

Optimization of Cultural Conditions for Production of Exopolysaccharide by *Halomonas marina* HMA 103 under Batch-Culture

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Abstract: A moderately halophilic bacterium, *Halomonas marina* HMA 103 (MTCC 8968) accumulating intracellular polyesters also produced significant amount of exopolysaccharides (EPS) under batch-culture. Production of EPS by the bacterium was more or less parallel with the growth in modified basal synthetic medium. During growth, it produced 0.46 g/L of EPS after 50 h of incubation and was accompanied by the complete utilization of glucose from the medium. EPS production was maximum in 2% (w/v) glucose followed by sucrose and maltose and was positively influenced by yeast extract, beef extract and peptone. Optimum concentrations of sulfate and phosphate for EPS production were 2.4% (w/v) and 0.03% (w/v) respectively. High concentration (10% w/v) of NaCl stimulated both growth and EPS production by *H. marina* HMA 103. Characteristically, this EPS is a heteropolymer of glucose and mannose and the Fourier Transform Infrared (FTIR) spectrum 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. Further, the detailed physico-chemical analysis will indicate the significance and potential importance of this EPS in biotechnology.

Keywords: *Halomonas*, Moderately Halophilic Bacteria, Exopolysaccharides, Solar Saltern, Batch-Culture

Introduction

Moderately halophilic bacteria that require 3-15% NaCl for satisfactory growth (Kushner, 1988) are widely distributed throughout the hypersaline environment and have been recognized as valuable sources of new biomolecules including exopolysaccharides (Margesin and Schinner, 2001). These exopolysaccharides (EPS) play important role in the attachment of bacteria with substrates, development of biofilms, adsorption of nutrients, conservation of enzymes, defense against attack and protection against changes in physico-chemical conditions (Wotton, 2004). In addition, EPS find applications in the field of pharmacy, food, cosmetics and petroleum industries (Sutherland, 2001; Flemming and Wingender, 2001).

Extensive search for EPS with novel properties have described several species of moderately halophilic

bacteria belonging to the genus *Halomonas*. Two of them, *Halomonas eurihalina* (Bejar *et al.*, 1998) and *H. maura* (Bouchotroch *et al.*, 2001) have produced technologically viable quantities of extracellular polysaccharides with unique chemical composition and functional properties (Quesada *et al.*, 2004). Amongst many others, novel species viz. *H. ventosae* (Martínez-Cánovas *et al.*, 2004), *H. anticariensis* (Martínez-Cánovas *et al.*, 2004) and *H. almeriensis* (Martínez-Checa *et al.*, 2005; Llamas *et al.*, 2012) deserve special mention for synthesizing copious amounts of exopolysaccharides in culture.

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 (Laws and Marshall, 2001;

Pal and Paul, 2008). Further, the fermentation conditions play important role in determining the conformation, molecular mass, monosaccharide ratios and functional properties of the EPS (Sutherland, 1994; Finore *et al.*, 2014). But until recently, there have been very few studies on the effect of nutrients and cultural conditions on the production and composition of EPS of moderately halophilic bacteria (Bouchotroch *et al.*, 2000; Arias *et al.*, 2003).

Halomonas marina HMA 103 (MTCC 8968), a moderately halophilic bacterium isolated in this laboratory from a hypersaline soil collected from solar salterns of coastal Odisha, India has been reported to produce significant amount biodegradable bioplastics, poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Biswas *et al.*, 2009). Along with accumulation of these intracellular polyesters, the isolate also produced substantial amount of exopolysaccharides. In this article, we report the influence of nutritional and environmental conditions for the production of exopolysaccharide by *H. marina* HMA 103 under batch-culture experiments.

Materials and Methods

Bacterial Strain and Cultural Conditions

Moderately halophilic bacterium, *H. marina* HMA 103 (MTCC 8968) previously isolated from multi-pond solar salterns of Odisha, India (Biswas *et al.*, 2009) was used throughout the present study. The strain was maintained on Ventosa's agar medium (Ventosa *et al.*, 1998) by regular subculturing at an interval of 30 days. The medium contained (g/L): MgSO₄.7H₂O, 1.0; CaCl₂.2H₂O, 0.36; KCl, 2.0; NaHCO₃, 0.06; NaBr, 0.23; FeCl₃, 0.005; peptone, 5.0; yeast extract, 10.0; glucose, 1.0 and NaCl, 100.0 (pH 7.2).

