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Full Length Research Paper

Characterization of an exopolysaccharide-producing marine bacterium, isolate *PSEUDOALTEROMONAS* sp. AM

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A marine exopolysaccharide (EPS) producing bacterium was isolated from a sponge sample at about 16 m depth in red sea (Huraghada, Egypt). Phenotypic characterization demonstrated that, the bacterium is a gram negative rod motile by means of single polar flagellum, showing wide range of carbohydrate utilization. Phylogenetic analysis based on 16S Ribosomal deoxyribonucleic acid (rDNA) sequence revealed 99% homology with *PSEUDOALTEROMONAS* sp., accordingly the name *PSEUDOALTEROMONAS* sp. AM was proposed. Maximum EPS productivity was achieved after 7 days at pH 7, agitation of 150 rpm in a fermentation medium containing glucose, meat extract and 3% NaCl. The partially purified EPS had emulsifying activity about 78% and retained its stability for about 13 days. Extended emulsion stability for up to 22 days was shown by increasing the concentration of EPS from 0.01 to 0.1% w/v. The EPS elicited a marked antiviral activity against herpes simplex (HSV-I) and showed lysis of plasma clots comparable to pentosan sulphuric polyester as a standard. Preliminary characterization of EPS by complete acid hydrolysis then analysis on paper chromatography revealed the presence of a single brown spot equidistant with authentic glucose indicating that EPS is mainly composed of glucose units. Further investigations will be carried out to realize the full structure and chemical composition of the produced EPS and other possible biotechnological potential.

Key words: Exopolysaccharide, *Pseudoalteromonas* sp., emulsifying activity, antiviral activity, fibrinolytic activity.

INTRODUCTION

Owing to the wide ecological and physiological diversity of marine environment, a renewed interest on marine microorganisms has emerged and gained considerable attention. Marine microorganisms were found to have unique metabolic and physiological capabilities to cope with the extreme variations in pressure, salinity, temperature, biological habitats, together with low nutrient availability. Hence, the marine microorganisms offer a potential for the production of novel bioactive metabolites that might not be produced by their terrestrial counterparts (Annarita et al., 2010). Marine micro-organisms have been isolated from seawater, sediment and surface associated, both animate and inanimate. Marine surfaces are continually bathed by an assem-blage of microorganisms like algae, fish and many forms of marine invertebrates such as sponges and molluscs (Kelecom, 2002). Bacterial exopolysaccharides have found wide range of applications in food, pharmaceutical, petroleum, textile, ceramics, paper, ink, adhesives and other industries. Owing to the various functions of EPSs in marine ecosystem such as adhesion of bacteria, stabilization of biofilms and maintenance of symbiotic association with different species, investigation of EPSs producing marine bacteria was reviewed by many authors (Okutani, 1982, 1984, 1985; Boyle and Reade, 1983;

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Abbreviations: rDNA, Ribosomal deoxyribonucleic acid; **DNA,** deoxyribonucleic acid; **PCR,** polymerase chain reaction; **NCBI,** national center for biotechnology information; **EPS,** exopolysaccharide; **BLAST,** basic local alignment search tool; **rRNA,** ribosomal ribonucleic acid; **CDV,** challenge dose virus; **HSV-I,** herpes simplex; **MEM,** minimal essential medium.

Christensen et al., 1985; Decho, 1990; Holmestrom and Kjelleberg, 1999). Many marine-exopolysaccharide producers already have been isolated including *Alteromonas*, *Pseudoalteromonas*, *Cyanothece*, *Pseudomonas*, *Vibrio* and *Zoogloea* (Lee et al., 2001; Annarita et al., 2010). The present work describes the isolation, identification, optimization of EPS production from a marine bacterium and explores the biological activities of the produced EPS.

MATERIALS AND METHODS

Bacterial strain

Samples were obtained from Huraghada, Red Sea, (Egypt). Seawater samples were collected in polypropylene bottles at about 8 meters depth, algae samples were collected at 1 m depth, sand samples were collected from seashore and from depth about 2 m, soft corals, hard corals as well as sponge samples were collected from depth about 16 meters. The solid samples were washed three times with sterile seawater to remove loosely attached bacteria. The sample was then placed in another 100 ml of sterile seawater and homogenized by shaking at 200 rpm for 15 min and a serial dilution was performed. Finally, 50 µl of the supernatant of each dilution was inoculated on: Marine agar 2216 E (Difco laboratories) (Oppenheimer and Zobell, 1952) and Glucose-yeast extract agar. Isolated microorganisms showing mucoidal growth on these media were further screened for EPS production by inoculation into shake flasks containing 50 ml of casein hydrolysate glucose broth consisting of (g/L): Casein hydrolysate 2.5; K_2HPO_4 4.0; $MgSO_4.7H_2O$ 0.7; $MnSO_4.7H_2O$ 0.05; and Glucose 30.0, dissolved in sterile sea water and adjusted at pH 7.0. Flasks exhibiting thickening of broth were tested for the presence of EPS by precipitation of the culture supernatant using 3 volumes ethyl alcohol 97%. Yield of EPS was expressed as weight (g/L). Bacterial isolate showing the highest yield of EPS was chosen for further characterization. The pure culture was preserved by freezing in 20% glycerol (v/v) at -70°C for long- term storage.

