H.saccharevitans*AB2 and high production of EPS was 2% maltose producing47.55 g/l by using isopropanol in EPS

precipitation (data not shown). Lactose was the best carbon source for EPS production of strain SMas was stated by Liu *et al*. (2011).

In the same manner, different nitrogen sources were evaluated for the highest EPS production.

The preferable nitrogen source for growing *H.saccharevitans*AB2 with high production of EPS was 2% malt extract producing 38 gl^{-1} using isopropanol in EPS precipitation (data not shown).

Plackett-Burman design for optimization of EPSs production by *H. saccharevitans*AB2

The Plackett-Burman design (Plackett and Burman, 1946) has been frequently used for screening of multiple factors at a time and for finding out the key factors by estimating only the main effects prior to optimization (Ooijkaas*et al.*, 1998). This technique cannot determine the exact quantity, but can provide indication and tendency regarding the necessity of each factor in relatively few experiments.

Seven independent variables were chosen to be evaluated for EPS production by *H. saccharevitans*AB2 using Plackett-Burman design. All experiments were performed in duplicates and the averages of results (dry weight of EPS/gl^{-1}) were presented as the response. The main effect of each variable on the production of the EPSs as well as t-values were estimated for each independent variable as shown in Table 1 and graphically presented in Figure 4.

However, results indicated that incubation period and potato water extract had negative main effects, while the other tested variables exhibited positive main effects on the EPSs production. These results indicated that the use of low incubation period value and potato water extract concentration will realize maximum production. Also, high concentration of all other medium components will give maximum production of EPS. Statistical analyses of the results (t-test) showed that variations in incubation period and culture volume in the tested ranges had the most significant effects on the production of EPSs.

According to obtained results, the predicted optimized medium composition for cultivation of *H. saccharevitans* AB2, was as follows: (gl^{-1}) malt extract, 15; (gl^{-1}) lactose, 25; inoculum size (ml), 1.5; culture volume (ml),75; potato water extract concentration, 100%, adjusted to pH 9 and incubation period 5 days at 25^{o}C.

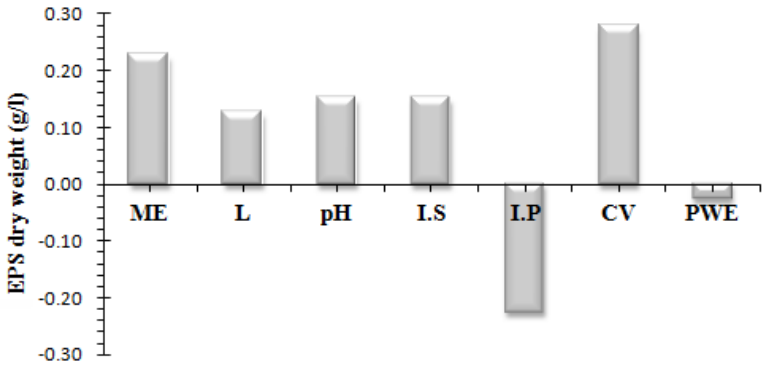

Fig. 4: Elucidation of the culture factors that affect the production of EPSs by *H. saccharevitans* AB2

In order to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was applied to compare between the predicted optimum levels of independent variables and the basal condition settings. Results in

Table 2 confirmed that the production of the EPSs (expressed in grams of dry weight) increased to 138 gl^{-1} with 1.4 fold increase when compared to cells grown under the basal conditions.

A verification experiment was applied also, to compare between the anti-optimum levels of independent variables and the basal condition settings. Results in Table3 confirmed that the production of the EPS (expressed in grams of dry weight) increased to 138 gl^{-1} with 1.7 fold increase when compared to cells grown under the basal conditions.

Table 3: A verification of Plackett-Burman results

Response	Dry weight of EPS (g/l)
Basal medium	97
Optimized medium	138
Anti-optimized medium	85

Optimization using Box-Behnken design

To identify the optimum response region for EPSs production, the significant independent variables (malt extract X_1, incubation period X_2 and culture volume X_3) suggested by the Plackett-Burman design were further investigated at three levels (-, 0 and +) in Box-Behnkendesign (Table 2). Data in Table 3presents the design matrix for the variables and the response of each trial. To predict the optimal point, a second order polynomial function was fitted to the experimental response results (non-linear optimization algorithm):

$Y=6+ 0.91875X_1 +0.925X_2 + 2.64375X_3 + 1.9125X_1X_2 + 0X_1X_3 +0.2125X_2X_3 + 1.0375X_{11} +0.95X_{22} - 0.7875X_{33}$

On the model level, the correlation measures for estimating the regression equation are the multiple correlation coefficient*R* and the determination coefficient R^2. In this experiment, the value of R^2 was 0.86 for the EPS efficacy on dry weight, indicating a high degree of correlation between the experimental and the predicted values.

