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STRATEGIES FOR ENGINEERING OF MARINE BACTERIA PRODUCING POLYSACCHARIDES

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ABSTRACT

Exopolysaccharides (EPS) are used in many industrial applications. Some microorganisms have the capability to excrete them with interesting properties. A marine bacterium, which has the ability to produce exopolysaccharide (EPS), was isolated from marine sediments, Huraghada, Red Sea, Egypt. Phenotypic characterization demonstrated that, the strain is Paracoccus sp. KSI (U58015), based on 16S (rRNA) sequence. The maximum production of EPS was done at pH 7 with agitation of 150 rpm after seven days, in a fermentation medium containing glucose, as carbon source, and 3% NaCl. The emulsifying activity of purified EPS, showed a remarkable stability of 69% that stable for 11days. The study indicated that the produced EPSs is a homopolysaccharide due to the occurrence of glucose as a sugar component. Fibrinolytic activity of the selected bacterium was exhibited a score of +2 compared to +3 for pentosan sulphuric polyester according to a standard preparation of pentosan sulphuric polyester. The EPS showed a remarkable antiviral activity of 52.77% against (HBV). This study indicates the capability of Paracoccus sp. KSI to produce EPS with biological activities that will be efficient to explore other applications.

Keywords: Paracoccus sp.KSI, antiviral activity, fibrinolytic activity, biological activities

INTRODUCTION

There has been renewed interest in marine microorganisms and has gained considerable attention because they have developed various strategies to live in extreme conditions, such as metabolic path-ways adaptation (Annarita et al., 2010). Marine bacteria excrete polysaccharides to protect themselves against extreme variations such as temperature, pH and salinity (Kelecom, 2002). Bacterial Polysaccharides have a lot of remarkable properties that used in many industries such as food, industrial waste treatment, pharmaceutical, health industry petroleum, and mining industries (Okutani, 1982, 1984, 1985). Bacteria have the capability to increase the rate of growth so they offer an advantage to increase the fermentation production of polysaccharides (Boyle and Reade, 1983). These polymers produced are not affected by climatic events, marine pollution or crop failure. They are highly reproduced from another (Christensen et al., 1985). Thus, these biotechnological resources systems are valuable (Decho, 1990; Holmstrom and Kjelleberg, 1999). Bacteria producing polysaccharides have a great variation of chemical properties that are not found elsewhere, they are always characterized by their superiority in polysaccharides production more than

other sources. There are many marine bacteria have the ability to produce exopolysaccharide such as *Pseudoalteromonas, Vibrio Cyanothece and Alteromonas* (Lee, 2001). The present study describes the isolation and identification of a marine bacterium, which has the ability to produce exopolysaccharide and explores the optimum parameters and the biological applications of the EPS produced.

MATERIAL AND METHODS

Isolation of Bacterial strain

Sediment samples were estimated from ten sites along the coast of Huraghada, Red Sea, Egypt. One gram of each sample was diluted with 10 ml of sterilized seawater and serial dilutions were performed. Finally, 50 µl of each dilution was inoculated on: Marine agar medium and Glucose-yeast extract agar (Oppenheimer and Zobell, 1952). The bacterial isolates were screened for EPS production by inoculation into 50 ml of casein hydrolysate glucose broth(g/L) ($MgSO_4.7H_2O$ 0.7, K_2HPO_4 4.0, $MnSO_4.7H_2O$ 0.05, Casein hydrolysate 2.5 and Glucose 30.0) dissolved in sterile sea water at pH 7.0. Bacterial isolate that produced a significant yield of EP was stored for further analysis.

Biochemical characterization

The potential bacterial strain was identified by using Biolog's third generation system to detect the biochemical properties and some antimicrobial agents.

16S rRNA Gene sequencing

The potential strain which showed significant EPS production, was identified by 16s rRNA sequencing. The PCR Clean-Up of the resulted product was done by using Gene JET™ PCR Purification Kit at Sigma Scientific Services Company, Egypt. The sequencing of the product was made by using ABI 3730xl DNA sequencer by the GATC Company. Genotypic characterization was done using 16S sequence analysis. Sequences of rRNA genes were obtained from (NCBI) database (Hall, 1999).

