



Isolation and Characterization of Biofilm Producing Bacteria from Arabian Sea

Nisha.P¹ and M. Thangavel²

¹Dept of Biochemistry and Biotechnology, S.S.V. College, Kerala, INDIA

²Dept of Microbiology, Sree Narayana Guru College, K.G. Chavady, TN, INDIA

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Abstract

Nowadays many studies have been carried out to investigate the occurrence of novel microbial bioactive compounds. Biofilms can be produced by microbial species and having fascinating industrial applications. In this present study, water samples from 'Arabian Sea', isolated organisms were screened for biofilm formation. Potent biofilm producer was identified as *Halomonas* (MP) by morphological and biochemical characteristics based on Bergey's manual of determinative bacteriology, and also by sequencing. Antibacterial activity of EPS from MP was done by disc diffusion method with some pathogenic organisms and characterized. The EPS produced in different environmental factors like incubation temperature 27°C and 37°C, pH 6 - 8 and in incubation time 24hr, 48hr, 72hr and 96 hr. Maximum EPS production was at pH 8, 27°C and at 96 hrs of incubation time. EPS supernatant and Dry EPS produced in each parameter, quantified by total carbohydrate and total protein).

Keywords: EPS, *Halomonas*, Biofilm, antibacterial activity, bioactive compound.

Introduction

Many bacterial species produce biofilms, are surface attached, densely packed cells of microbial communities and secreting polymers surround themselves. Bacteria can be able to adhere on natural or artificial surfaces and form sessile communities and are called biofilms¹.

Most of the biofilm bacteria in marine environment having novel sources of EPS and these biofilms are useful to screening putative EPS producers². Exopolysaccharides from bacteria has vital roles in the bacterial primary attachment and later development of matured complex matrix of bacterial biofilm. Bacterial colonization on surfaces of a biotic materials, biotic materials and artificial surfaces provide microbial survival. These mature biofilms tolerate against toxins and antibiotics, improved entry to nutrients, self-protection from predation and care of some extracellular enzyme activities are the survival strategies of biofilm colonization³. EPS produced mainly during the exponential/log phase of bacterial growth are capsular while EPS produced during the stationary phase are slime⁴. EPS are high molecular weight polymers consists of exopoly saccharides, proteins, lipids, nucleic acids and humic substances⁵ and are high molecular weight substances has gluey in nature⁶. Bacterial exopoly saccharides which are essentially involved in the interaction between bacteria and their environment. These biofilm forming bacteria may produce innovative bioactive compounds including EPS with unique structures. The extracellular materials (polysaccharides, lipids, glycoproteins and lipopoly saccharides) can be used as

stabilizers, crystallizing agents, adhesives, solidifying agents, emulsifying agents, flocculants and flushing agents⁷. In this study bacteria were isolated from Arabian Sea and isolated 12 pure culture colonies were screened for the biofilm synthesis. MP was a potent strain which used for further studies and identified as *Halomonas* based on morphological, biochemical and physiological characteristics and 16SrRNA sequencing. EPS production was assessed and quantified at pH 6 - 8, temperature at 27°C and 37°C and at incubation time such as 24hr, 48hr, 72hr and 96 hr. Antibacterial activity of Crude EPS was checked and subjected to FT-IR spectroscopy.

Materials and Methods

Isolation of bacteria: Water samples were collected from ARABIAN SEA in sterile bottles and aseptically transfer to the laboratory immediately. The isolation of organisms was done by using serial dilution method.

Screening of biofilm bacteria: Isolated bacteria were screened for biofilm formation by using glass rods and lancets as surfaces. The surfaces were washed with acetone, immersed in a detergent for 1hr, thoroughly washed out with distilled water and dried for 1hr at 160°C. Surfaces were separately immersed in a conical flask containing 100 ml of Yeast Malt Glucose media (yeast extract 3g, malt extract 3g, glucose 10g, peptone 5g, distilled water 1000 ml) and inoculated with 12 selected organisms. After 7 days at 37°C incubation, glass rods and lancets were taken out and washed with phosphate buffer solution to remove unadhered cells. Once again surfaces were

transfer to a fresh media which was inoculated with the same amount of culture and incubate for 7 days in order to achieve the biofilm formation. Superior biofilm producer (MP) in both surfaces was used for further studies.

Identification: MP was identified based on morphological, biochemical and physiological characters and 16S rRNA sequencing.

Production of EPS from MP: The pre inoculum was prepared in Yeast Malt Glucose broth by incubating at 25°C for 24 hours and 200µl of this culture inoculated into 50ml of yeast malt glucose broth. After incubation at room temperature, 120 rpm for 5 days, the culture broth centrifuged 10,000 rpm for 20 min, after removing pellet, supernatant mixed 3 volumes of ethanol /isopropyl alcohol and well shaken during addition of ethanol / isopropyl alcohol to prevent local high concentration of the precipitate and left overnight at 4°C. Precipitated EPS weighed after drying at 80°C for 24 hour. EPS was extracted according to the method followed by⁸.