The optimal levels of the three factors as obtained from the maximum point of the polynomial model, were estimated using the *solver* function of the Microsoft Excel tools, and found to be: malt extract, 20gl^{-1}; incubation period, 7d and culture volume,100ml with a predicted dry weight of EPS 153 gl^{-1}.

In addition, the simultaneous effects of the three most significant independent factors on each response using three-dimensional graphs generated by STATISTICA 10.0 software. As shown in the surface plots of the Box-Behnken design variations in malt extract (X_I), incubation period (X_2) and culture volume (X_3) within the examined concentration ranges and under the present experimental conditions, were clearly effective. Data in Figure 5A suggests that the high the concentration ofmalt extract and short in incubation period increase production of EPSs. While in Figure 5B, it suggests the high concentration of culture volume and malt extract the increase production of EPSs. On the other hand, the high concentration of culture volume and day in incubation period increase the production of EPSs (Figure 5C).

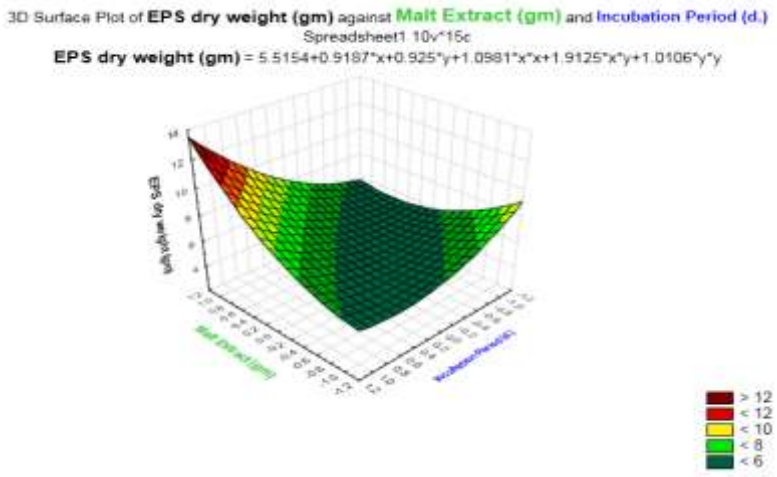

Fig. 5A: The interaction of malt extract with incubation period as independent variables that affect in production of EPS *H.saccharevitans*AB2based on the results of Box-Behnkendesign.

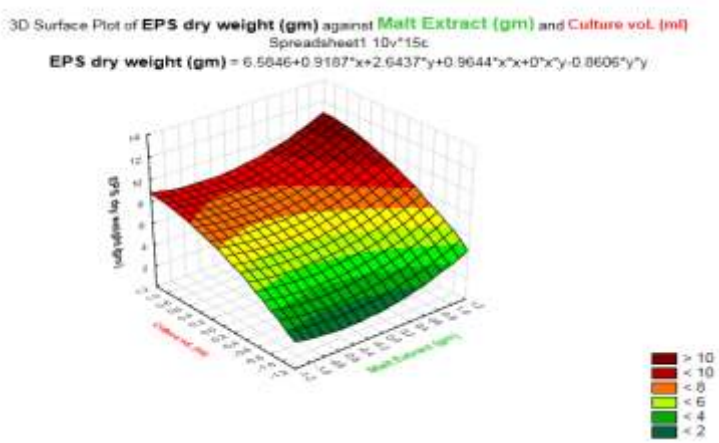

Fig. 5B: The interaction of culture volume with malt extract as independent variables that affect in production of EPS by *H.saccharevitans*AB2based on the results of Box-Behnkendesign.