Growth and Production of Exopolysaccharide

Modified basal synthetic medium (Lillo and Rodriguez-Valera, 1990) which contained (g/L) NH₄Cl, 1.0; KH₂PO₄, 0.3; FeCl₃, 0.005; NaCl, 100.0; MgCl₂.6H₂O, 16.0; MgSO₄.7H₂O, 24.0; CaCl₂.2H₂O, 1.0; KCl, 5.0; NaHCO₃, 0.2; NaBr, 0.5; glucose, 10.0 and yeast extract, 1.0 (pH 7.2) was used for growth and EPS production. The medium (20 mL/100 mL Erlenmeyer flask) was inoculated with 1.0 mL of freshly prepared inoculum in Ventosa's medium and incubated at 30°C on rotary shaker (120 rpm). Growth was determined by measuring cell dry weight at regular interval. The cell mass was harvested by centrifugation (10,000xg, 10 min, 20°C), washed twice with normal saline, cell pellet transferred to pre-weighed aluminum cups and dried to constant weight at 80°C. The

supernatant was cooled to 4°C and the soluble EPS was precipitated with double volume pre-chilled acetone, kept overnight at 4°C and recovered by centrifugation (12,000xg, 4°C, for 20 min). The cell bound EPS was extracted with 2% EDTA and precipitated with chilled acetone as usual. The soluble and bound EPS were pooled and dissolved in known volume of distilled water prior to quantification.

Purification of Exopolysaccharide

The pooled EPS dissolved in distilled water was subjected to dialysis in sterile water for 72 h at 4°C with regular change of dialysate. On completion of dialysis, the EPS was again treated with chilled acetone at 4°C, the precipitate was collected by centrifugation (12,000xg, 4°C, 20 min.) as the partially purified EPS and lyophilized at -56°C to obtain white powdered polymer.

Estimation of Exopolysaccharide

EPS estimation was carried out following the phenol-sulphuric acid method (Dubois *et al.*, 1956) using glucose as the standard. EPS obtained from 1.0 mL of fermented broth was dissolved in 1.0 mL of distilled water. To this 1.0 mL of 5% (w/v) phenol was added, mixed thoroughly and incubated for 5 min. Following this, concentrated sulphuric acid (5.0 mL) was purged in using adjustable repeater pipette (Socorex Stepper TM 411), mixed immediately and incubated at 30°C for 10 min. Optical density of the reaction mixture was measured at 490 nm using Systronics colorimeter.

Transmission Electron Microscopy Studies

Cells from actively growing culture were stained with freshly prepared 2% uranyl acetate and observed under Philips Transmission Electron Microscope (FEI Tecnai 12 BioTwin).

Analysis of EPS

The partially purified EPS was analyzed for its sugar, protein, uronic acid and nucleic acid contents. Total sugar content was estimated following the phenol-sulphuric acid method of Dubois *et al.* (1956) using glucose as standard. Estimation of sugar monomers was achieved by gas chromatography (Shimadzu GC-17A, Japan) following methanolysis of EPS. Protein content of EPS was estimated by folin-phenol reagent using bovine serum albumin as standard (Lowry *et al.*, 1951). Nucleic acid was estimated by measuring the absorbance at 260 nm (Jenway UV-Vis spectrophotometer) using purified DNA as standard. Quantitative estimation of the uronic acids was spectrophotometrically performed following the method of Blumenkrantz and Asboe-Hansen (1973).

Fourier Transform Infrared Spectroscopic Analysis

The Fourier-Transform Infrared (FTIR) spectrum of the partially purified EPS was recorded in a Perkin

Elmer RX-1 FTIR spectrometer. The purified sample after lyophilization at -56°C was prepared as KBr pellet and scanned in the range of 500 to 4000 cm^{-1} .

Statistical Analysis

All experiments were performed in triplicates and the results represent mean \pm standard error.

