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Production, Characterization and Valuable Applications of Exopolysaccharides from Marine *Bacillus subtilis* SH1

SAHAR W.M. HASSAN* and HASSAN A.H. IBRAHIM

Marine Microbiology Department, National Institute of Oceanography and Fisheries, Alexandria, Egypt

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Abstract

Exopolysaccharides (EPSs) are high molecular weight polymers consisting of different sugar residues they are preferable for replacing synthetic polymers as they are degradable and nontoxic. Many microorganisms possess the ability to synthesize and excrete exopolysaccharides with novel chemical compositions, properties and structures to have potential applications in different fields. The present study attempt to optimize the production of EPS by marine *Bacillus subtilis* SH1 in addition to characterization and investigation of different valuable applications. Effect of medium type, incubation period and pH were studied using the one factor at a time experiments. It was shown that the highest productivity (24 gl^{-1}) of exopolysaccharides was recorded by using yeast malt glucose medium with pH 9 at the fourth day of incubation. Experimental design using Response Surface Methodology (RSM) was applied to optimize various nutrients at different concentrations. The finalized optimized medium contained (gl^{-1}) glucose (5), peptone (2.5), yeast extract (4.5) and malt extract (4.5) increased the production of EPS to 33.8 gl^{-1} with1.4 fold increase compared to the basal medium. Chemical characterization of the extracted EPS showed that, FTIR spectra exhibited bands at various regions. Moreover, HPLC chromatogram indicated that the EPS was a heteropolysaccharide consisting of maltose and rhamnose. The study was extended to evaluate the potentiality of the extracted polysaccharides in different medical applications. Results concluded that, EPS exhibited antibacterial activity against *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Streptococcus faecalis* and the highest antibacterial activity (7.8, 9 and 10.4 AU/ml) was against *S. faecalis* at 50, 100 and 200 mg/ml respectively. The EPS exhibited various degree of antitumor effect toward the tested cell lines (MCF-7, HCT-116 and HepG2). In addition, EPS exhibited antiviral activity at 500 µg/ml. The antioxidant capacity increased with increasing the concentration of the sample. Scanning electron microscopic analysis showed that EPS had compact film-like structure, which could make it a useful in the future applications as in preparing plasticized film.

Key words: *Bacillus subtilis* SH1, anticancer, antiviral, exopolysaccharides, response surface methodology

Introduction

Exopolysaccharides (EPSs) are defined as; high molecular weight, biodegradable polymers which biosynthesized by a wide range of organisms (Vijayabaskar *et al*., 2011; Sanlibaba and Çakmak, 2016). The EPS are often found in the surroundings of the outer structures of prokaryotic as well as eukaryotic microbial cells. They are either closely associated with the cell in the form of discrete capsules or else are excreted as slime unattached to the cellular surface. They exist in a wide variety of unique and complex chemical structures and are believed to provide self-protection against antimicrobial substances growing nearby (Nanda and Raghavan, 2014). Exopolysaccharides have major roles in different processes viz., formation of biofilm (Ohno *et al*., 2000; Vimala and Lalithakumari, 2003), protection of bacterial cell from desiccation, for maintaining primary cellular functions and antibacterial activity against predators, gelling ability, pollutant degradation kinetics and cement based construction industry (Bhaskar and Bhosle, 2005; Rawal *et al*., 2016). There are two major types of EPSs according to the constituents; homo- and hetero-EPSs. Recently, microbial polysaccharides have been attended due to their unique properties and the possibility of quick mass production. Today, EPSs find wide range of applications in food, pharmaceutical, other industries (Patil *et al*., 2009; Nwodo *et al*., 2012). Also, they have proved various physiological activities in human beings as anti-tumor, anti-viral and anti-inflammation agents, as well as being inducers for interferon, platelet aggregation inhibition, colony stimulating factor synthesis, coagulants and lubricants (de Godoi *et al*., 2014; Li *et al*., 2016; Venkateswarulu *et al*., 2016). A number of microbial strains have the potentiality to produce polysaccharides with widely varying compositions. On the other

* Corresponding author: S.W.M. Hassan, National Institute of Oceanography and Fisheries (NIOF), Marine Microbiology Department, Kayet Bay, El-Anfushy, Alexandria, Egypt; **e-mail: saharwefky@yahoo.com**

side, EPSs production from marine microorganisms such as; *Zoogloea* sp. (Ikeda *et al*., 1982), *Pseudomonas* sp. (Matsuda and Worawattanamateekul, 1993), *Vibrio fischeri* (Rodrigues and Bhosle, 1991), *Cyanothece* sp. (Philippis *et al*., 1993), and *Alteromonas macleodii* (Raguenes *et al*., 1996) were reported and their properties were studied well. Furthermore, *Bacillus* sp. produces complex EPSs and widely studied species were; *Bacillus licheniformis*, producing levan (Ghaly *et al*., 2007), *Bacillus coagulans*, *Bacillus polymyxa* (Lee *et al*., 1997) and *Bacillus mucilaginous* (Lian *et al*., 2008). *Bacillus subtilis* is also one of the major producers of EPS among *Bacillus* sp. In major reports, *B. subtilis* is mostly found to produce a biopolymer poly-γ-Glutamate (PGA) (Jane and HsiuFeng, 2007; Robert *et al*., 2011), levan, fructan (De Melo *et al*., 2010). The optimization process of fermentation is very critical and parameters affecting could be carried out using a statistical tool; Response Surface Methodology (RSM). This method has many advantages over the conventional method which involves numerous experiments by changing one variable at a time with keeping other independent variable constant. The RSM is a reliable tool and a fast experimentation technique which deals with individual effects of nutrients and their interactive effects (Wu *et al*., 2008; Rabha *et al*., 2012; Prathima *et al*., 2014).

The main goal of the present study was the production, maximization and characterization of EPSs from the marine *B. subtilis* SH1. Moreover, different medical applications were investigated.

Experimental

Materials and Methods

Microorganism and culture conditions. Marine *B. subtilis* SH1 was kindly provided by Dr. Hassan A.H. Ibrahim; Marine Microbiology Dep., NIOF, Alexandria, Egypt. It was isolated upon seawater agar medium from Suez Gulf and completely identified by phenotypic and genotypic means. The nucleotide sequence was deposited to GenBank sequence database and has EU107759 accession number.