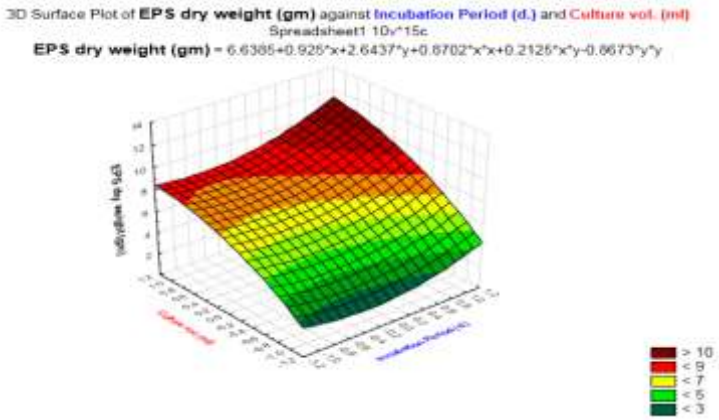

Fig. 5C: The interaction of culture volume with incubation periodas independent variables that affect in production of EPS by *H.saccharevitans*AB2based on the results of Box-Behnkendesign

A verification experiment was applied also, to compare between the optimum levels of independent variables and the basal condition settings. Results confirmed that the production of the EPSs (expressed in dry weight gl^{-1}) decreased to 153 gl^{-1} with1.6 fold decrease when compared to cells grown under the basal conditions. The components of optimized medium are: malt extract (gl^{-1}); 20, lactose conc. (gl^{-1}); 25,

pH; 9, inoculum size (ml); 1.5, incubation period (days); 7, culture volume (ml); 100, and potato water extract conc. (gl^{-1}); 100.

Chemical analyses of EPS produced by *H.saccharevitans*AB2

Chemical characterization of the produced EPSs from *H. saccharevitans* AB2 was carried out. UV–Vis spectroscopy analysis showed that the maximum wave-length area of absorption spectra was 290nm as shown in Figure 6. Hassan and Ibrahim (2017) showed the maximum absorption of EPS produced by *Bacillus subtilis* HS was at 190 nm.

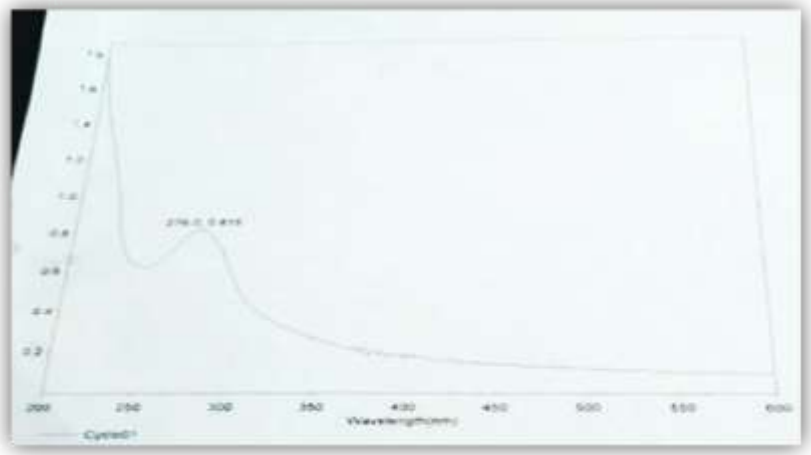

Fig. 6: UV spectrum of the purified EPS by *H.saccharevitans*AB2

The FTIR spectra of the EPS from *H. saccharevitans* AB2 (Figure 7) exhibited bands at various levels. A dominant absorption that is often attributed to O-H stretching vibration. The bandsat 3387.00 cm^{-1}, of O-H in carboxylic acid which is accompanied with the bands at 2927.49 cm^{-1} corresponds to H stretching in carboxylic group. In such manner, Kennedy and Sutherland (1987) refereed to that exopolymers produced by marine bacteria generally contain 20-50% of the polysaccharide as uronic acid. Typically, the EPS from *Halomonas* strains are characterized by a high presence of sulfate groups and uronic acids as was reported by Finore *et al.* (2014).