Results

Growth Associated Exopolysaccharide Production

Halomonas marina HMA 103 (MTCC 8968), the moderately halophilic heterotrophic bacterium previously isolated from solar slatterns of Odisha, India produced characteristic opaque, slimy colonies with profound accumulation of extracellular polysaccharides (EPS) when grown on Ventosa's agar medium containing 5 to 10% NaCl. Negatively stained cells of *H. marina* HMA 103 showed distinct accumulation of EPS from the early stages of growth (Fig. 1), which remain loosely adhered to the cells and was easily extracted with 2% (w/v) EDTA. During growth under shake flask condition in modified basal synthetic medium (Lillo and Valera, 1990), the extracellular polymer accumulation was found to be more or less parallel with the growth of the bacterium and the highest EPS production (0.46 g/L) was obtained during the late stationary phase (Fig. 2). Production of EPS was accompanied by gradual utilization of glucose and decline of pH of the medium (final pH 5.0, 55 h).

Optimization of Cultural Conditions for EPS Production

Growth and production of extracellular polysaccharide by *H. marina* HMA 103 was influenced by the presence different carbon sources like glucose, sucrose, fructose, maltose, acetate, citrate and malonate in the medium. Although, acetate followed by citrate appeared to be most suitable for growth of the bacterium, maximum exopolysaccharide production was detected in presence of glucose followed by sucrose and maltose (Table 1). Variation in the concentration of glucose in the fermentation medium clearly indicated that polymer production by *H. marina* HMA 103 was more or less parallel with the growth accounting maximum EPS production (0.69 g/L) at 2 % (w/v) glucose (Fig. 3).

Supplementation of both inorganic and organic nitrogenous substances in the growth medium at 0.1% (w/v) level showed discrete variation in the growth and polymer production by the isolate HMA 103. Amongst the nitrogenous sources, yeast extract, peptone, beef extract and ammonium chloride enhanced the polymer production significantly (0.47 g/L to 0.50 g/L) (Table 2).

Table 1. Effect of different carbon sources on growth and exopolysaccharide production by *H. marina* HMA 103

Carbon source 1% (w/v)	Growth (CDW g/L)	EPS yield (g/L)	Final pH of the medium
Glucose (control)	4.25 \pm 0.01	0.46 \pm 0.01	5.05
Sucrose	3.75 \pm 0.01	0.34 \pm 0.02	5.03
Fructose	1.18 \pm 0.01	0.22 \pm 0.01	6.70
Maltose	3.5 \pm 0.01	0.33 \pm 0.01	5.81
Sodium acetate	5.5 \pm 0.01	0.18 \pm 0.01	8.68
Sodium citrate	5.35 \pm 0.01	0.23 \pm 0.01	8.78
Sodium malonate	2.75 \pm 0.01	0.04 \pm 0.02	8.38

CDW = Cell Dry Weight, EPS = Exopolysaccharides

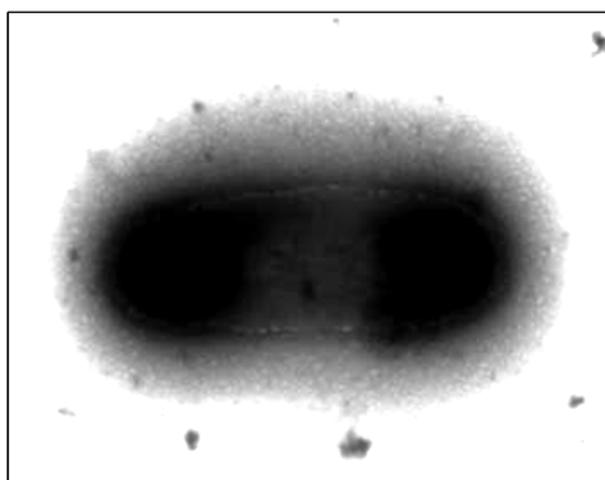


Fig. 1. Transmission electron micrograph of negatively stained cell of *H. marina* HMA 103 displaying cell bound extracellular polysaccharides (EPS)

Growth and EPS production by HMA 103 increased with increasing concentration of ammonium chloride, highest growth and exopolysaccharide production being achieved at 0.1% (w/v) NH_4Cl . However, in yeast extract, growth and EPS production exhibited a different relationship. While, maximum EPS production was recorded at 0.1% (w/v) yeast extract, biomass formation increased steadily with increasing concentration [0.2% (w/v)] of yeast extract (Fig. 4A and B).

The optimal concentrations for both growth and EPS production by *H. marina* HMA 103 were estimated to be 2.4% (w/v) and 0.3% (w/v) of sulphate (Fig. 5A) and phosphate ((Fig. 5B) respectively. Increasing concentrations of sulphate as well as phosphate retarded both biomass formation and extracellular polymer production.