Cytological and biochemical characterization

Bacterial cell was examined by light microscope and transmission electron microscope. Automated Biolog's third generation system (BIOLOG, Inc. USA) was used to detect different metabolic, biochemical properties, and susceptibility to some chemicals and antimicrobial agents.

16S rDNA Gene sequencing

The bacterial chromosomal deoxyribonucleic acid (DNA) was extracted using UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA). 16S-rDNA was amplified by Polymerase chain reaction (PCR) (Mullis and Falloona, 1987) using universal forward primer 16F27 (5´- AGA GTT TGA TCC TGG CTC AG-3´) and reverse primer 16R1525 (5´- AAG GAG GTG ATC CAG CCG CA-3´) derived from *Escherichia coli* 16SrDNA sequence (Lane, 1991). The reaction mixture of 50 µl contained at least 100 ng of genomic DNA (in 10 mM Tris-HCl, pH 8), 0.2 µM of each primer and PCR Supermix High fidelity (Taq and Go, Promega, CA).The amplified 16S-rDNA product (466 bp) was sliced off from the agarose with sterile razor blade. The DNA was purified from the agarose by QIAquick Gel Extraction Kit (Qi agen, Valencia, CA) and sequenced on an ABI 377 automated sequencer using the PRISM Ready Reaction Kit (Applied BioSystems, Foster City, CA). Sequence data was compared with the non-redundant sequence data base at the National Center for Biotechnology Information (NCBI) using basic local alignment search tool (BLAST) for homology. The sequence was multiple aligned with 16S**,** ribosomal ribonucleic acid (rRNA) gene sequences of some representatives having different identity percentage using MEGA version 5 software. A phylogenetic tree was constructed by the neighbor joining method using the same software.

Optimization of fermentation parameters

Production of bacterial polysaccharides was achieved by fermentation in 250 ml shake flasks containing 50 ml casein hydrolysate glucose broth at 30°C. The flasks were inoculated with 2.5 ml of 48 h old bacterial culture adjusted to an optical density of 1.0 at 600 nm. The effect of different carbon sources including: galactose, fructose, lactose, sucrose, dextrin, glycerol, molasses and citric acid were tested. Different nitrogen sources including: meat extract, yeast extract, tryptone, peptone, ammonium sulphate, ammonium oxalate and sodium nitrate were also studied. The effect of NaCl concentrations on the EPS production using casein hydrolysate broth prepared with sterile distilled water was also examined. The effect of initial pH on EPS productivity was evaluated over pH range of 4 to 9, moreover the effect of the agitation rate and different fermentation periods were also studied. Fermentation parameters such as dry weight of EPS, the total carbohydrates (using the phenol-sulphuric acid method according to Fournier (2001), dry weight of cells as well as the relative viscosity of the culture filtrate (ratio between specific viscosity of broth and specific viscosity of water at 25°C in an Ostwald viscometer) were monitored.

Isolation and partial purification of the produced EPS

Crude broth was centrifuged at 9000 rpm for 30 min. The supernatant fluid was decanted and was slowly stirred with 3 volumes of cold ethanol 97%. The polysaccharide was then separated by centrifugation at 9000 rpm for 30 min at 4°C. The precipitated polysaccharide was dissolved in least amount of water and then trichloroacetic acid solution was added to give final concentration of 10%. The precipitated proteins were centrifuged out and excess trichloroacetic acid was removed by extraction with equal volumes of ether. The aqueous layer was then separated and dialyzed for 2 days against distilled water. The dialyzed solution was concentrated, under reduced pressure at 40°C, to half its volume and treated with 4 volumes of ethanol. The partially purified polysaccharide material was separated by centrifugation, washed with ethyl alcohol, ether and finally dried under vacuum at 40°C.

Chemical analysis and characterization of EPS

Qualitative examination of the hydrolysis products of EPS was performed by paper chromatography of the resulted hydrolyzate after complete acid hydrolysis and by using the solvent system: n-butanol-acetone-water (4:5:1, v/v) and aniline phthalate as spraying agent (Jayme and Knolle, 1956).

Lipid emulsifying test

Emulsifying activity was measured according to a modified method of Cooper and Goldenberg (1987). Oil was added to aqueous phase containing the EPS (in a ratio of 1:1, v/v) and agitated vigorously for 2 min. The oil, emulsion and aqueous layers were

measured every 24 h interval and emulsification index (E) was calculated according to the following equation:

Emulsification index = [Volume of emulsion layer / Total volume] × 100

Emulsion formed with sunflower oil using 0.01 and 0.1 % w/v of EPS was checked at different time intervals for stability.