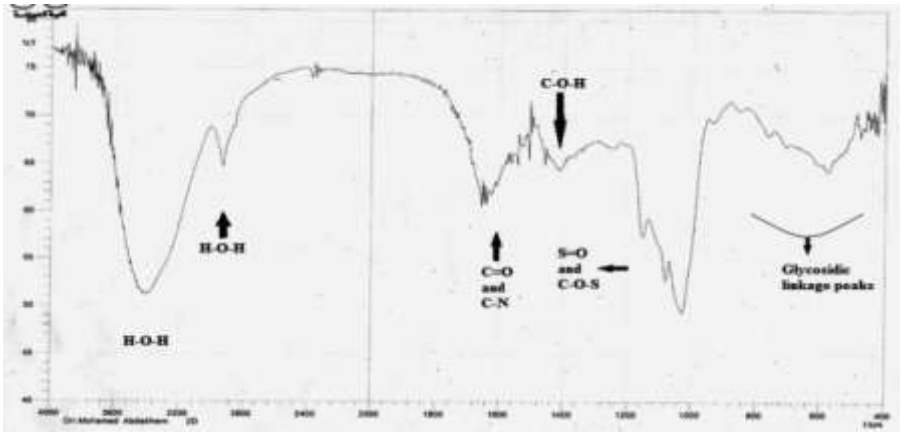

Fig. 7: FTIR spectra of the EPS produced by *H.saccharevitans*AB2

The band in FTIR spectra of the EPS from *H. saccharevitans* AB2 at 1763.00 cm^{-1}, approves the stretching vibration of C=O carbonyl group of an aldehyde or

ketone. Quite a spectral peak was obtained at 1608.63 cm^{-1} indicated amide NH_2 bending vibration or C=O, C=N stretching vibration of $RCONH_2$. The peak at 1377.17 cm^{-1}, identifies the vibration stretching of alkyl hydrogen (CH_2-CH_3) in aliphatic alkyl group (R-CH_2-CH_3). The sharp band was observed at 1246.02 cm^{-1} strongly suggesting the stretching vibration of O-H group in a phenol. The peak at 1053.43 cm^{-1} is assigned to stretching vibration of (C-O, alcohol, ester, ether and phenol) groups.

Orsod *et al.* (2012) also reported that the EPS extracted from marine bacteria showed absorption indicated alkenes, ketones, isocyanate and isothiocyanate groups, alcohols, ethers, esters carboxylic acids and phenols groups. Another study by Pal *et al*. (2015) stated that the Fourier Transform Infrared (FTIR) spectrum of EPS produced by *Halomonas marina* HMA 103 showed distinct absorption peaks at 3424.2, 1638.7, 1122.9 and 622.3 cm^{-1} indicating the presence of free hydroxyl, phenyl or carbonyl groups of carbohydrate as was stated by Pal *et al.* (2015).

The XRD profile of EPSs obtained from *H. saccharevitans* AB2 exhibited the characteristic diffraction peaks at 37.5°, 43.7° and 63° with inter-planar spacing (d-spacing) 2.41426, 2.03174 and 1.47974 A◦, respectively (Figure 8). Crystalline parts give sharp narrow diffraction peaks while amorphous component gives a broad peak. It is difficult to interpret broad amorphous peaks of several amorphous EPS in X-ray scattering profile (Shimizu *et al.*, 2000; Mandal *et al.*, 2011).

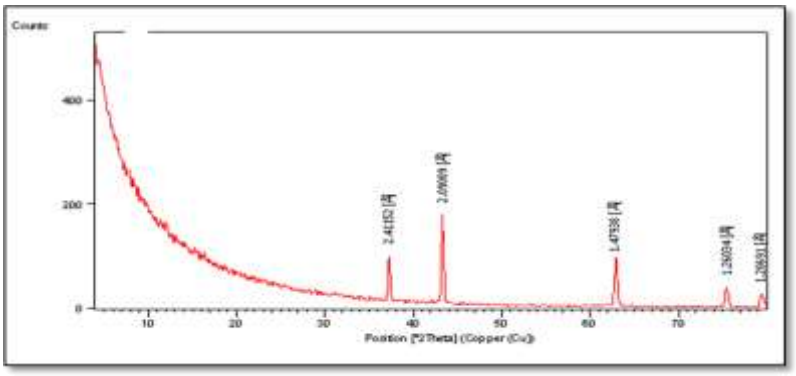

Fig. 8: XRD profile of EPSs isolated from *H. saccharevitans*AB2

HPLC analysis

The produced EPS after being hydrolyzed and dissolved with methanol was analyzed for its sugar composition by HPLC. By comparing the retention time found on with the standard retention time of different carbohydrates, the distinct peaks obtained for each EPS produced by *H.saccharevitans*AB2 was found to be 3.910, which indicated the presence of Rhaffinose (Figure 9).