Halomonas HMA 103 showed a wide degree of tolerance to sodium chloride (25% w/v) in the growth medium. Growth and EPS production by the isolate followed a more or less parallel behavior with respect to NaCl concentration and maximum exopolymer yield (0.42 g/L) was recorded at 10% (w/v) NaCl (Fig. 6).

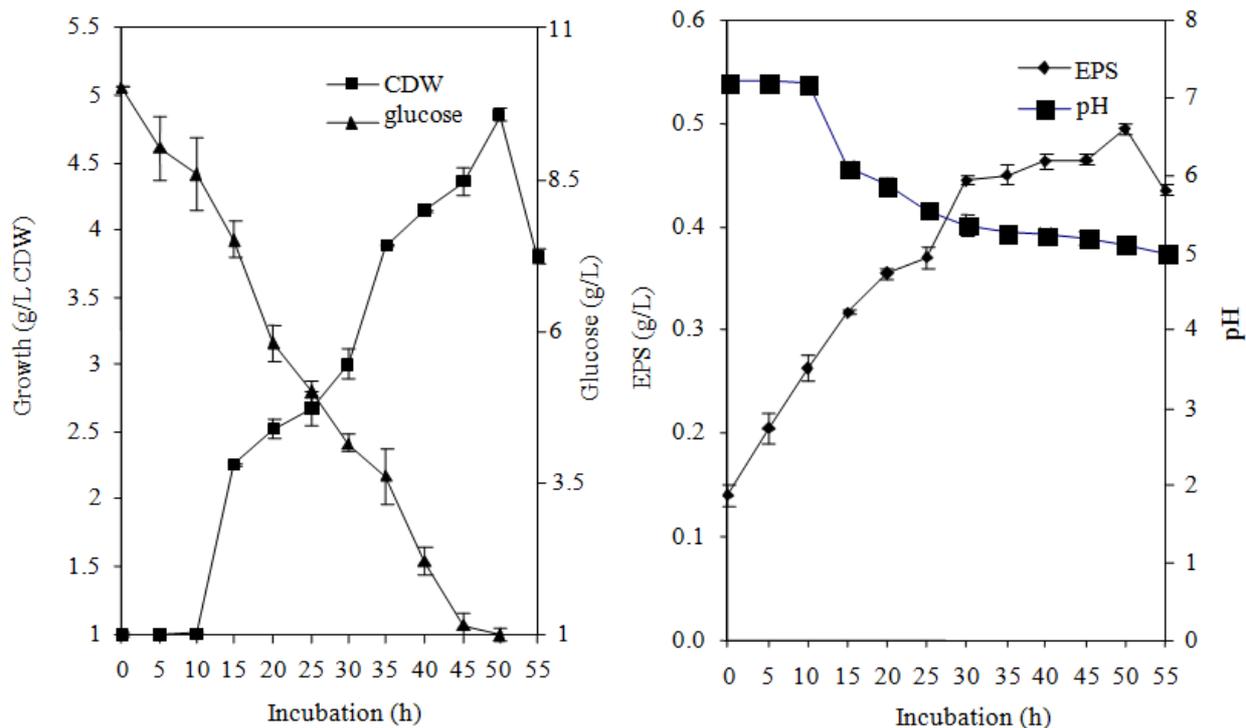


Fig. 2. Time course of growth and EPS production by *H. marina* HMA 103 in modified basal synthetic medium. Residual glucose concentration and final pH of the medium were also estimated. CDW = Cell Dry Weight