Antiviral activity

0.1 ml of (10^3) concentration of challenge dose virus (CDV) were mixed with 0.1 ml of (10%) EPS. The mixtures were incubated at room temperature for one hour in sterile screw-capped vials. Non-treated virus infected control set was prepared by diluting 0.1 ml of (10^3) of virus suspension, with 0.1 ml of minimal essential medium (MEM). 12-well plates seeded with Vero cells (clone CCL-81) were washed twice with phosphate buffered saline and incubated at room temperature for 5 to 10 min. 0.2 ml of either test or control were inoculated. Negative control well of non treated cells was also included. Plates were incubated for one hour at 37°C to allow virus adsorption. Cell monolayers were washed twice with phosphate buffered saline, then overlaid with MEM agarose mixture and the experiment was carried out according to plaque reduction assay (Markland et al., 2000). The antiviral activity was defined as the percentage of plaque inhibition as follows.

% Plaque inhibition= [1-(Number of plaque in test / Number of plaque in control) × 100]

Fibrinolytic activity

This was determined according to a modified method of USP 28-NF 23 Pharmacopeia (2005) for the determination of anti-coagulation activity of heparin sodium. Briefly, to each of 13 × 100 mm test tube, 0.8 ml saline solution (0.89% w/v), 1 ml human plasma and 0.2 ml calcium chloride solution (1 % w/v) were added. After mixing, the tubes were placed in water bath at 37°C, and when clotting was complete, tubes are divided into three sets (each of 3 tubes) corresponding to negative control, standard and test. 1 ml of either saline solution, hemoclar preparation (2 mg/tube) (Pentosan Sulphuric Polyester a product of ClinMidy Paris, France), or the tested EPS sample (2 mg/tube) was added individually to each set of tubes. The lysis of the plasma clots at 37°C was recorded visually after 24 h. recognizing 5 grades (+1, +2, +3, +4 and +5), where +5 corresponds to full lysis.

RESULTS AND DISCUSSION

Marine bacteria have become increasingly popular and novel sources of EPSs. Although many known marine bacteria can produce EPSs, few of the produced EPSs are of biotechnological importance, so the search of EPSs that might have innovative applications is still of potential interest (Llamas et al., 2010). A total of 21 bacterial isolates collected from various marine samples and exhibiting mucoidal morphology on marine agar and glucose yeast extract agar media were inoculated into shake flasks containing 50 ml of casein hydrolysate glucose broth. Marine bacterial isolates were screened for their capacity to produce EPS. The highest yield of EPS (9.01 g/L) was obtained by a marine bacterium isolated from a sponge sample. The strain isolated from the sponge sample was Gram negative strictly aerobic, rod-shaped cells. Cells occur singly, cells are motile by means of a single polar flagellum. Biochemical characterization showed that, the bacterial isolate had positive oxidase, protease and beta-glucosidase activity. The isolated bacterium utilized many carbohydrates as a sole carbon source including lactose, fructose, glycerol, tween 40 and acetic acid (Table 1). The bacterium was resistant to fusidic acid, lincomycin, nalidixic acid and was sensitive to minocycline, rifamycin, niaproof 4, vancomycin and aztreonam and could be classified as slightly halophilic since it required 3 to 4% Na^+ ions for growth.

The phylogenetic analysis of 16S rDNA demonstrated that the bacterium belonged to the gamma subdivision of the *Proteobacteria* phylum and is closely related to *Pseudoalteromonas* sp. (Figure 1). The nucleotide sequence of the marine bacterial isolate has been submitted to the NCBI Database under accession number JF415135 and the name of *Pseudoalteromonas* sp. AM. This newly created genus has attracted significant interest for two reasons: First, *Pseudoalteromonas* species are frequently found in symbiotic association with eukaryotic hosts in the marine environment and studies of such associations will elucidate the mechanisms important in microbe-host interactions. Secondly, many of the species produce biologically active metabolites. During batch fermentation, *Pseudoalteromonas* sp.AM produced EPS during the log phase and increased along the stationary phase reaching its maximum concentration during this phase at the 7^{th} day of incubation. No significant increase in the production was observed after this period, hence during the stationary phase, dry weight of EPS, relative viscosity of the culture filtrate increased progressively (Figure 2). Recently it was found that most bacteria release the highest quantity of EPS in the stationary phase of growth, this finding might be justified by the competition occurring during the growth phase between EPS and cell- wall polymer biosynthesis (Sutherland, 1982), however there are microorganisms that produce the maximum amount of EPS during the exponential phase (Annarita et al., 2010). With the aim of improving EPS production from *Pseudoalteromonas* sp. AM, the influence of different culture parameters were studied. In that context, Kumar et al. (2007) reported that polysaccharide synthesis and yields largely depends on environmental and nutritional conditions. Production of EPS increased reaching its maximum level (9.8 g/L) as the initial culture pH ranged between 6 to 7.

However pH values higher or lower the optimal range resulted in lower growth and lower EPS yield. Relative viscosity was significantly increased when pH was shifted from 6 to 7 (Figure 3). Moreover, it was found that production of EPS increased substantially as the incubation changed from static to aerated condition, also

Table 1. Phenotypic properties of *Pseudoalteromonas* sp. AM. Computer-based analysis of the results of Biolog's third generation system microplates indicated that the isolated strain was closely related to *Pseudoalteromonas* sp.