Optimization of fermentation parameters

Polysaccharides bacterial production was studied at variant carbon sources such as glycerol, fructose, galactose, lactose, molasses, sucrose, citric acid and dextrin and variant nitrogen sources such as yeast extract, peptone ,meat extract, tryptone, ammonium oxalate, sodium nitrate and ammonium sulphate. Also, the effect of variant NaCl concentrations and pH, in a range of 4 to 9, on EPS production was examined. The effect agitation of the fermentation periods was also studied. Moreover, fermentation parameters including dry weight of cells ,the relative viscosity and , dry weight of EPS, the total carbohydrates (Lane, 1991; Fournier, 2001), were examined.

Isolation of EPS

The broth was centrifuged at 9000 rpm for 30 min then added to ethanol 97%. The polysaccharide was then separated by using the centrifuge at 8000 rpm for 45 min at 4°C. Some droplets of water were added to dissolve the resulted polysaccharide then trichloroacetic acid solution was added to reach 10% final concentration. The reslulted proteins were centrifuged and excess trichloroacetic acid was eliminated by extraction with ether by equal volumes. The purified polysaccharide material was separated by centrifugation, washed with ethyl alcohol, ether and dried at 40°C.

Characterization of EPS

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

Lipid emulsifying test

A modified method was used for measuring the emulsifying activity (Cooper and Goldenberg BG, 1987). Tested oil was added to aqueous phase containing exopolysaccharide by a ratio of 1:1(v/v) and agitated for 2 min. Every 24 hours, the oil, emulsion and aqueous layers were measured then emulsification index (E) was estimated according to the following equation:
E = (Volume of emulsion layer / Total volume) × 100
The emulsion formed, by mixing of 0.01(w / v) corn oil with 0.1% (w / v) of EPS, was checked at different times for stability

Antiviral activity

Hepatitis B virus (HBV), human cytomegalovirus (HCMV), bovine viral diarrhea virus (BVDV), Human T-lymphotropic virus-I (HTLV-I) (10^3) concentration were mixed with of (10%) EPS in a ratio of 0.1: 0.1 (v/v), then incubated at 25^0C for one hour. The plates, were inoculated with Vero cells, were washed by using phosphate buffered saline and incubated at 25^0C for ten min. These plates were inoculated and incubated for 1 hour at 37°C to let the virus to be adsorbed. The cell monolayers were washed two times with phosphate buffered saline, then covered with MEM agarose mixture and the experiment was approved by using plaque reduction assay
(Markland et al., 2000).The inhibition percentage of plaque was measured as the following:
% Plaque inhibition= [1-(Number of plaque in test /Number of plaque in control) × 100]

Fibrinolytic activity

Anti-coagulation activity of heparin sodium was determined according to a modified method (USP 28-NF 23.Pharmacopoeia, 2005). A mixture of 1 ml human plasma, 1 ml saline solution (1% w/v) and 0.2 ml calcium chloride solution (1 % w/v) were added, to each test tube, They were placed in water bath at 37°C, and when clotting was complete, they are

divided into three sets; negative control, standard and test. 1 ml of either saline solution, hemoclar preparation (2 mg/tube), or the tested EPS sample (2 mg/tube) was added individually to each set of tubes. The lysis of the plasma clots was observed after 24 h at 37°C.

RESULTS AND DISCUSSION

Marine microorganisms such as bacteria have become very important in the production of sources of EPSs which are of biotechnological importance, are of vital importance to biotechnology, so the research of EPSs may have innovative applications is still of potential benefit (Llamas et al., 2010). Seventy two marine bacterial isolates (MB) were collected from sediment samples and were screened for their cabability to produce EPS. Biochemical characterization was done for a marine bacterium isolate (MB59) that showed the highest yield of EPS (8.11 g/L) (Table 1). The nucleotide sequence 16S rDNA of the marine bacterial isolate (MB59) is closely related to *Paracoccus sp.KSI* with an accession number of *U58015* according to the NCBI Database. *Paracoccus sp.KSI* produced EPS at the 7th day of incubation without any production after this period. Thus, the dry weight of EPS was observed to be directly proportional to its relative viscosity of the culture filtrate (Figure1). This result might be due to the competition between biosynthesis of cell wall polymer and EPS (Sutherland, 1982; Sutherland, 1972). Different parameters were studied to improve EPS production from *Paracoccus sp.KSI* (Kumar, 2007). The Production of EPS increased reaching its maximum level (8.7 g/L) as the initial culture pH ranged between 6 to 7. Conversely, pH values lower than 6 or higher than 7 showed lower EPS yield. When pH was ranged from 6 to 7, relative viscosity was increased (Figure 2).