Quantification of EPS Production at different growth parameters: Estimation of Total Carbohydrate in EPS: The total carbohydrate content of crude EPS and dried EPS were determined at pH 6 - 8, different time of incubation 24, 48, 72 and 96 hours and different temperatures 27°C, 37°C by Anthrone method by using glucose as the standard⁹.

Estimation of Total Protein in EPS: The total protein of crude EPS and dried EPS were estimated by Lowry's method¹⁰ by using Bovine serum albumin as the standard. The estimation was carried out at pH 6- 8, different incubation time 24, 48, 72 and 96 hours and different temperature 27°C, 37°C to quantify EPS production.

Antimicrobial activity of Crude EPS - Kirby Baeur method: Crude EPS (supernatant) after 3 days of incubation was used as a sample to determine the antimicrobial activity. Test organisms such as *Escherichia coli*, *Klebsiella spp.*, *Salmonella typhi* and *Staphylococcus sp.* Zone of inhibition on MHI agar plate (Mueller hinton Agar) was measured in millimeter after 24 hr of incubation at 37°C.

Characterization of Crude EPS: Crude EPS was characterized by FT-IR spectroscopy (Analyzed at STIC-Cochin University).

Result and Discussion

In this work, the biofilm producing bacterial strain isolated from ARABIAN SEA. Biofilm production ability was checked based on the adherence on two solid surfaces such as glass rod and lancet, MP was selected for further biofilm studies, this result is related with the reports^{11,12}. Another study reported that glass and stainless steel are surfaces that provide a greater bacterial adherence^{13,14}. The process of biofilm formation is influenced by various factors including nutrients level, pH, temperature,

incubation period, ionic strength, culture concentration, etc., but the bacterial cell surface appendages and the contact surface characteristics are the most important among all of them as its formation begins when bacterial cells encounter a suitable surface and its outer surface adheres to the substratum. In the case of interactions between microbes and adhering surfaces are also varies for an attachment onto hydrophilic or hydrophobic surfaces for attachment¹⁵. More attachment on both surfaces showed by MP and identified as *Halomonasp. Halomonasp* is a gram negative, motile bacilli, moderately halophilic, non fermenting bacteria have growth in between 17°C -30°C.

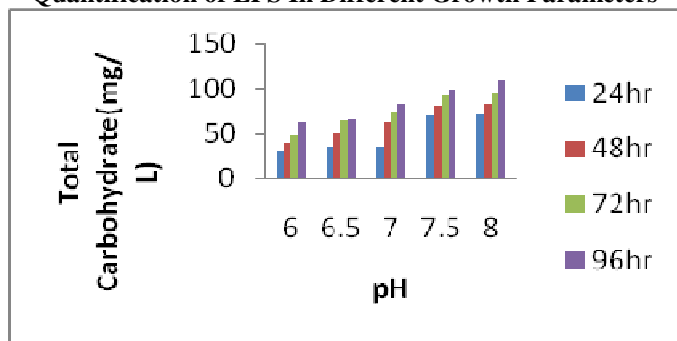
Table-1
Antimicrobial Activity of Crude EPS

Sample	Test Organisms	Zone of Inhibition(mm)
Crude EPS	<i>Escherichia coli</i>	12
	<i>Klebsiellasp</i>	9
	<i>Salmonella typhi</i>	8
	<i>Staphylococcus sp</i>	8

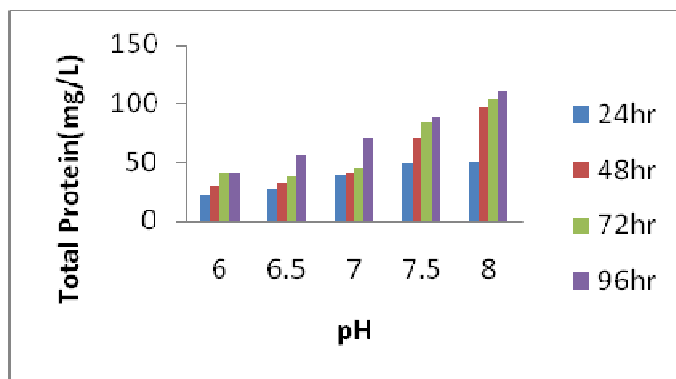
The total carbohydrate (figure-1) and total protein (figure-2) of Crude EPS and Dry EPS were estimated at different incubation period (24hr, 48hr, 72hr and 96hr) and temperature (27°C, 37°C) and at pH 6 - 8. From the quantification results of Crude EPS, higher carbohydrate content was at 96 hrs, at pH 8 and at 27°C of incubation time, was 138.3 mg/L. The higher content of total protein in Crude EPS was 128.1mg/L at 96 hrs. Total carbohydrates and total protein contents of dry EPS were comparatively lower than crude EPS and both were maximum at incubation temperature 27°C and 96hrs of incubation time and pH 8. Total carbohydrates and total protein contents of crude and dry EPS were lower at all other parameters studied. *Bacillus polymyxa* had produced EPS in the presence of sucrose (61g /g of sugar) in 31 hr of cultivation¹⁶. EPS production was increased in the log phase and maximum at stationary phase that was in 96hr of incubation time. The optimum conditions for maximum production of EPS from MP was at incubation temperature 27°C and 96hrs of incubation time and pH 8.