CHARACTERISTIC	REACTION OF ISOLATE
PRODUCTION OF:	
Indole	-
Melanine dark pigment	-
Enzyme activity	
Nitrate reductase	-
Arginine dihydrolase	-
Urease	-
Oxidase	+
Hydrolysis of:	
Gelatine	+
Esculin	+
Agar	-

UTILIZATION AS SOLE CARBON SOURCE:

Control negative	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose
-	+	?	-		-	+	-	-
D-Raffinose	D-Lactose	D-Melibiose	β-Methyl D-Glucoside	D-Salicin	N-Acetyl D-Glucosamine	N-Acetyl-â-D-Mannosamine	N-Acetyl D-Galactoseamine	N-Acetyl Neuraminic Acid
-	+	-	-	-	-	-	-	-
Glucose -D	D-Mannose	D-Fructose	D-Galactose	3-Methyl glucose	D-Fcuose	L-Fucose	L-Rhamnose	Inosine
+	-	+	?	-	-	-	-	-
D-Sorbitol	D-Mannitol	D-Arabitol	Myo-Inositol	Glycerol	$_4$D-Glucose- 6 -po	$_4$D-Fructose- 6 -po	D-Aspartic Acid	D-Serine
-	-	-	-	+	-	-	-	-
Gelatin	Glycyl-L-proline	L-Alanine	L-Arginine	L-aspartic acid	L-Glutamic acid	L-Histidine	L-pyroglutamic acid	L-Serine
+	-	-	-	-	-	-	-	-
Pectin	D-Galacturonic Acid	L-Galactonic Acid Lactone	D-Gluconic Acid	D-Glucoronic Acid	Glucoronamide	Mucic Acid	Quinic Acid	D-Saccharic Acid
-	-	-	-	-	-	-	-	-
p-Hydroxy Phenyl acetic acid	Methyl Pyruvate	D-Lactic Acid Methyl Ester	L-Lactic Acid	Citric Acid	α-Keto - Glutaric Acid	D-Malic Acid	L-Malic Acid	Bromo Succinic Acid
-	-	-	?	?	-	-	-	-
Tween-40	γ-Amino Butyric Acid	α-Hydroxy Butyric Acid	β-Hydroxy D,L Butyric Acid	α-Keto Butyric Acid	Acetoacetic Acid	Propionic Acid	Acetic Acid	Formic Acid
+	-	-	?	-	-	-	+	-
L-Arabinose	D-Mannose	Capric acid						
-	-	-						

? intermediate, + growth, - no growth.