Reference bacterial strains. The bacterial indicator strains (*Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio damsela*, *Escherichia coli*, and *Streptococcus faecalis*) were kindly provided by Marine Microbiology Dep, NIOF, Alexandria, Egypt.

Production of EPS. *B. subtilis* SH1 was inoculated in 50 ml aliquot of nutrient broth medium dispensed in 250 ml Erlenmeyer flask, the flask was incubated at 37°C under shake condition (120 rpm) for overnight. After overnight incubation, 500 µl was transferred to 50 ml of a fresh production media in a conical flask. Above step was carried out in an aseptic manner, the flask was incubated for 4 days at 37°C.

Extraction of EPS from *B. subtilis* SH1 culture. The marine *B. subtilis* SH1 culture was centrifuged at 10,000 g for 10 min at 4°C. The supernatant obtained was mixed with two volumes of ice cold ethanol and kept at 4°C for 24 h. The mixture was then centrifuged at 2500 × g for 20 min at 4°C. The obtained pellet was suspended in distilled water, which was centrifuged at 2500 × g for 30 min at 4°C with two volumes of ice cold ethanol. The process was repeated twice and the EPS obtained was dried, weighed and lyophilized (Savadogo *et al*., 2004).

One factor at time experiments

Effect of different media on EPS production. To study the effect of media on the production of EPS, different media (gl^{-1}) including nutrient broth (Abou-Dobara *et al*., 2014); yeast extract 2, peptone 5, NaCl, 5 (NB) (Abou-Dobara *et al*., 2014), soft brown sugar 40; $MgSO_4 \cdot 7H_2O$ 0.2; K_2HPO_4 9; KH_2PO_4 3; yeast extract 2 (SEM) (Tallgren *et al*., 1999); yeast extract 3, malt extract 3, glucose 10, peptone 5 (YMG) (Abou-Dobara *et al*., 2014); Yeast mannitol (YM), yeast extract 0.5, mannitol 4 (Mukherjee *et al*., 2011) were tested. A liquots of 1% (OD = 1 at 600 nm) of the active culture of the *B. subtilis* SH1 which was previously proliferated in marine nutrient broth, was used as constant inoculum size for each medium.

Effect of different incubation period on EPS production. The effect of incubation time was investigated by varying time periods (1–6) days. Culture with 1% was used as constant inoculum size.

Effect of different pH on EPS production. The initial pH for higher product yield was determined by adjusting pH with 1M HCl and 1M NaOH before sterilization at different pH as 5, 6, 7, 8, and 9. Culture with 1% (OD = 1 at 600 nm) was used as constant inoculum size.

Optimization of culture conditions using RSM. Response surface methodology was used to determine the optimum concentration of the tested variables for enhancement of EPSs production. The variables were (glucose, peptone, yeast extract and malt extract). The four independent variables were evaluated at five different levels (–2, –1, 0, +1 and +2) and 30 experiments, containing 6 replications at the center point as shown in Table I. The behavior of the system was explained by the following second order polynomial equation:

$$Y = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 4X4 + \beta 12X1X2 + \\ + \beta 13X1X3 + \beta 14X1X4 + \beta 23X2X3 + \beta 24X2X4 + \\ + \beta 34X3X4 + \beta 11X12 + \beta 22\ X22 + \beta 33X32 + \beta 44X42$$

Where, Y is the predicted response, β0 is the scaling constant, X1-X4 are the coded levels of the fac-

Table I
Central composite design and the EPSs production by *B. subtilis* SH1 obtained from the culture trials

Run order	Coded levels				Response		
	X1 (Glucose)	X2 (Peptone)	X3 (Yeast extract)	X4 (Malt extract)	EPS (gL^{-1})		Biomass (gL^{-1})
					Observed values	Predicted values	
1	1	−1	−1	1	24.6	22.8	1.8
2	0	0	0	0	17.6	22.8	1.8
3	0	2	0	0	12.8	21	1.8
4	0	0	0	0	20	17.6	2
5	1	1	1	−1	9.6	21	2.6
6	−1	−1	1	1	33.8	5.8	1.6
7	0	0	0	−2	21.6	25	3.2
8	0	0	0	2	21.2	18	2.4
9	0	0	0	0	24	1.8	1.4
10	1	1	−1	1	31.8	21	2
11	−1	−1	−1	−1	14.4	36.8	1.4
12	−1	1	1	1	29.8	18	2
13	1	−1	1	−1	6.2	36	0.6
14	0	0	2	0	17.8	5.8	1.6
15	−2	0	0	0	33	17.8	1.6
16	0	0	0	0	12	28.6	2.4
17	−1	1	−1	1	23	21	1.2
18	1	1	−1	−1	20.8	20.6	2
19	0	0	0	0	22	26.8	2.2
20	0	0	0	0	20	21	2.4
21	2	0	0	0	24.8	21	2.4
22	0	−2	0	0	24.8	25	1.8
23	−1	1	−1	−1	25.6	21.6	1.4
24	−1	−1	1	−1	15.6	23	1.8
25	1	−1	1	1	16	11.2	1.6
26	−1	−1	−1	1	10.4	10.4	0.4
27	0	0	−2	0	27.4	23.6	1.6
28	−1	1	1	−1	27.4	26.2	2.2
29	1	−1	−1	−1	26.6	26.8	1.8
30	1	1	1	1	24.4	18	1.8

tors, β1-β4 are the linear coefficients, β12-β34 are the interactive coefficients and β11-β44 are the quadratic coefficients. ANOVA and regression analyses were also carried out used the above software. The quality of the polynomial equation was confirmed by the determination of coefficient R^2 and its statistical significance was determined by Fisher's test, F value.

Morphological characterization of EPS. Morphology of dried EPSs was determined by scanning electron microscopy examination (SEM). EPS was coated on gold particles and microstructure was visualized under scanning electron micrograph at 3000X. Whole and surface view of EPSs was taken and structure was analyzed (Yada *et al*., 2011).

Structural analyses. Pellets for infrared analysis were prepared by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The Fourier transform-infrared (FTIR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4000–400 cm^{-1} region. Chemical characterization included UV was carried out using UV spectroscopy (Heλiosα).