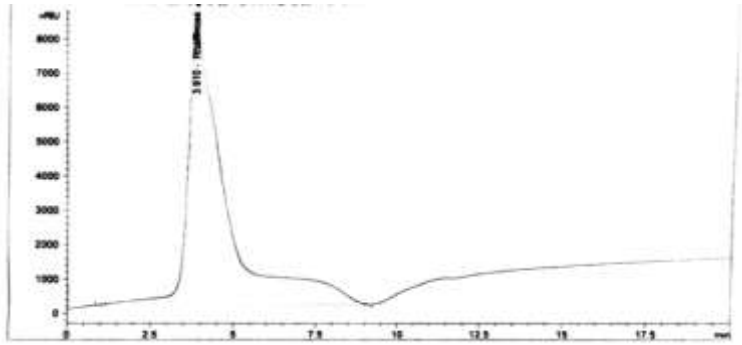

Figure 9: HPLC results of EPS produced by *H. saccharevitans* AB2

Applications of EPS produced by *H.saccharevitans*AB2

Many bacteria biosynthesize structurally diverse EPS and excrete them into their surrounding environment. The EPS functional features have found many applications in industries such as cosmetics and pharmaceutics (Delbarre-Ladrat *et al*., 2017). Potentially, EPS have proved various physiological activities in human beings as antitumor, antiviral and anti-inflammation agents, as well as being inducers for interferon, platelet aggregation inhibition, and colony stimulating factor synthesis (de Godoi *et al*., 2014).

Indeed, the genus *Halomonas* has received increasing interest as several species are able to produce significant quantities of EPS with high surface activity and/or rheological properties (Martinez-Checa *et al.,* 2002 and Pepi *et al.,* 2005). Intriguing properties of the EPS derived from *Halomonas* species, such as emulsification activity, appear to be worthwhile for an ample range of products and application.

The produced EPS was evaluated as antimicrobial agents against different types of Gram positive bacteria (*S. aureus* ATCC6538, *E.faecalis* and *B. subtilis* ATCC33018), Gram-negative bacteria (*E. coli, P. aeruginosa* ATCC9027, *V. damsela, V. fluvialis* and *K. pneumonia* ATCC43816) and fungi (*A. niger* ATCC16404 and *C. albicans* ATCC10231).

As shown in Table 4,the highest antibacterial activity (14.1 and 9.7 AU)of EPS produced from *H.saccharevitans* AB2 was recorded against *V.fluvialis* and *E. coli*, respectively. Antibacterial activity of EPS was proven in previous studies where Anju *et al.,* (2010) isolated EPS from a marine bacterium with antibacterial activity against some fish. In the same line the antibacterial activity of EPS produced from *B. subtilis* HS was tested against Grampositive bacteria (*S. aureus, S. faecalis*) and Gram negative bacteria (*E. coli, A. hydrophila, P. aeruginosa, V. damsela*). The highest antibacterial activity was documented against *S. faecalis* at 50, 100 and 200 mg/ml, respectively while no inhibitory activity was detected at lower concentrations as was reported by Hassan and Ibrahim (2017).

On the other hand, antifungal activity of EPS produced from *H. saccharevitans* AB2 was recorded against *A.niger* ATCC16404 and *C. albicans* ATCC10231 with the highest antifungal activity (25.1 AUml^{-1}) against *A.niger*.

Table 4: Antimicrobial activity of EPS produced by *H. saccharevitans* AB2 (expressed as absolute unit; AU).

Tested microorganism	**AU**
K. pneumonia ATCC43816	1.8
P. aeroginosa ATCC9027	2.25
S. aureus ATCC6538	Not detected
V. damsela	Not detected
V. fluvialis	14.1
E. coli	9.7
E. faecalis	Not detected
B. subtius ATCC33018	2.25
C. albicans ATCC10231	14.1
A.niger ATCC16404	25.1

The inhibitory effect of different concentrations (0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250 and 500 µgml^{-1}) of EPS was tested on eight different cell lines included HepG2, HCT-116, MCF-7, PC-3, A-549, CACO, HEP-2 and HELA. Results in Figure 10 showed that the produced EPSs exhibited various degree of antitumor effect toward the tested cell lines and increasing concentrations of EPS resulted in increased rates of tumor inhibition and the maximum inhibition was against A-549 cell line

(Figure 11) with IC_{50}=20.3 µgml^{-1}, which was comparable to the levels of tumor inhibition exhibited by the positive control.