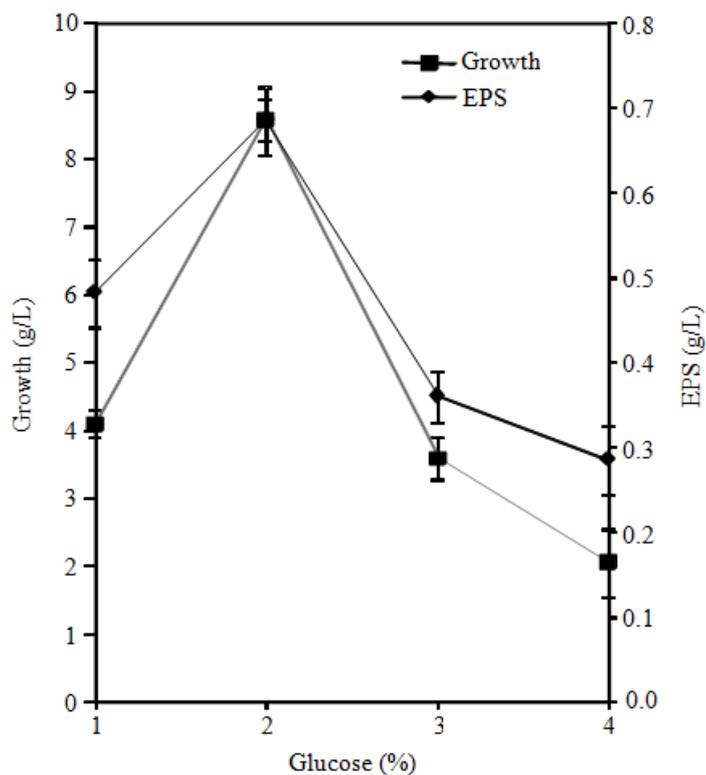


Fig. 3. Effect of increasing glucose concentration on growth and EPS production by *H. marina* HMA 103

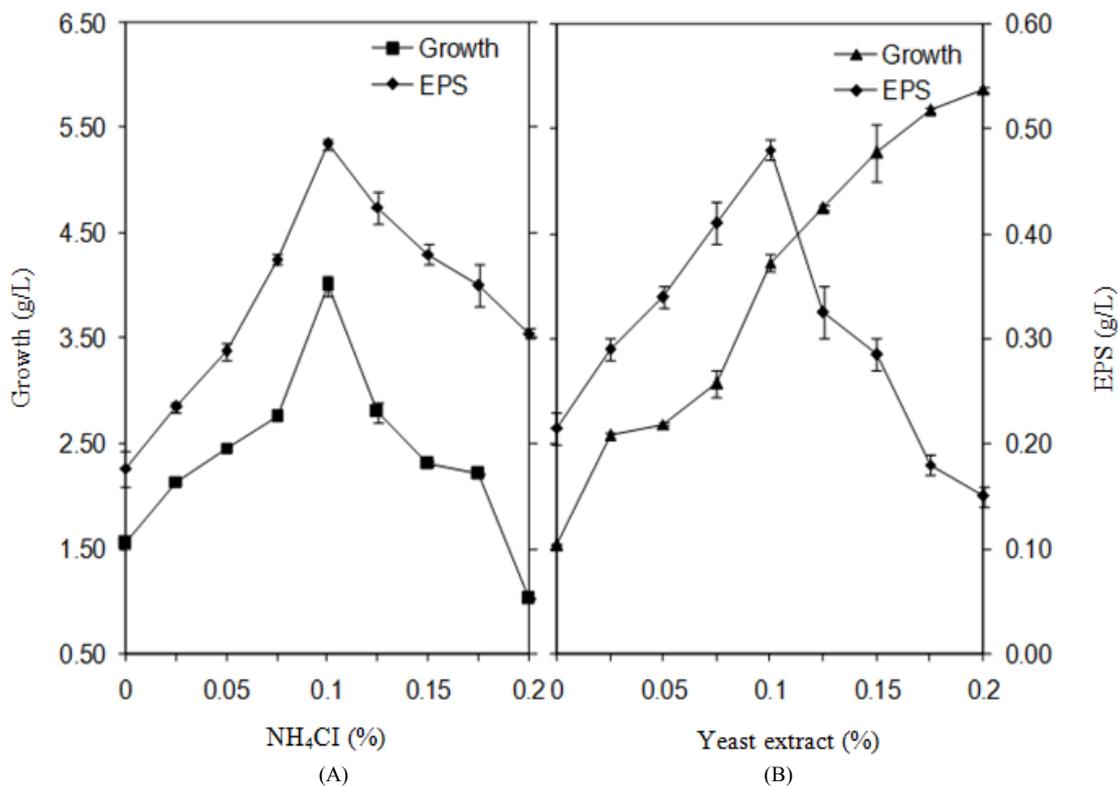


Fig. 4. Effect of increasing concentration of ammonium chloride (A) and yeast extract (B) on growth and EPS production by *H. marina* HMA 103