Composition of EPSs produced by marine *B. subtilis* SH1 was done using HPLC system. The EPS isolated from culture was dissolved in distilled water and EPS (1 ml) was hydrolyzed with 3 M Tri-fluoroacetic acid (TFA) for 1–2 h at 250°C using oil bath.

Hydrolyzed EPS was neutralized using 5 N NaOH and volume was made to 50 ml with MilliQ water. HPLC of hydrolyzed EPSs was done by using aminopropile column (HI-Plex, Agilent, 4.6 × 250 mm), acetonitrile and water was used as mobile phase in ratio of 75:25. The separation was carried out at 24°C with flow rate of 1 ml/min and sample volume of 20 µl. Monosaccharides such as glucose, arabinose, raffinose, rhamnose, maltose, *etc*. were used as standard. The column was calibrated with different molecular mass standard and a standard curve was then established (Yada *et al*., 2011).

Antimicrobial activity of EPS against indicator strains. Fifty milliliter of nutrient agar medium inoculated with indicator pathogenic bacteria (1% v/v) were poured into all plates. After solidifying, wells were punched out using 0.5 cm cork borer, and each of their bottoms was then sealed with two drops of sterile water agar. One hundred microliters of different concentrations (10, 25, 50, 100 and 200 mg/ml) of filtrated EPS were transferred into each well after sterilizing by ultrafiltration using 0.22 µm 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: $AU = Y^2/X^2$ (El-Masry *et al*., 2002).

Antitumor activity. Human Breast cancer (MCF-7), Human colon carcinoma HCT-116 and Hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% v/v inactive fetal calf serum and 50 µg/ml Gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week.

The antitumor activity was evaluated on tumor cells. The cells were grown as monolayers in growth medium supplemented with 10% inactive fetal calf serum and 50 µg/ml Gentamycin. The monolayers of 10000 cells adhered at the bottom of the wells in a 96-well microliter plate incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M, pH 7.2) and simultaneously the cells were treated with 100 µl from different dilutions of tested sample in fresh maintenance medium and incubated at 37°C. A control of untreated cells was made in the absence of tested sample. Six wells were used for each concentration of the test sample. Every 24 h the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet (Gangadevi and Muthumary, 2007) followed by cell lysing using 33% glacial acetic acid and read the absorbance at 590 nm using ELISA reader (SunRise, TECAN, Inc, USA) after well mixing. The absorbance values from untreated cells were considered a 100% proliferation.

The number of viable cells was determined using ELISA reader as previously mentioned before and the percentage of viability was calculated as (ODt/ODc) × 100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The 50% inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

Antiviral assay

Mammalian cell line. Vero cells (derived from the kidney of normal African green monkeys) were obtained from agle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, HEPES buffer and Gentamycin (50 µg/ml). All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

Evaluation of the antiviral activity using cytopathic inhibition assay. The screening of antiviral assay using cytopathic effect inhibition was carried out at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University. This assay was selected to show specific inhibition of a biologic function (Hu and Hsiung, 1989). Monolayers of 1000 vero cells adhered at the bottom of the wells in a 96-well microliter plate incubated at 24 h at 37°C in a humidified incubator with 5% CO_2. The plates were washed with fresh DMEM and challenged with 10^4 herpes simplex type I virus (HSV-1) doses and simultaneously the cultures were treated with two-fold serial dilutions of tested compound in fresh maintenance medium and incubated at 37°C for 3 days. An infection treatments as well as untreated vero cells control was made in the absence of tested compound. Six wells were used for each concentration of the tested compound. Every 24 h the observation under the inverted microscope was made until the virus in the control wells showed complete viral- induce cytopathic effects. Antiviral activity was determined by the inhibition of cytopathic effect compared to control (Vijayan *et al*., 2004).

Three independent experiments were assessed each containing four replicates per treatment. Acyclovir, which is clinically used for the treatment of hepatic viral disease, was used as a positive control under this assay system (Dargan, 1998).

After the incubation period, the media was aspirated, and the cells were stained with a 0.1% crystal violet solution for 4 h. The stain was removed and the plates rinsed using tap water until all excess stain was

removed. The plates were allowed to dry for 24 h and then glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc) at 620 nm. Viral Inhibition rate was calculated as follows:
[(ODtv-ODcd/ODcv)] × 100%, where ODtv, ODcv and ODcd indicate the absorbance of the test compounds with virus infected cells, the absorbance of the virus control and the absorbance of the cell control, respectively.

Data and statistical analysis. From these data, the dose that inhibited viral infection by 50% (EC50) was estimated with respect to virus control from the graphic plots using STATA modeling software. EC50, the effective concentration needed to restrain 50% virus infection compared to untreated infected cells, was determined directly from the curve obtained by plotting the inhibition of the virus yield against the concentration of the samples. To determine if each compound has sufficient antiviral activity that exceeds its level of toxicity, a selectively index (SI) was calculated. The selectivity index (SI) was measured from the ratio of the concentration at which 50% cytotoxicity was observed (CC50/EC50) (Zandi *et al.*, 2007). This index, also referred to as a therapeutic index, was used to determine if a compound warrants further study. Compounds that had an SI of 2 were considered active, 10 or greater was considered very active.

Antioxidant activity using DPPH stable free radical scavenging assay. The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) stable free radical scavenging assay was performed as previously described by Hu and Kitts, 2000 with modifications. Sample was dissolved in a methanol at different concentrations ranging from 1 to 128 µg/ml and then of the sample (2 ml) was incubated with 0.5 ml (0.2 mM) DPPH in 100% methanol. Sample absorbance at 519 nm was recorded after 30 min of incubation at room temperature. Inhibition of the DPPH stable free radical was calculated as follows:

$$\text{Inhibition } \% = (Ab_{Control} - Ab_{Sample})/(Ab_{Control} - Ab_{Blank}) \times 100$$

Where:

Ab control = absorbance of 0.1 mM DPPH alone in methanol;
Ab sample = absorbance of 0.1 mM DPPH + sample in methanol; and
Ab blank = absorbance of methanol solvent control in absence of DPPH or sample.

Results and Discussion

B. subtilis is one of the major producers of exopolysaccharides (EPSs) among *Bacillus* sp. Therefore, the current study was suggested to evaluate the production and characterization of EPSs from marine *B. subtilis* SH1, as well as, to detect some medical applications of EPSs.