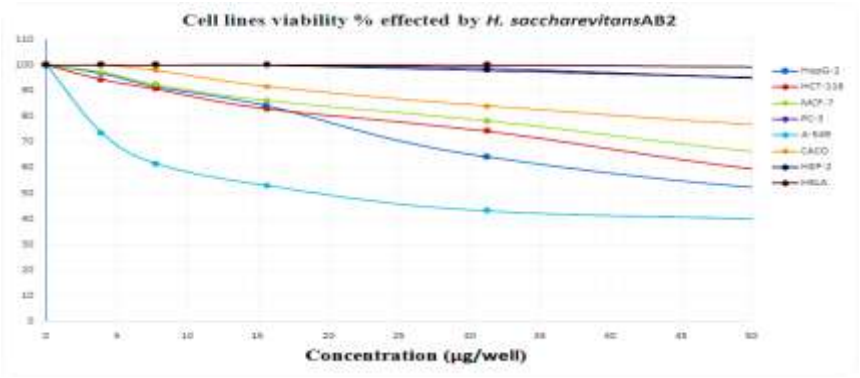

Fig. 10: Anticancer activity for different concentrations of EPS produced by *H. saccharevitans* AB2 against different cancer cell lines (measured by MTT assays after 24 h exposure).

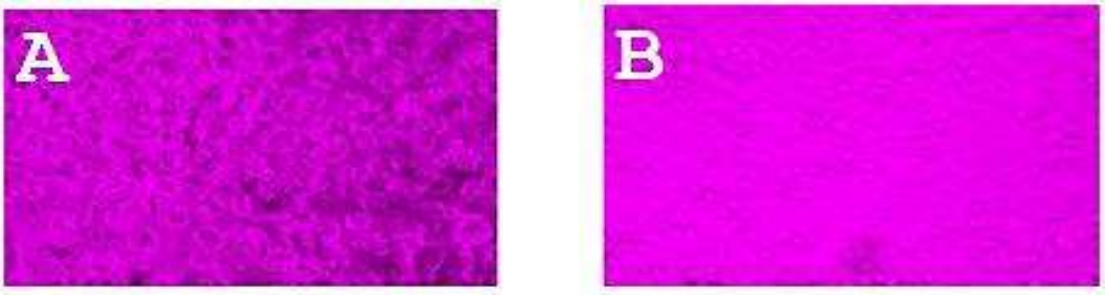

Figure 11: Photographs illustrating the difference between the tested compound (B) on the growth inhibition of A-549 tumor cell line compared to control (A).

The same result was reported by Yang *et al.* (2014), who reported that SBT-A shows significant potent growth inhibitory effects on A549 cells *in vivo* and *in vitro*.

Antitumor activity of EPS from marine *Halomonas* spp. was proven in different studies, such as sulphated EPS from *H. eurihalina* H2-7, which enhances the unspecific proliferation of human lymphocytes in response to the presence of the anti-CD3 mononuclear antibody (Pérez-Fernández*et al*., 2000) and the sulphated EPSs from *Halomonasstenophila* (B100 and N12T) (Llamas *et al*., 2011) which blocks the growth of human T-lymphocyte tumours (Ruiz-Ruiz *et al*., 2001) phosphate groups, which have also been observed in other EPSs from halophilic bacteria (Mata *et al*. 2008) could also confer important properties on them because they are required for the activation of lymphocytes (Nishimura-Uemura *et al*., 2003) and in some antitumoural processes.

CONCLUSION

A number of surface attached bacteria isolated from the algae, rocks in addition to biofilm formation device showed potential for EPSs production. The most promising *Halomonas saccharevitans* AB2 is a good extremophilic candidate for EPS production. Optimization of the culture conditions for EPS production realized increase in the yield. The produced EPS showed good antibacterial and anticancer activity to be promising for different pharmaceutical applications.

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