Table 1: Biochemical and sugar utilization tests of bacterial strain KSI

Characteristics	Bacterial strain KSI
Catalase	–
Oxidase	++
Urease	+
Citrate utilization	–
Nitrate reduction	++
Glucose	++
Adonitol	++
Lactose	–
Sorbitol	++
Esculin hydrolysis	–
Xylose	–
Maltose	++
Fructose	++
Galactose	–
ONPG	–
Raffinose	–
Trehalose	++

Melibiose	−
Sucrose	++
L-Arabinose	++
Mannose	++
Inulin	++
Sodium gluconate	++
Glycerol	++
Salicin	++
Dulcitol	++
Inositol	++
Mannitol	+
Arabitol	++
Erythritol	+
Malonate utilization	+
α-Methyl-D-glucoside	++
Rhamnose	+
Cellobiose	+
Melezitose	+
α-Methyl-D-mannoside	−
Xylitol	−
D-Arabinose	+
Sorbose	−

++: positive, +: weak, and −: negative.

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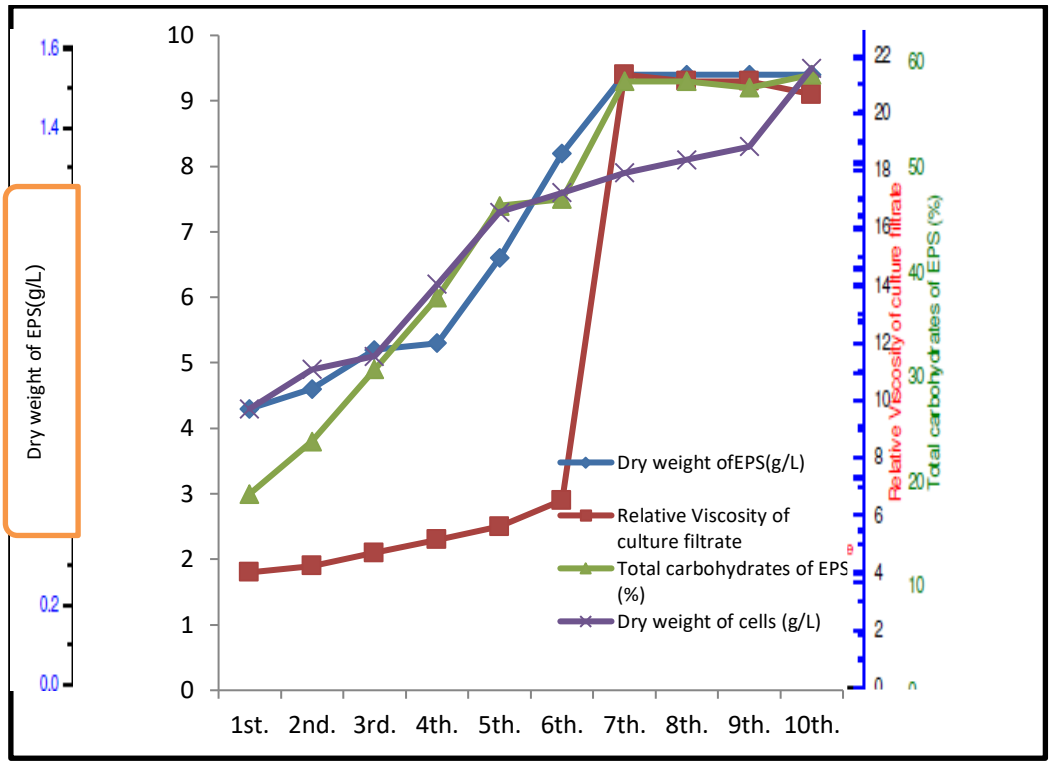
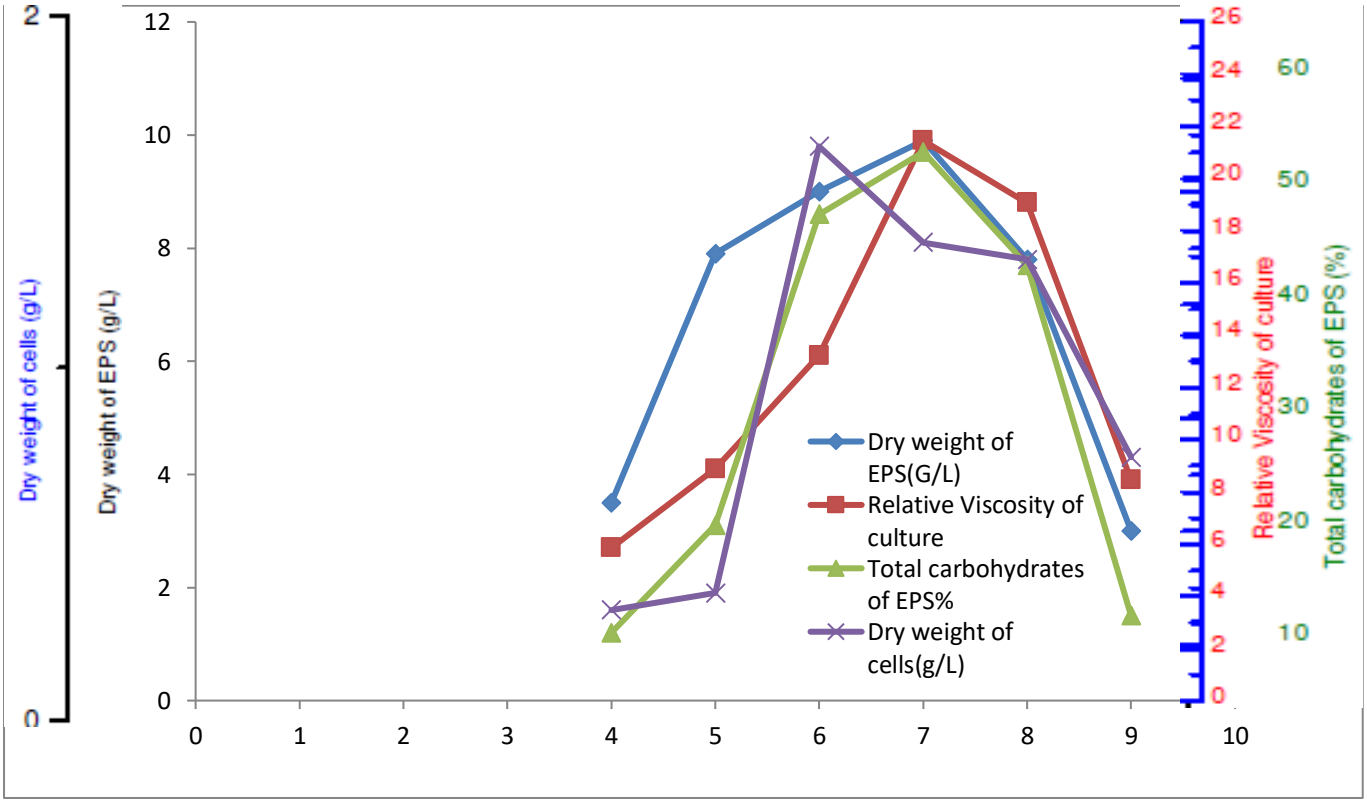


Figure 1. Production of EPS at various Incubation periods.