Optimization of fermentation conditions for EPS production by marine *B. subtilis* SH1

Effect of different media on EPS production. Different media including NB, SEM, YMG and YM were evaluated for the production of EPS by *B. subtilis*. As shown in Fig. 1, YMG medium was the most suitable for higher production of EPS with yield of 18 gl^{-1} followed by NB (11.2 gl^{-1}) and YM (8.8 gl^{-1}), while the lowest production (8.4 gl^{-1}) was recorded up on using SEM medium. Similar results were recorded by Abou-Dobara *et al.* (2014), who stated that among seven tested media, YMG medium gave the highest production of EPS by *B. subtilis*. They also reported that glucose, malt and mannitol support good biomass production.

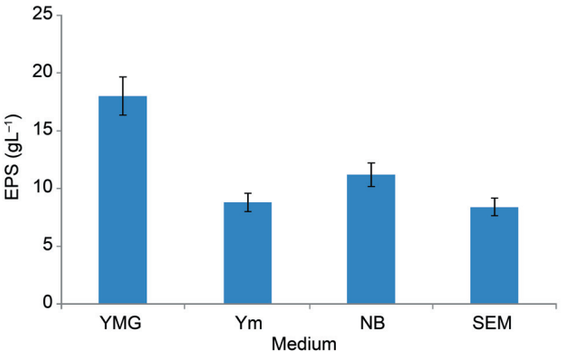

Fig. 1. Effect of different media on the production of the EPSs by marine *B. subtilis* SH1.

Effect of incubation time on the production of EPS by *B. subtilis* SH1. Incubation time is an essential factor determining the enhancement of EPS synthesis in the culture. Observation of time course of EPS production in association with growth of *B. subtilis* SH1 at different time interval (1–6 days) (Fig. 2) indicated that the production of EPS increased gradually from 17.2 gl^{-1} at the first day with biomass of 20 gl^{-1} to 19.8 gl^{-1} at the fourth day which represented the highest productivity of EPS and also the highest biomass production (44 gl^{-1}), while the production decreased after that till the last day to reach 18 gl^{-1}. This can be explained by the fact that EPS is highly synthesized during late exponential growth phase and decrease in incubation time may lower the production. Higher incubation time might affect the yield due to the production of certain enzymes, saccharases, along with EPS, might act upon polysaccharides, thus deteriorating the product formation. The result of the present work is consistent with similar studies carried out earlier which reported the production of

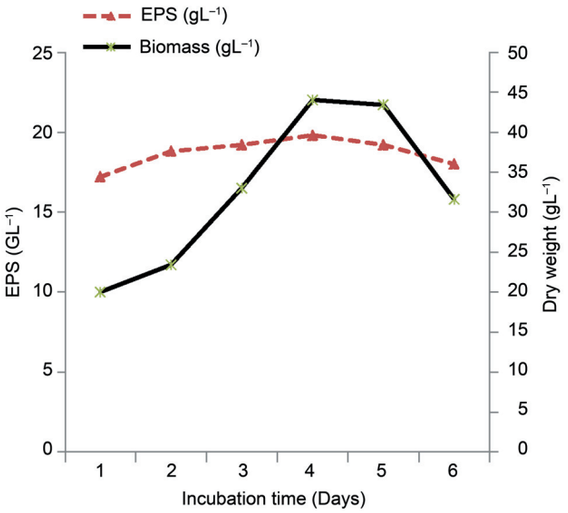

Fig. 2. Effect of incubation period on growth and EPSs production by marine *B. subtilis* SH1.

EPS by *B. subtilis* at late phase of growth (72 h) (Lee *et al.*, 1997; Vijayabaskar *et al.*, 2011).

Effect of pH on EPS production by *B. subtilis* SH1. The pH is a significant factor influencing the physiology of a microorganism by affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology, by product formation and oxidative-reductive reactions (Bajaj *et al.*, 2009). Production of EPS by *B. subtilis* SH1 was estimated at different pH (5–9). As shown in Fig. 3, the production of EPS increased from 19 gl^{-1} at pH 5 to 19.8 gl^{-1} at pH 7 and the highest productivity of EPS was recorded at pH 9 which realized 24 gl^{-1} which means that the production was favorable at the alkaline level. These results coincide with those reported by Ko *et al.* (2000), who reported that the highest production of EPS by *Bacillus* sp. was produced at pH 8 and reduced in lower pH values. Relatively low concentration of EPS produced by *B. subtilis* was obtained in other studies at pH 7 and reduced at lower pH values (Abdul Razack *et al.*, 2013). Also, other studies reported that the maximum production of EPS by *B. subtilis* was obtained at pH 7 and decreased gradually at pH 8 to10 (Abu-Dobara *et al.*, 2014).

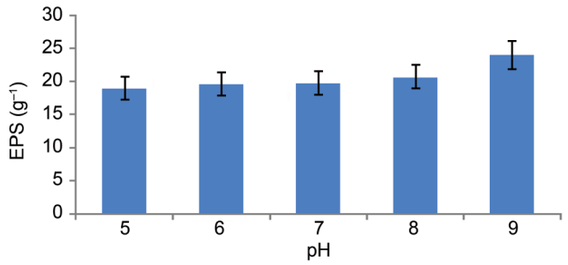

Fig. 3. Effect of different pH on the EPSs production by marine *B. subtilis* SH1.

Optimization of EPS production by *B. subtilis* SH1 using Response Surface Methodology (RSM). Response Surface Methodology (RSM) was used to investigate the influence of different components on the yields of biomass and extracellular EPS by *B. subtilis* SH1. Central composite design with 4 factors at five levels was used for fitting data on a second-order polynomial model (Table II). The mathematical method describing the relationships between the process responses (the yield of EPS) and the medium contents was developed. Different trials and the response were illustrated in Table I.

Table II
Levels of process variables used in central composite design.

Variables (gL^{-1})	Coded levels				
	−2	−1	0	1	2
Glucose (X1)	2.5	5	10	15	20
Peptone (X2)	1.25	2.5	5	7.5	10
Yeast extract (X3)	0.75	1.5	3	4.5	6
Malt extract (X4)	0.75	1.5	3	4.5	6

Results in Table I showed that the EPS yield varied between 6.2–33.8 gl^{-1}. The maximum productivity of EPS was achieved at conditions of low concentrations of glucose and peptone (5 g and 2.5 g) respectively but with high concentrations of yeast extract and malt extract (4.5 gl^{-1}). In agreement with the present study, Looijesteijn *et al.* (2000) studied the influence of different substrate limitations on EPS production by closely related organism namely *Lactococccus lactis* subsp. *cremoris*. They observed that reduction in glucose resulted in slightly higher production of EPS.