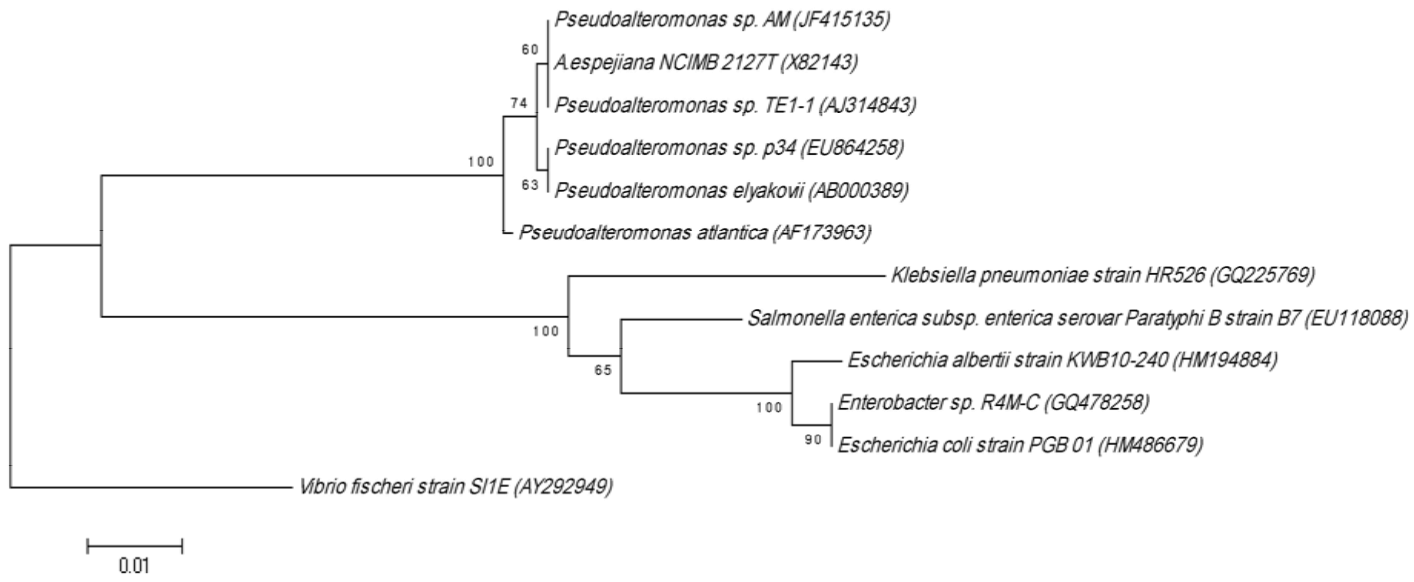

Figure 1. Phylogenetic relationships among *Pseudoalteromonas* sp. AM and selected gamma-proteobacteria showing the position of the marine bacterium isolate among some representatives having different identity percentage based on almost complete sequences of 16S rDNA. Genbank accession numbers are indicated for each strain. The percentage numbers at the nodes indicate the levels of bootstrap support for the branch point in the topology shown based on neighbor-joining analyses of 1000 resampled data sets.

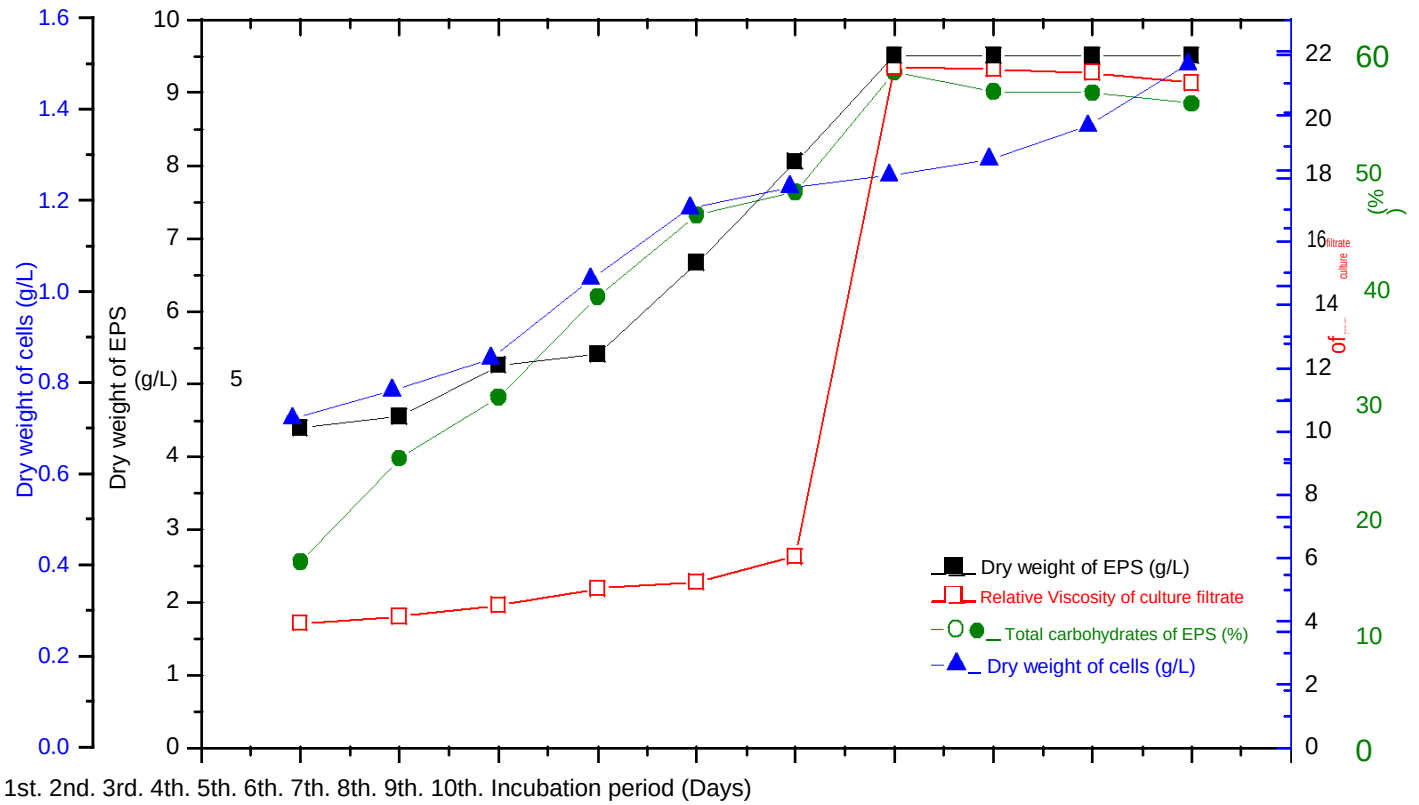

Figure 2. Production of EPS by *Ps eu do al t er omon as* sp. AM at various fermentation periods. Results are average of three replicate flasks per experiment.