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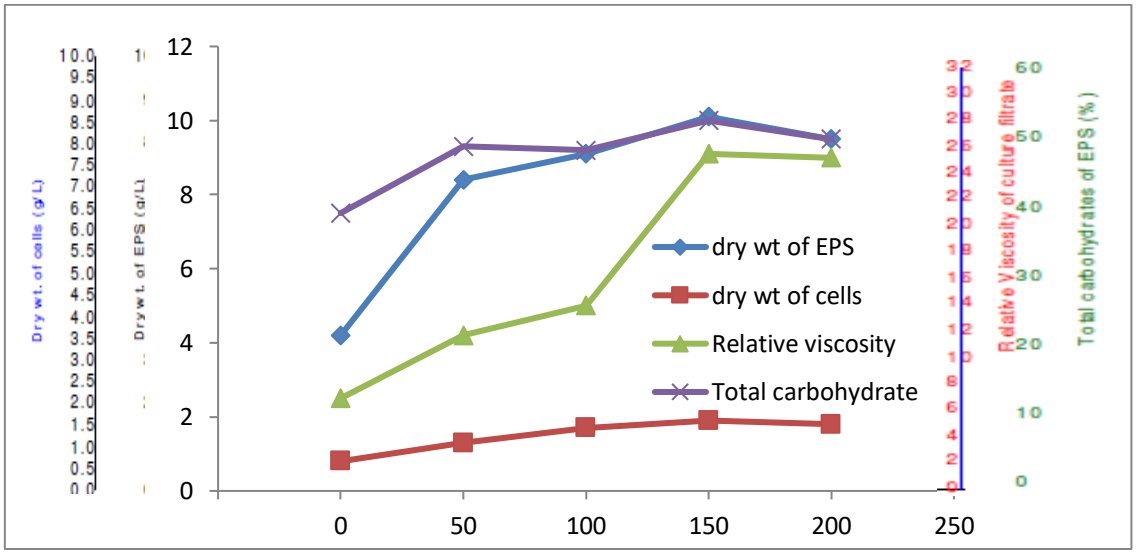
Initial pH

Figure 2. Effect of Initial pH on the production of EPS.

The maximum EPS yield and biomass were at agitation speed of 150 rpm (Figure 3). By increasing the speed of agitation up to 150 rpm, they remained constant. Among various sugars tested, glucose showed the highest yield of EPS (9.5 g/L) (Figure 4), while molasses, lactose, glycerol. Galactose, fructose and Sucrose gave nearly a yield of 2.3, 3.9, 3, 2.7, 4 and 2.9 g/L, respectively, finally, citric acid and dextrin showed the least EPS yield of 1.1 and 1.3 g/L, respectively. Many studies confirmed that glucose has been shown the same results (Lu et al., 2007). By using different tested nitrogen sources, it was observed that organic nitrogen sources were suitable for polysaccharide production and growth but inorganic nitrogen sources were weakly supported them, resulted in a decrease in EPS yield to 1 g/L (Farres et al., 1997; Pomeroy, 1974). Yeast extract, as organic nitrogen source, showed a maximum productivity of 9.61 g/L(Figure5), while meat extract , tryptone, casein and peptone showed lower production of EPS . By using different NaCl concentration, it was observed that EPS yield cell and growth increased when NaCl concentration was increased from 10 to 30 g/L. Further increase in NaCl concentration to 40 g/L, showed closely yields (Figure6). Similar reports confirmed the same results (Egan, 2001; Choi, 2009). A considerable increase in total carbohydrates content of EPS from 60.3 to 71.25% decrease in the yield of EPS from 10.1 to 7.41 g/L during the purification process . After acid hydrolysis of EPS, identification of the building units of them was estimated by paper chromatography.



Agitation speed (rpm)

Figure 3. Effect of agitation speed on EPS production by *Paracoccus sp.KSI*.