Kimmel *et al.* (1998) reported that higher glucose concentration (Carbon source) provides the higher yield of EPS for *L. lactis* sp. Abou-Dobara *et al.* (2014) reported that the highest production of EPS was recorded by using yeast extract with a concentration of 0.22%. Conversely, reports suggested that nitrogen limitation and higher amounts of carbon in the medium could yield a maximum amount of EPS. A study showed that EPS production from *Rhizobium meliloti* was higher when the nitrogen source was in minimal quantity. Similarly, pullulan was generated by *Aureobasidium pullulans* when it was grown in a medium with lesser amounts of nitrogen source. On the other hand, EPS production was observed under nitrogen-limited conditions (Mengistu *et al.*, 1994; Marshall *et al.*, 1995). It was observed that the growth culture conditions that realized the highest EPS production do not realize the highest growth. It was stated that biosynthesis of biomass and EPS biosynthesis follow roughly the same metabolic pathways. This results in the same meta-

bolic control for EPS production and for growth. At higher growth rates, more intermediates per time unit are needed for the biosynthesis of cell surface polysaccharides, and the intermediates are apparently used in favor of the synthesis of these polysaccharides; this may explain the reduction of the EPS yields at higher growth rates (Prathima *et al*., 2014).

It can be concluded that the present study achieved good yield of EPS (33.8 gl^{-1}) using the marine *B. subtilis* SH1 under the optimized conditions which is more yield in shorter time than other previous studies such as Shukla *et al*. (2015), who reported that the highest production of EPS was 12 gl^{-1}at 144 h. Also, Berekaa (2014) stated that the highest yield of EPS by *B. licheniformis* strain-QS5 was 22.5 gl^{-1}at 96 h under the optimized conditions. Thus the marine *B. subtilis* SH1 is promising candidate for EPS production as it is cheap and ecofriendly marine source.

Based on the results of the experimental designs, a second order polynomial equation was developed, describing the correlation between the variables used for study. The EPS yield could be represented as:

$$Y = 8.40 + 0.553\ X1 + 3.738\ X2 + 4.510\ X3 - 4.191\ X4 + \\ + 0.081\ X21 - 0.341 \times 22 - 0.0001\ X23 - 0.261 \times 24 - \\ - 0.065\ X1\ X2 - 0.868\ X1X3 + 0.118\ X1 \times 4 - \\ - 0.003\ X2X3 + 0.450X2X4 + 1.039\ X3X4.$$

Where, Y is the response *i.e*. EPS; X1, X2, X3 and X4 are the uncoded values of the test variables peptone, glucose, yeast extract and malt extract. Regression equation for the levels of EPS production (Y) as functions of; glucose concentration (X1), peptone concentration (X2), yeast extract (X3), and malt extract (X4) suggested that all the four factors influenced the EPS production by such organism.

ANOVA and regression analysis done for the experimental designs are tabulated (Table III). There was a significant difference ($p < 0.05$) and the second order model showed fit with $R^2 = 0.8$.

Table III
Statistical analysis of RSM.

Terms	EPS
F-value	1.67
P > F	0.167
R^2	0.8
Lack of fit	10
Pure error	5

Three dimensional response surface plots represent regression equations and illustrate interaction between the response and experimental levels of each variable was illustrated as shown in Fig. 4a–4f.

The results showed that the EPS yield varied between 6.2–33.8 gl^{-1}. The maximum productivity of EPS was achieved at concentrations (gl^{-1}): glucose, 5; peptone, 2.5; yeast extract, 4.5 and malt extract, 4.5. The production decreased under other conditions, irrespective of higher or lower than these levels.

These figures showed that the highest yield of EPS was recorded upon decreasing the concentrations of glucose and peptone (Fig. 4a) or decreasing the concentration of glucose and increasing the concentration of yeast extract (Fig. 4b). The same will be realized at low glucose concentration and high concentration of malt extract (Fig. 4c). Our finding is in accordance with Prathima *et al*. (2014), who reported the production of EPS by *L. lactis* NCDC 191 in a whey based medium under glucose limitation using Response Surface Methodology.

Results in Fig. 4d showed that interaction between low concentration of peptone and high concentration of yeast extract cause increase in the EPS yield, which also can be achieved in case of low concentration of peptone and high concentration of malt extract (Fig. 4e). On the other hand, increasing concentrations of both yeast extract and malt extract achieved the highest yield of EPS (Fig. 4f).

Morphological characterization of EPS. Textural and rheological properties of EPS are contributed by its structure beside its quantity and composition (Yada *et al*., 2011). The EPS obtained from *B. subtilis* SH1 was observed by SEM (Fig. 5). As it is seen from the microstructure of EPS surface view, the produced EPS is highly compact in structure. This indicates the potential of EPS as viscosifying, as a thickener or as stabilizing agent for novel food products (Yada *et al*., 2011).

Structural analyses. The FTIR spectra of the EPS (Fig. 6) exhibited bands at various levels. A dominant absorption that is often attributed to O-H stretching vibration at 3361.74 of O-H in carboxylic acid which is accompanied with the bands at 2923.88 cm^{-1} corresponds to H stretching in carboxylic group. Exopolymers produced by marine bacteria generally contain 20–50% of the polysaccharide as uronic acid (Kennedy and Sutherland, 1987).