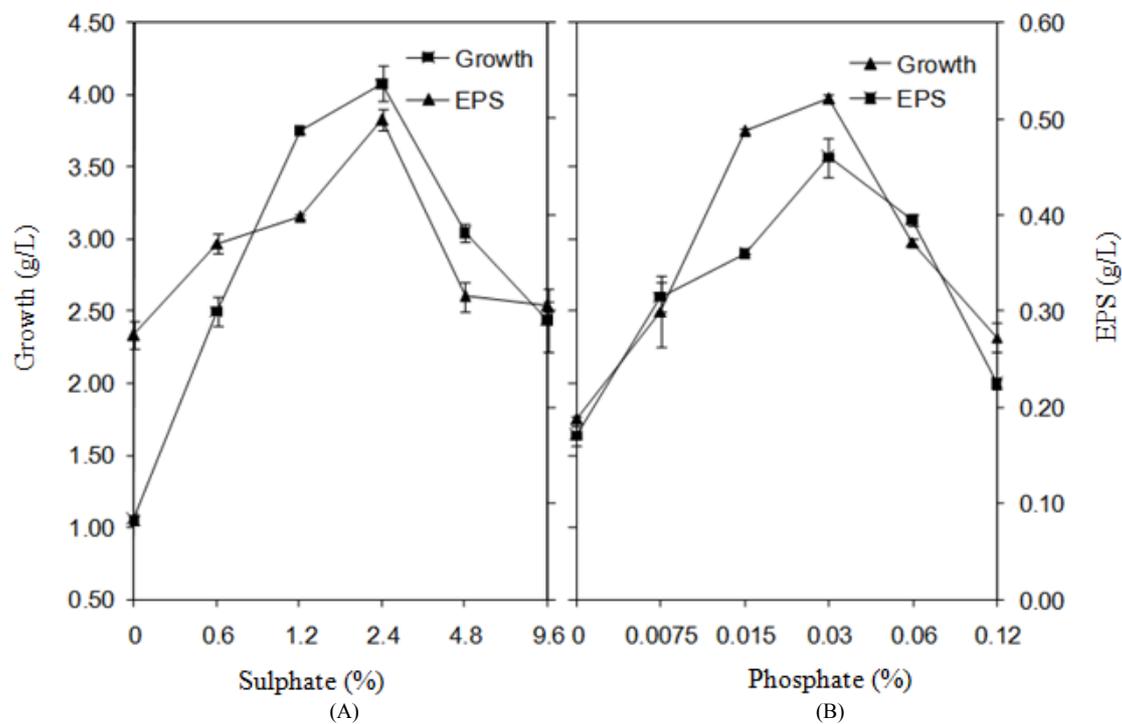


Fig. 5. Effect of sulphate (A) and phosphate (B) on growth and EPS production by *H. marina* HMA 103

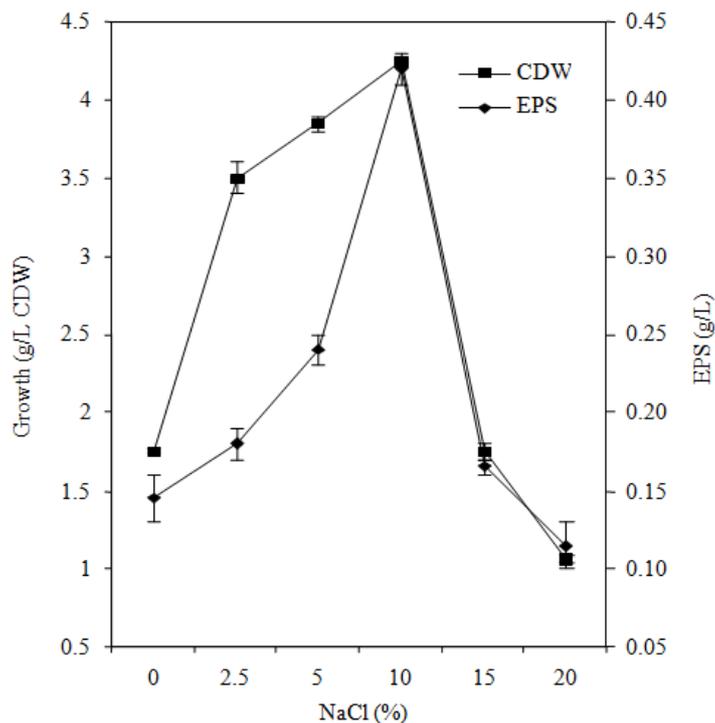


Fig. 6. Effect of increasing concentrations of sodium chloride on growth and EPS production by *H. marina* HMA 103