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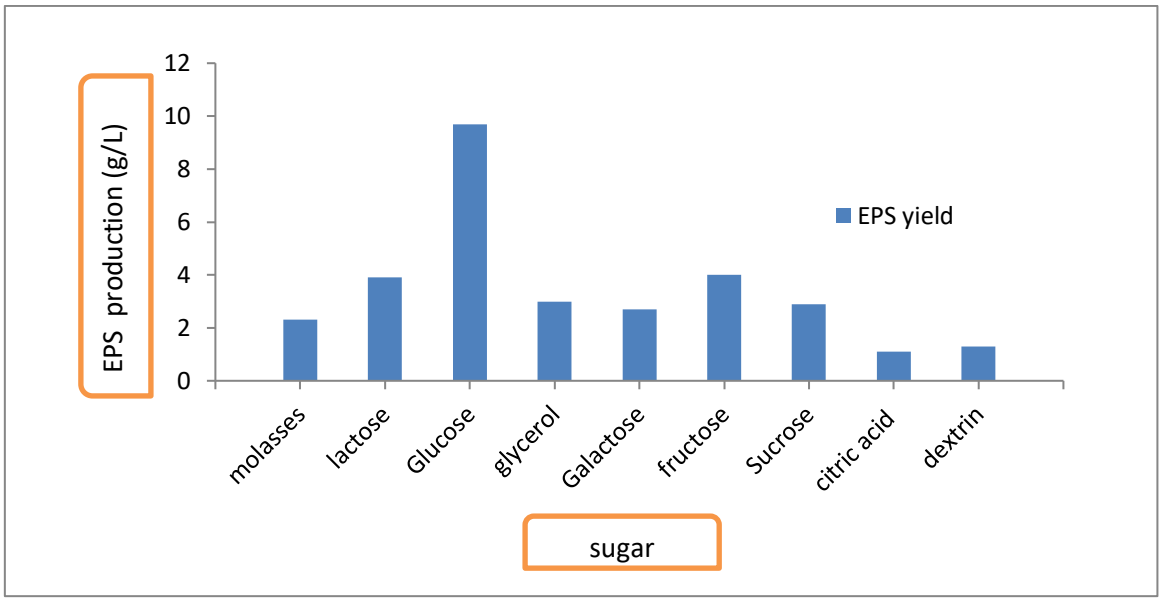