The band at 1740.04 cm^{-1} approves the stretching vibration of C = O carbonyl group of an aldehyde or ketone. Quite a spectral peak was obtained at 1628.99 cm^{-1} indicated amide NH_2 bending vibration or C = O, C = N stretching vibration of $RCONH_2$. The peak at 1370.45 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 1222.62 cm^{-1} strongly suggesting the stretching vibration of O-H group in a phenol. The peak at 1055.33 cm^{-1} is assigned

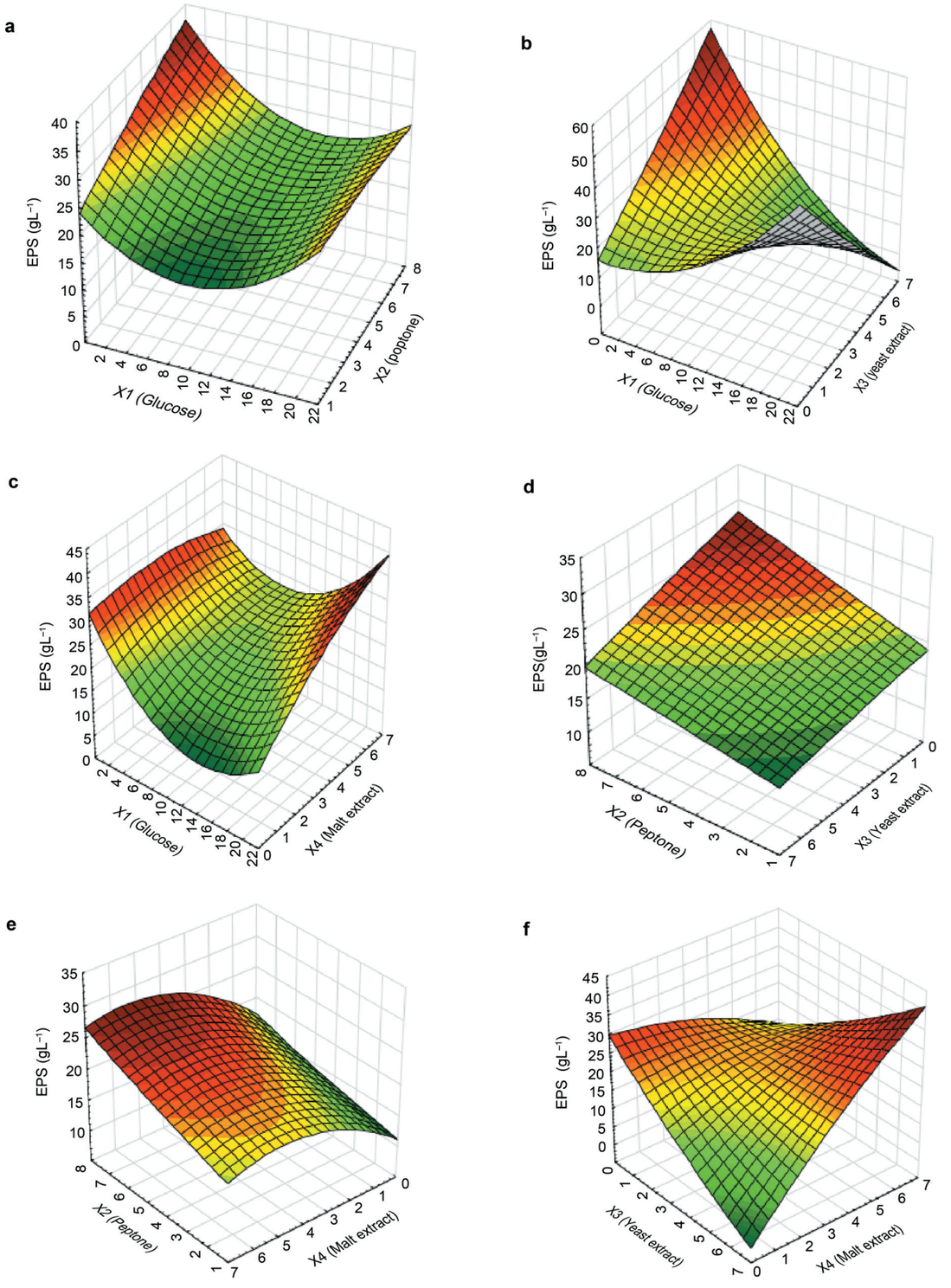

Fig. 4. Response surface plots of interaction between process variables in the EPSs production by *B. subtilis* SH1.

to stretching vibration of (C-O, alcohol, ester, ether and phenol) groups. It was 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 (Orsod *et al.*, 2012).

UV spectrum analysis of EPSs (Fig. 7) indicated that there was a significant amount of absorption in

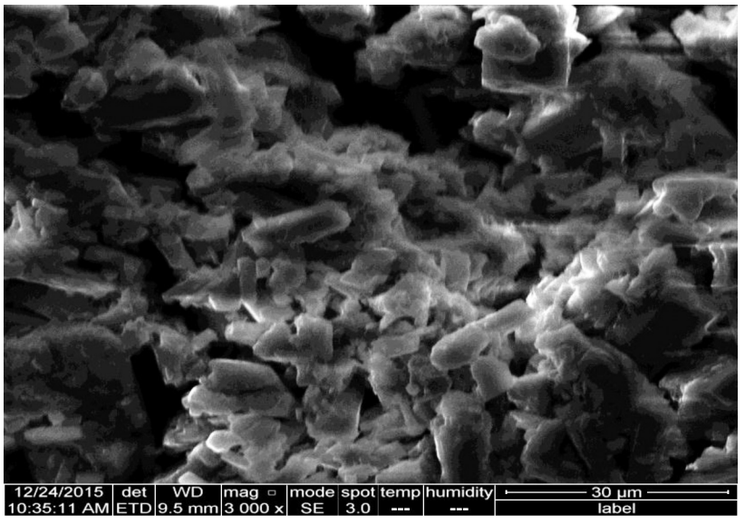

Fig. 5. Electron micrograph showing the surface of *B. subtilis* SH1 EPSs at 3000 X.

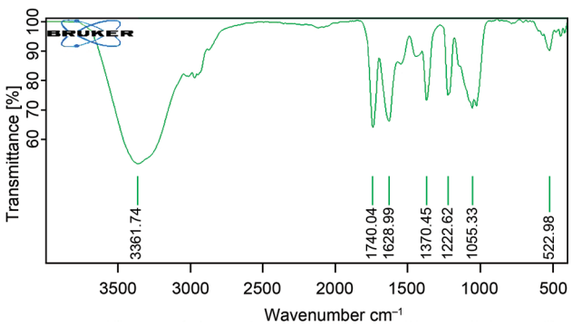

Fig. 6. FTIR spectra of the EPSs produced by *B. subtilis* SH1.