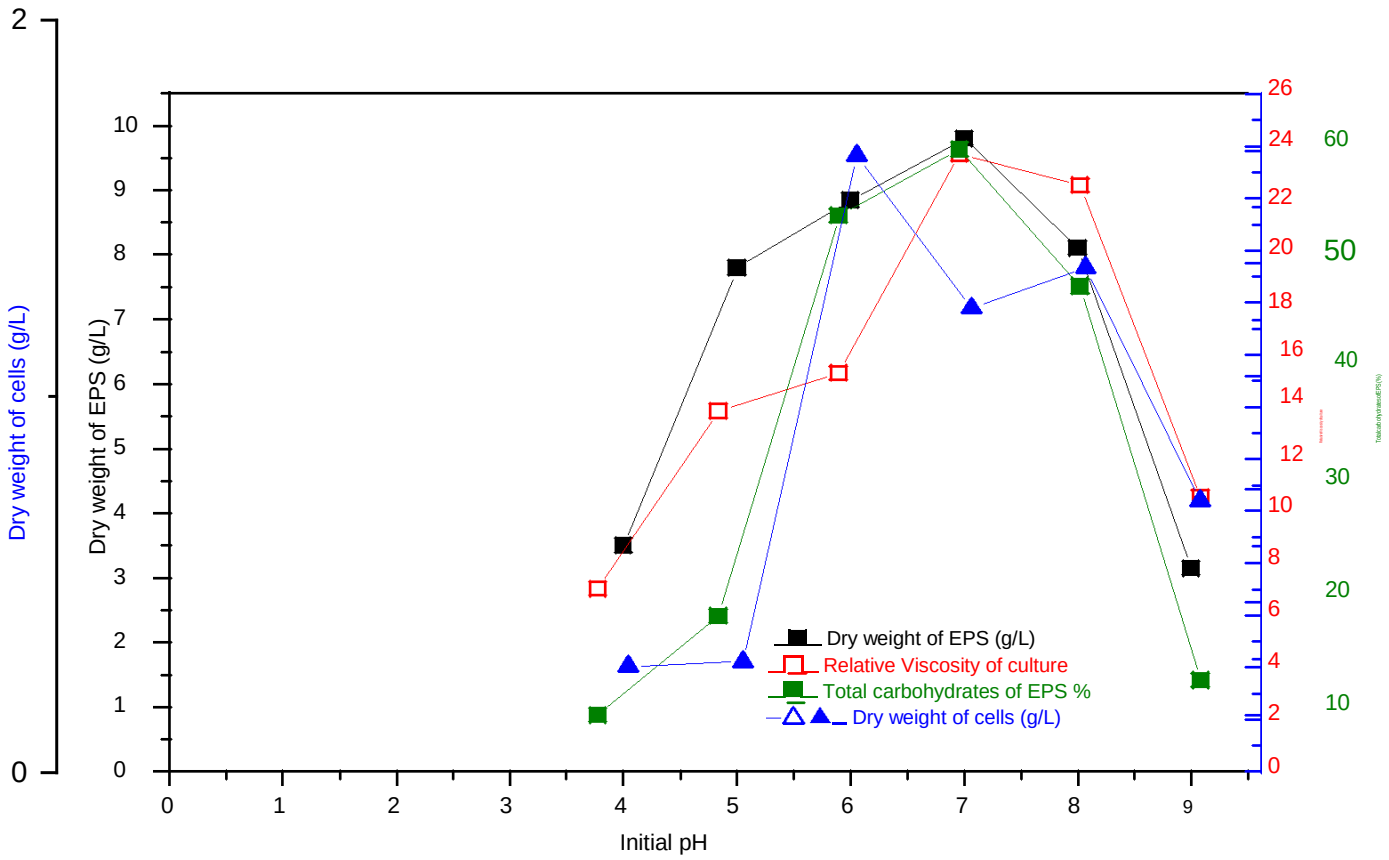

Figure 3. Effect of Initial pH on the production of EPS by *Ps eu doal t er omo nas* sp.AM. Results are average of three replicate flasks per experiment.

biomass increased progressively as the agitation speed increased reaching their maximum values at 150 rpm. However, upon further increase of the agitation speed to 200 rpm, EPS yield and biomass remained constant (Figure 4). In the present work, among different sugars tested, glucose exhibited the highest EPS yield of 9.8 g/L followed by glycerol. Galactose, lactose, molasses. Sucrose and fructose gave nearly the same yield of 3 g/L, while dextrin and citric acid showed the least EPS yield (Data not shown). This observation was also reported by Lu et al. (2007) who reported that glucose has been shown to be the best carbon source for EPS production and energy generation. Concerning different tested nitrogen sources, it was observed that inorganic nitrogen sources weakly supported growth, polysaccharide production and resulted in a substantial decrease in biomass and EPS yield to 2 g/L, on the other hand organic nitrogen sources favors both growth and polysaccharide production. These results were in agreement with Farres et al. (1997) and might be explained by Pomeroy (1974) who confirmed that the primary role of heterotrophic bacteria is classically considered to be the decomposition and mineralization of dissolved particulate organic nitrogen. Amongst tested organic nitrogen sources, meat extract was found to be the most suitable nitrogen source resulted in a slightly higher productivity of 10.51 g/L, followed by yeast extract, as compared to casein hydrolysate in the control medium, while tryptone and peptone showed lower EPS productivity (Data not shown). Upon studying the effect of NaCl concentration, it was revealed that the marine bacterium *Pseudoalteromonas* sp. AM grew only in presence of NaCl.