Figure 4. Effect of different sugars on EPS production by *Paracoccus sp.KSI*

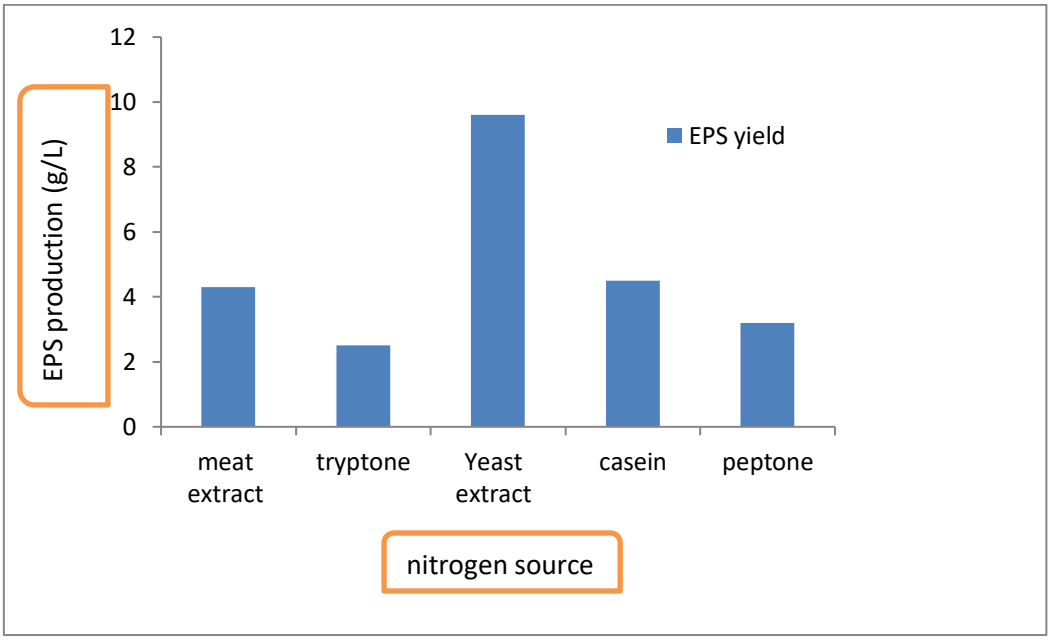

Figure 5. Effect of organic nitrogen sources on EPS production

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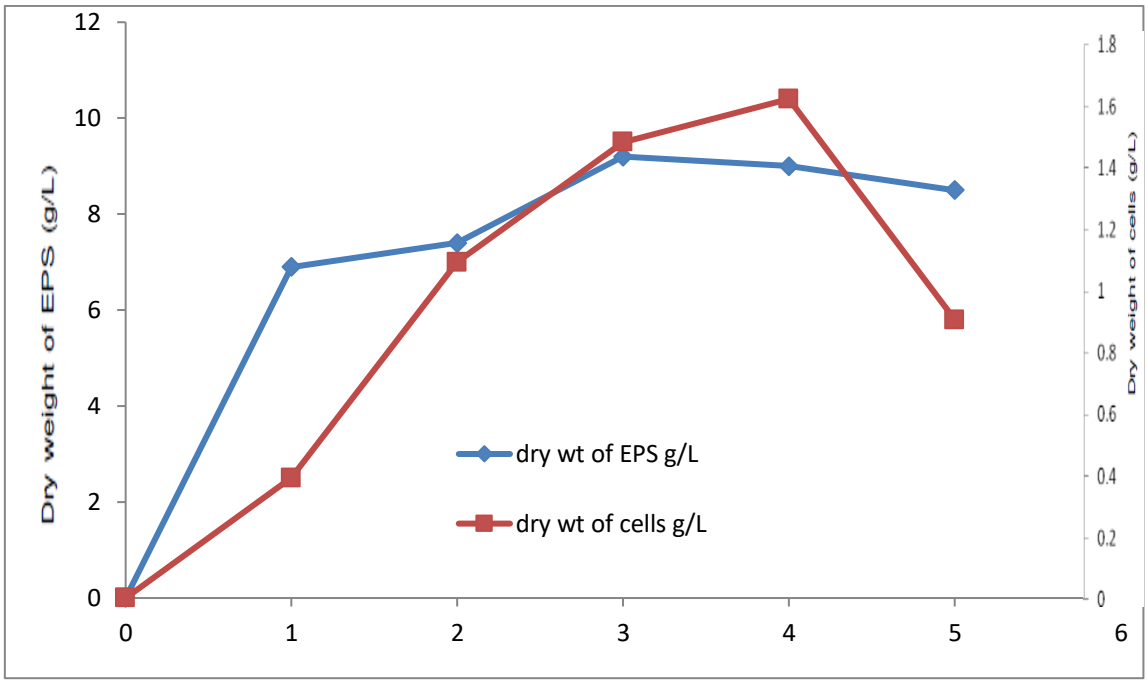

Figure. 6. Effect of Different concentrations of NaCl on the production of EPS

The study indicated that the produced EPSs is a homopolysaccharide due to the presence of glucose as sole sugar constituent. This result was supported by similar reports (Perepelov et al., 2004; Qin et al., 2007). The efficiently of EPS emulsified corn oil showed a remarkable stability of 69% that stable for 11days. Similar reports of the emulsifying ability of the EPS were closely to this result (Abbasi and Amiri, 2008; Llamas et al., 2010). Antiviral activity of bacterial polysaccharide showed a remarkable inhibition of 52.77% against HBV resulting in the number of plaques when 10% EPS was treated with HBV by a ratio of 1:1 (v/v). On the other hand, it showed neither activity toward (HCMV), (HTLV-I) nor (BVDV). Similar reports showed the same result (Arena, 2009). In the present study, fibrinolytic activity of bacterial polysaccharide was exhibited a score of +2 compared to +3 for pentosan sulphuric polyester according to a standard preparation of pentosan sulphuric polyester. The fibrinolytic and antiviral activities of EPS produced from marine bacteria were used for their ability to inhibit virus particle adsorption to host cells (Llamas et al., 2010). This study indicates the ability of *Paracoccus sp. KSI*. to produce EPS with biological activities that will be efficient to explore other biotechnological potential.

CONCLUSION

This study indicates the capability of *Paracoccus sp.KSI* to produce EPS with biological activities. The maximum production of EPS was done at pH 7 with agitation of 150 rpm after seven days, in a fermentation medium containing glucose, as carbon source, and 3% NaCl. The emulsifying activity of purified EPS, showed a remarkable stability of 69% that stable for 11days. Fibrinolytic activity of the selected bacterium was exhibited a score of +2 compared to +3 for pentosan sulphuric. The EPS showed a remarkable antiviral activity of 52.77%

against (HBV). The study showed that the produced EPS will be efficient to explore more applications in the future.

Acknowledgments: Sincerely thanks to Marine Microbiology Lab, National Institute of Oceanography and Fisheries, Alexandria, Egypt and Microbiology and Immunology Lab (SIN). for encouragement, facilities and support.

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