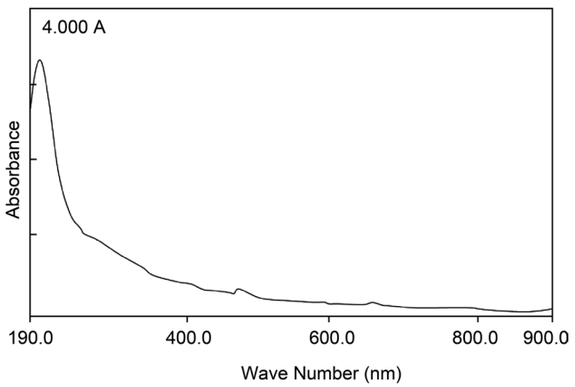

Fig. 7. UV analysis of the EPSs produced by *B. subtilis* SH1.

the UV area and revealed that the maximum absorption was at 190 nm. Trabelsi *et al.* (2009) stated that, the 190 ~ 230 nm wavelength area often results from n-σ* and/or π-π* transitions, which are found in many functional groups such as carboxyl, carbonyl, and ester.

The HPLC chromatogram of EPS in Fig. 8 showed the appearance of 2 major peaks, the first peak was at retention time 3.08 min with area 5.83% and the second peak was at retention time 9.65 min with area 91.23%. Based on the chromatogram, it was indicated that the EPS is a heteroploysaccharide contains maltose and rhamnose.

Different medical applications of the EPS produced by *B. subtilis* SH1. There are four types of activities detected and estimated for EPSs to be used as medical applications; antibacterial, antitumor, antiviral, and antioxidant activities.

Antibacterial activity. Different concentrations of the extracted EPS (10–200 mg/ml) were screened for antibacterial activity against different types of Gram-positive bacteria (*S. aureus*, *S. faecalis*) and Gram-negative bacteria (*E. coli*, *A. hydrophila*, *P. aeruginosa, V. damsela*). Data in Table IV showed that EPS had different range of antibacterial activity against the tested bacteria. The highest antibacterial activity (7.8, 9 and 10.4 AU/ml) was recorded against *S. faecalis* at 50, 100 and 200 mg/ml respectively while no inhibitory activity was detected against all the tested bacteria at lower concentrations (10 and 25 mg/ml). Antibacterial

Table IV
Antibacterial activity of the EPS from *B. subtilis* SH1 expressed as absolute unit (AU).

Indicator bacteria	AU/concentration (mg/ml)				
	10	25	50	100	200
A. hydrophila	ND	ND	3.6	5.4	5.4
P. aeroginosa	ND	ND	2.8	4.0	4.6
S. aureus	ND	ND	ND	ND	ND
V. damsela	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	ND	ND
S. faecalis	ND	ND	7.8	9.0	10.4

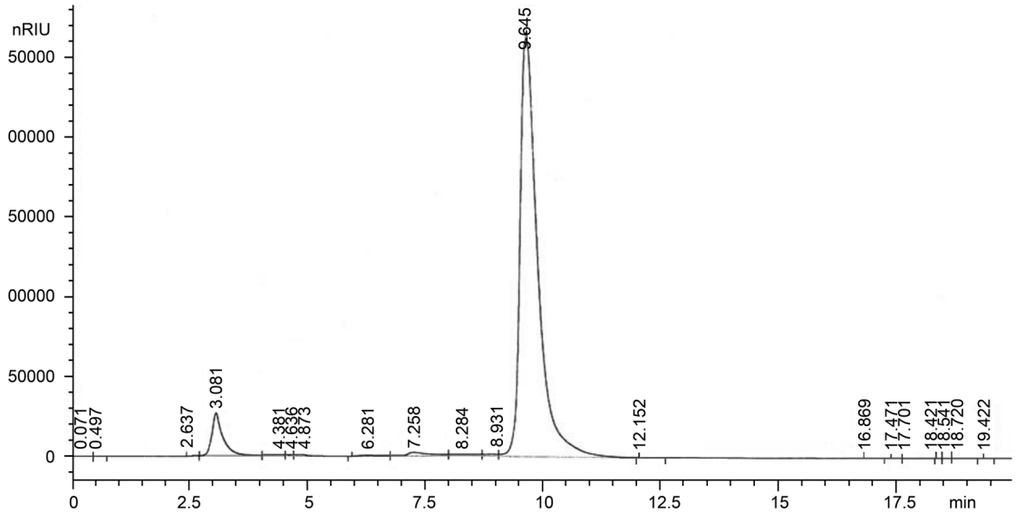

Fig. 8. Sugar analysis of EPS produced by *B. subtilis* SH1 using HPLC.

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 pathogens. Also, Shankar *et al.* (2010) isolated the EPS from four biofilm bacteria and the isolated EPS exhibited antimicrobial activity against different pathogens. In addition, Orsod *et al.* (2012) reported that the extracted EPS from the marine bacteria which associated with Asian sea bass has potential antimicrobial activities against different pathogens.

Antitumor activity. The inhibitory effect of different concentrations (1.56, 3.13, 6.25, 12.5, 25 and 50 µg/ml) of EPS was tested on three different cell lines included MCF-7, HCT-116 and HepG2. Results showed that the produced EPS exhibited various degree of antitumor effect toward the tested cell lines and increasing concentrations of EPS resulted in increased rates of tumor inhibition. Results in Fig. 9A showed that, the cell viability of HCT-116 was 34.53–9 5.64% which exhibited inhibition % of 65.47–4.36% with IC_{50} = 35.8 µg which is consistent with the finding of You *et al.* (2011), where the obtained polysaccharide revealed a marked inhibition of proliferation of HCT 116. Regarding the antitumor effect of EPS against MCF-7, results in Fig. 9B showed that the