When NaCl concentration was increased from 10 to 30 g/L, cell growth and EPS yield increased as well, however further increase in NaCl concentration to 40 g/L showed nearly the same yields (Figure 5). Other *Pseudoalteromonas* sp. was shown to produce EPSs under similar condition of NaCl concentration (Egan et al., 2001; Choi et al., 2009). Under optimum growth conditions, the yield of EPS produced in our work was higher compared to other yields reported in literature (Annarita et al., 2010). The purification process was accompanied with considerable decrease in the yield of EPS from 11.2 to 7.52 g/L and an increase in total carbohydrates content of EPS from 61.2 to 70.25% as a response for this purification. Identification of the building units of the present polysaccharide was achieved after

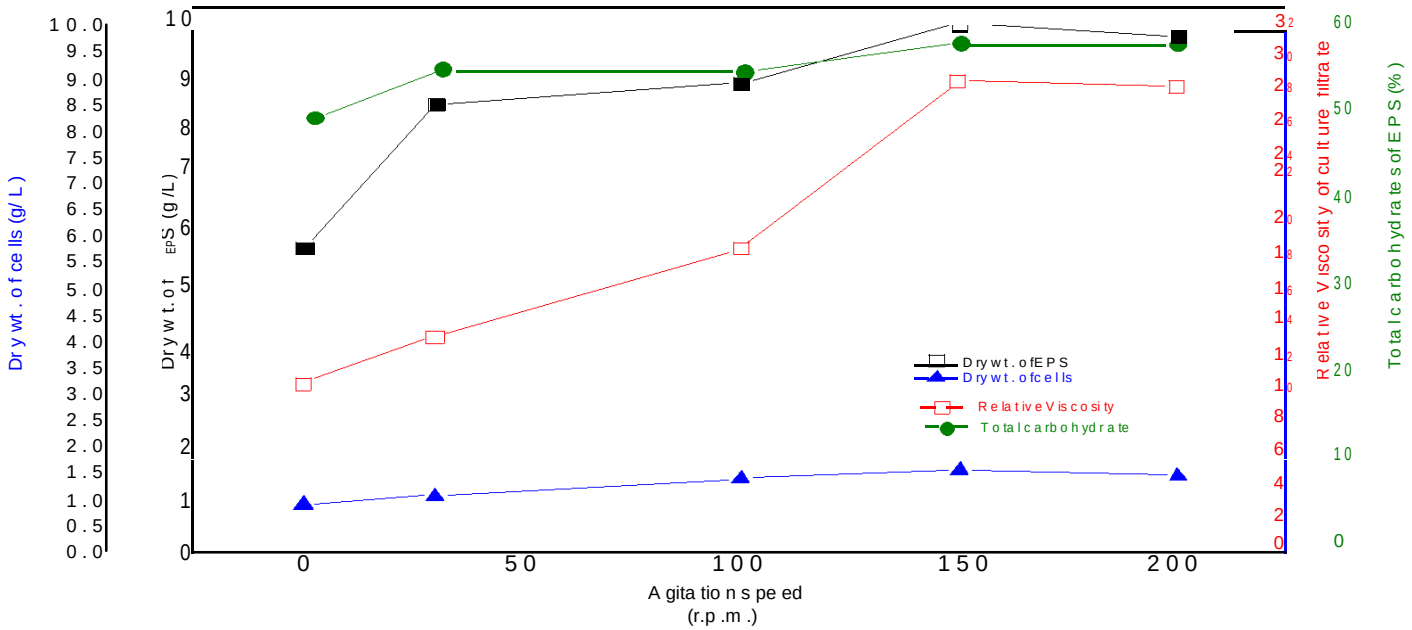

Figure 4. Effect of agitation speed on EPS production by *Pseudoalteromonas* sp.AM. Results are average of three replicate flasks per experiment.