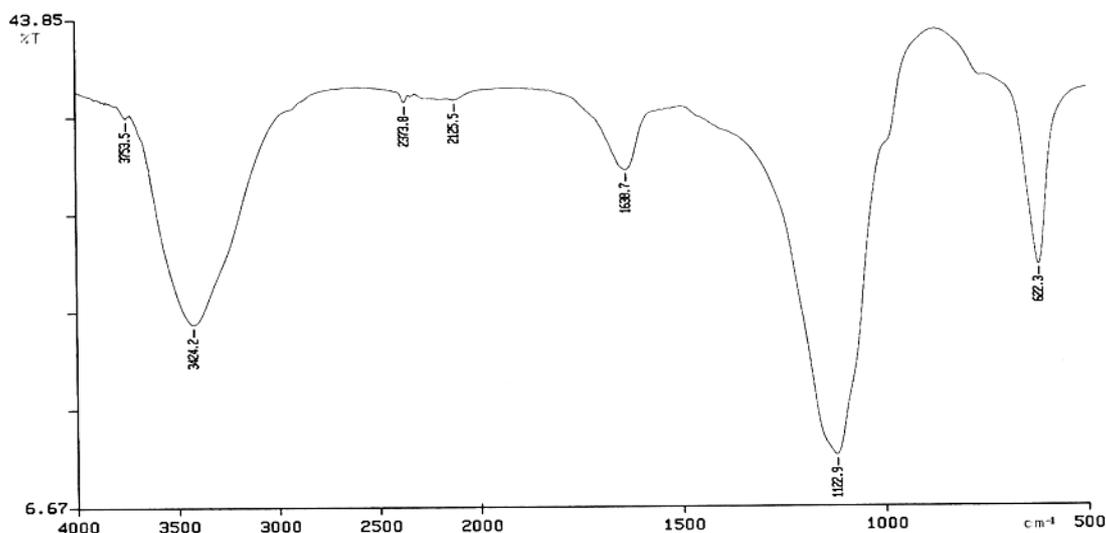


Fig. 7. Fourier transform infrared spectra of purified EPS produced by *H. marina* HMA 103. Purified EPS powder was prepared as KBr pellet and scanned in the range 4000 to 500 cm^{-1}

Characterization of EPS

The purified EPS of *Halomonas* HMA 103 was subjected to compositional analysis and it was found to be a heteropolymer of glucose and mannose. Protein, nucleic acids and uronic acids were not detected. FTIR spectrum of purified exopolysaccharide prepared as KBr pellet is illustrated in Fig. 7. The spectrum shows

characteristic absorption peaks at 3424.2, 1638.7, 1122.9 and 622.3 cm^{-1} ; along with few weak vibrations at 3753.5, 2373.8 and 2125.5 cm^{-1} . The broad O-H stretching vibration observed at 3424.2 cm^{-1} indicated presence of free hydroxyl groups of carbohydrates. The medium C=C stretching vibration at 1638.7 cm^{-1} might indicate phenyl ring or presence of conjugated carbonyl groups. The strong stretching band at 1122.9

cm^{-1} may be assigned to -COC- group indicating presence of cyclic structures of carbohydrates. The minor bands at 2373.8 and 2125.5 cm^{-1} might indicate presence of alkynes in the exopolysaccharide.

Discussion

Production of extracellular polysaccharide by moderately halophilic *Halomonas* spp. has long been recognized as a character of taxonomic significance (Mata *et al.*, 2006) and commercial importance (Flemming and Wingender, 2001). The quality and quantity of EPS produced by an organism is greatly influenced by fermentation conditions and medium composition (Sutherland, 1998). We describe here the production of extracellular polysaccharide by *H. marina* HMA 103, part of which remain in cell-bound form (Fig. 1), while the rest is released into the growth medium as soluble EPS. Nutritional and cultural conditions have been optimized in the modified basal synthetic medium (Lillo and Rodriguez-Valera, 1990) under shake flask culture. The high yield of EPS accompanied by increasing cell mass formation indicated a balanced growth of *Halomonas* HMA 103 in synthetic medium until the glucose as sole source of carbon was consumed and caused cessation of EPS production (Fig. 2). Unlike many other organisms (Pham *et al.*, 2000), there was no significant decrease in the quantity of EPS during the subsequent phases of incubation which indicated possible lack of EPS degradation and utilization in the culture.