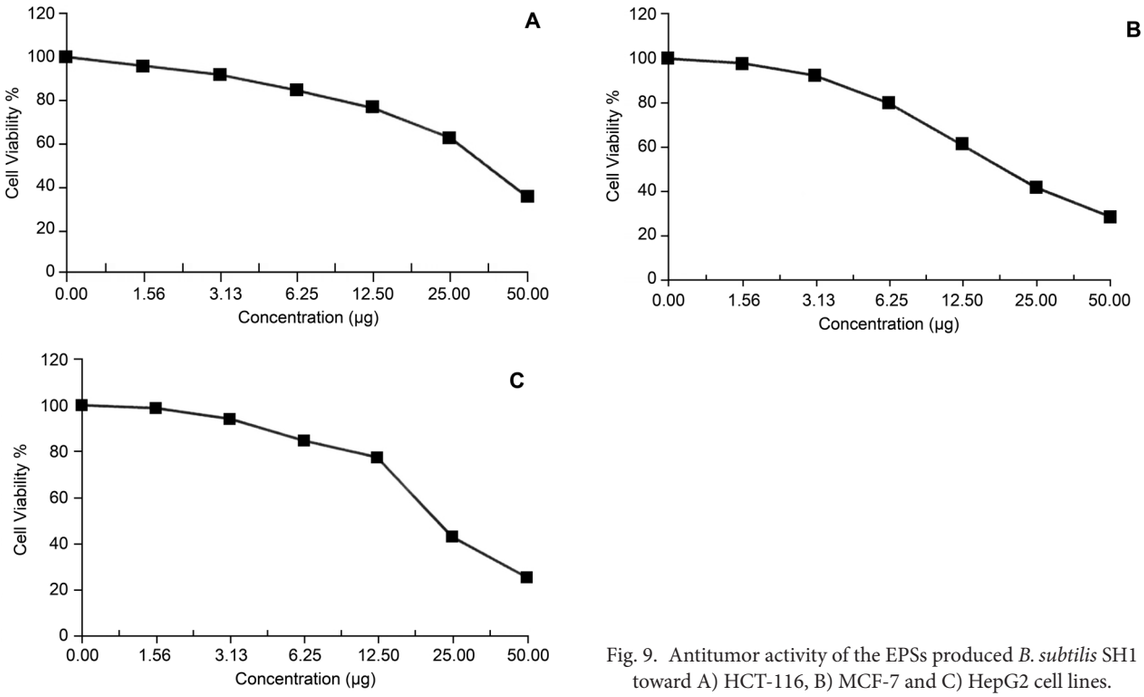

Fig. 9. Antitumor activity of the EPSs produced *B. subtilis* SH1 toward A) HCT-116, B) MCF-7 and C) HepG2 cell lines.

cell viability was 28.12 to 97.84% with inhibition range (71.88–2.16%) and exhibited $IC_{50} = 19.4$ µg

Fig. 9C shows that the viability % of the HepG2 cells ranged from 24.63 to 98.56% upon using EPS with different concentrations (0.39–50 µg) which means that it recorded inhibition range of (1.44–75.37%) and exhibited the highest antitumor effect compared to MCF-7 and HCT-116 cell lines with $IC_{50} = 22.3$ µg. The present work agree with that reported by You *et al.* (2011), who studied antitumor activity of different types of EPS toward 7 cell lines and reported that EPS exhibited the highest antitumor activity toward HepG2 (49.93–61.82%). The same result was reported by Dahech *et al.* (2012). It has been reported that the difference in antitumor effects of polysaccharides depends on their molecular weight, chemical composition, structure of the polymeric backbone, and degree of branching (You *et al.*, 2011).

Antiviral activity. Antiviral activity of EPS produced by *B. subtilis* SH1 was proven in different studies (Ahmed *et al.*, 2010). Polysaccharides are thought to inhibit the very early step of viral replication, *i.e.*, virus attachment to the cell surface (de Godoi, 2014). In the present study, EPS exhibited weak antiviral activity at 500 µg/ml against HSV-1, suggesting that this polysaccharide blocked a step in virus replication subsequent to virus attachment and entry. This result is in good agreement with the finding that increased internalization of heparin occurs during HSV-1 entry into cells to suggest that viral particles and the polysaccharide are co-internalized in endosomes. The polymer may inhibit a step in virus replication occurring between virus release and the synthesis of late proteins.

Antioxidant activity. The antioxidant capacity of EPS was determined by DPPH (2, 2-Diphenyl-2-picryl-hydrazyl) and revealed that, the sample has a significant antioxidant activity compared with ascorbic acid as control (Fig. 10). The different concentrations of the EPS sample (128, 64, 32, 16, 8, 4, 2, and 1 µg/ml) showed different percentages of inhibition (89.89, 88.86, 87.95, 81.03, 69.35, 38.49, 23.68, and 14.92%), respectively. These results revealed that, the antioxidant capacity was increased with increasing the concentration of the sample. These results agree with that recorded by Abdel-Fattah *et al.* (2012), who stated that the antioxidant activity of levan and their derivatives (SL1 and SL2) produced by *B. subtilis* exhibited a strong free radical scavenging activity with DPPH. You *et al.* (2011) also reported the antioxidant activity of different types of polysaccharides.

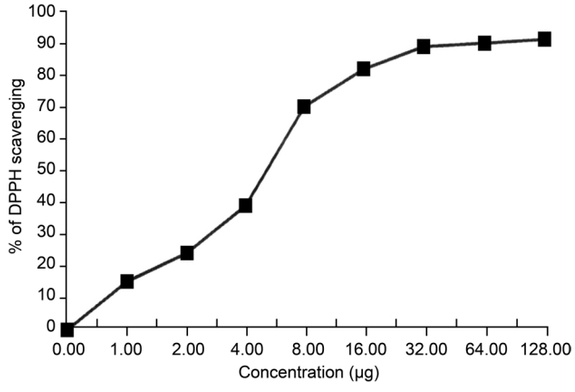

Fig. 10. Antioxidant activity of the EPSs produced by *B. subtilis* SH1.

Conclusions

The present study is spotlight on the importance of EPS from *B. subtilis* SH1 and the data confirmed the following points:

1. The YMG medium was the most suitable for higher production of *B. subtilis* SH1 EPSs.
2. The production of EPS increased gradually from the first day to the fourth day recording the highest productivity and the highest productivity of EPS was also favorable at the alkaline level.
3. The maximum productivity of EPS was achieved using RSM design at conditions of low concentrations of glucose and peptone but with high concentrations of yeast extract and malt extract.
4. The structural characterization was done by FTIR spectroscopy which exhibited bands at various levels. HPLC revealed the presence of maltose and rhamnose as major component in EPS.
5. EPS produced by *B. subtilis* SH1 showed valuable activities for medical purposes such as antibacterial, antitumor, antiviral, and antioxidant.

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