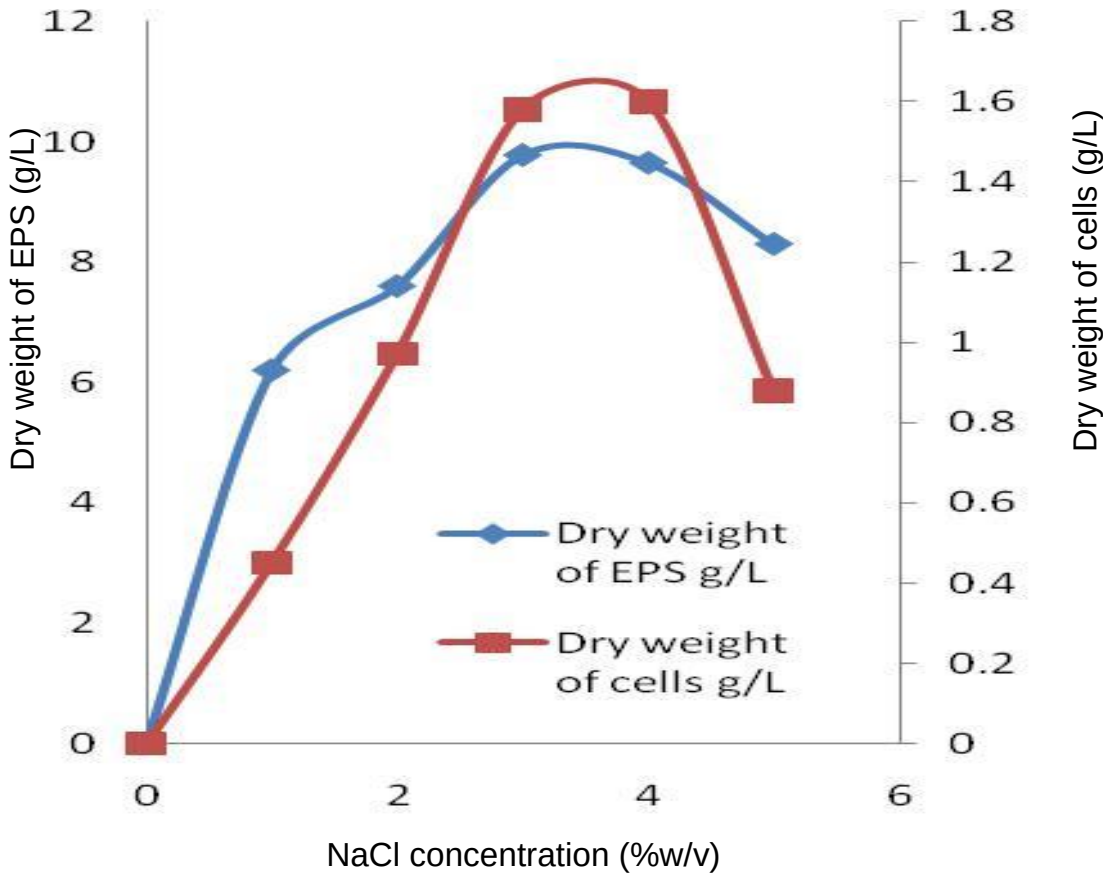

Figure 5. Effect of Different concentrations of NaCl on the production of EPS by *Pseudoalteromonas* sp.AM. Results are average of three replicate flasks per experiment.

acid hydrolysis of this polymer followed by paper chromatography of the resulted hydrolysates. The results indicated the presence of glucose as sole sugar constituent of the studied bacterial polysaccharide and suggested that the produced EPS is a homopolysaccharide. This result was supported by Perepelov et al. (2004) and Qin et al. (2007) who examined the structure of polysaccharide obtained from *Pseudoalteromonas* sp. and found that it was formed mainly of glucose, also Qin et al. (2007) reported that *Pseudoalteromonas* sp. SM9913 produced EPS, its structure was a linear chains of {alpha}-(1, 6) glucose residues. However, the single hydrolysis procedure followed might be not sufficient to reveal the exact composition of the EPS owing to the structural complexity of most EPSs and current study of the full structure and chemical composition of the EPS is under progress.

It became an interest to examine various biological activities of the produced EPS. The EPS efficiently emulsified sunflower oil even in very low concentration (0.01% w/v) and emulsion showed an exceptional stability. The emulsifying activity was 78% and remained stable for 13 days. Lower emulsion index of 68% was reached on the 14^{th} day, but it was found that increasing the concentration of the partially purified polysaccharide solution to 0.1%w/v showed the same emulsion index of 78% for 22 days as it was allowed to stand at 28°C. The emulsifying ability of the EPS was comparable to that of natural gums and superior to those produced from various microorganisms (Abbasi and Amiri, 2008; Llamas et al., 2010). Fibrinolytic activity of the presently studied bacterial polysaccharide was investigated and compared to that of a standard preparation of the fibrinolytic drug "Hemoclar" (pentosan sulphuric polyester). The investigated polysaccharide preparation exhibited a score of +3 compared to +4 for pentosan sulphuric polyester. Moreover, one of the most important novel applications of the microbial EPS produced is its remarkable antiviral activity against HSV1 resulting in inhibition of 60.3% in the number of plaques when HSV-I was treated with equal volume of 10% EPS. On the other hand it showed neither activity toward COX B4 nor toward HAV. This finding could be supported by (Arena et al., 2009) who showed for the first time that EPS obtained from a marine bacterium could hinder HSV-2 replication.

The fibrinolytic and antiviral activity of the produced EPS suggest the presence of a sulfate content, since various reported medicinal use were assigned for sulfated EPSs including; anticoagulant, antiangiogenic, antiproliferative and antiviral activities. In that context, the antiviral activity of sulfated EPSs was attributed to their ability to inhibit virus particle adsorption to host cells (Llamas et al., 2010). Finally, it is worthy to add *Pseudoalteromonas* sp. AM is a promising candidate for the production of EPS with interesting novel biological activities. Further investiga-tions will be carried out to characterize the EPS and to explore other possible applications.

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