The presence of a suitable carbon source providing energy for cellular growth is an essential requirement for the EPS production. The bacterium, HMA 103 grew and produced EPS in a variety of carbon sources, reflecting variations in the carbon source utilization pattern. The isolate preferred acetate and citrate for growth, while glucose was best utilized for polymer production (Table 1). However, glucose above the optimum concentration (2% w/v) impedes both growth and EPS production (Fig. 3). In haloalkalophilic *Halomonas* sp. CRSS, acetate was the most efficient carbon source producing a fructo-glucan polysaccharide (Poli *et al.*, 2004), while *Halomonas* sp. TG39 yielded a high molecular weight extracellular polymeric substance (HMW-EPS) when grown on glucose (1% w/v) and a peptone : yeast extract ratio of 5:1 (Gutierrez *et al.*, 2009).

During the course of optimization of nitrogen sources, individual organic and inorganic nitrogen sources were supplemented in the basal medium. In comparison with inorganic nitrogen sources, organic nitrogenous compounds supported higher biomass and EPS production (Table 2). Production of EPS was triggered by lower concentrations of NH_4Cl and yeast extract, however, the excess of organic and inorganic nitrogen appeared to be utilized for generating the biomass of *H. marina* HMA 103 (Fig. 4).

Table 2. Effect of different nitrogenous substances on growth and exopolysaccharide production by *H. marina* HMA 103

Nitrogen source	Growth (CDW g/L)	EPS (g/L)	Final (pH)
Organic			
Yeast extract	4.45±0.50	0.50±0.01	6.06
Beef extract	4.45±0.25	0.47±0.01	5.74
Tryptone	4.25±0.75	0.43±0.01	6.24
Peptone	4.00±0.10	0.49±0.02	6.35
Casein hydrolysate	4.00±0.01	0.35±0.06	6.39
Inorganic			
Ammonium chloride	4.15±0.01	0.47±0.05	5.33
Ammonium sulphate	4.00±0.01	0.41±0.01	5.57
Ammonium nitrate	3.25±0.25	0.36±0.03	5.49
Potassium nitrate	1.50±0.01	0.10±0.01	7.58

Nitrogen sources were used at 0.1% (w/v) level

Stressful culture condition in presence of excess carbon often enhances the production of EPS. Limiting phosphate ion concentrations in the growth medium appeared to be a triggering factor for enhanced production of EPS by the bacterial strain HMA 103 (Fig. 5B). However, sulfate at sub-optimal concentration failed to show any such induction (Fig. 5A). High NaCl concentrations (10% w/v) also resulted in more water-soluble EPS production by HMA 103 (Fig. 6), more or less identical to that of the photosynthetic bacterium, *Rhodospseudomonas acidophila* (Sheng *et al.*, 2006).

The Fourier Transform Infrared spectroscopy has been used as a rapid and sensitive analytical technique for qualitative characterization of microbial cells and cell components like EPS (Sheng *et al.*, 2006). The major peaks in the FTIR spectra (Fig. 7) of EPS obtained from *Halomonas* HMA 103 at 3424.2, 1122.9 and 622.3 cm^{-1} confirmed the presence of carbohydrates, while absence of vibrations at 2860, 1660 and 1250 cm^{-1} confirmed the absence of proteins, uronic acids, nucleic acids and lipids (Sheng *et al.*, 2006; Jiao *et al.*, 2010), which also confirmed the data of the biochemical analysis of the EPS. Gas chromatography, on the other hand has confirmed the heteropolymeric nature of the EPS from HMA 103 being composed of glucose and mannose.

Conclusion

It is, therefore, apparent that the moderately halophilic bacterium *H. marina* HMA 103 is capable of producing considerable amount of exopolysaccharide utilizing an array of carbon and nitrogen sources under high saline conditions suggesting its implication in the production of the exopolymer under extreme saline environment. The organism thereby could be a potential source of biopolymer possessing unique properties for future biotechnological applications. However, an in-depth physico-chemical analysis of HMA 103 exopolysaccharide is essentially required to explore such potentials.

Author's Contributions

All authors equally contributed to this work.

Ethics

The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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