Antibacterial activity of crude EPS was checked with *Escherichia coli*, *Klebsiellasp*, *Salmonella typhi* and *Staphylococcus sp* (figure-3) as test organisms. Crude EPS produced zone of inhibition against all test organisms. By interpreting the FTIR spectra of the Crude EPS (figure-4), the value 3366.07 cm⁻¹ indicates cell proteins delivered several amide related bands 1°, 2° amines, 2260–2100 cm⁻¹ –C≡C– stretch alkynes. 1631.7 cm⁻¹ indicate 1° amines/amide, 1001.36 cm⁻¹ indicates C-O and 900–675 cm⁻¹ indicates C–H aromatics.

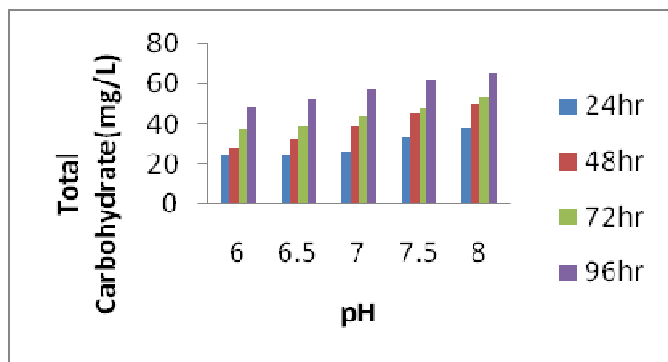
Quantification of EPS In Different Growth Parameters



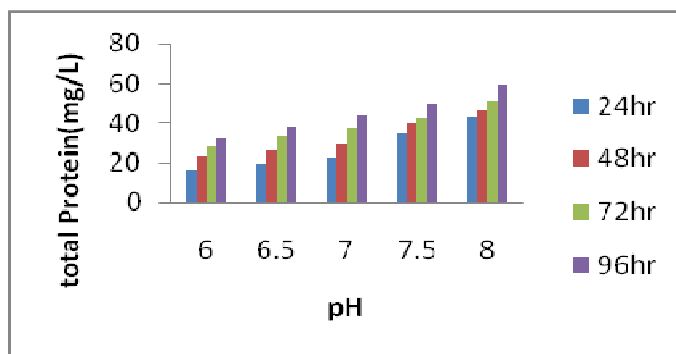
CRUDE EPS- Total Carbohydrate in 37°C



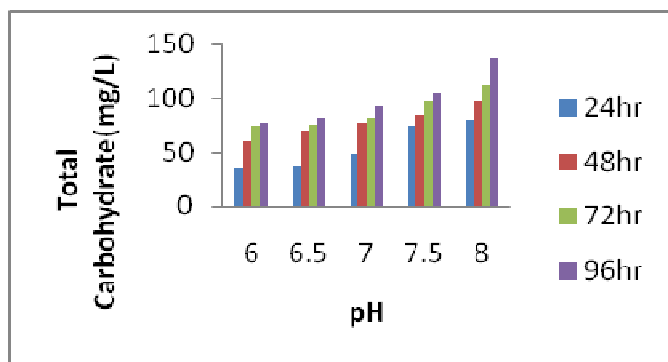
CRUDE EPS Total Protein in 37°C



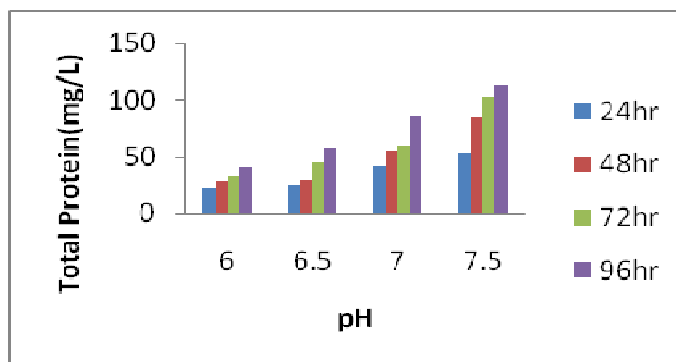
DRY EPS -Total Carbohydrate in 37°C



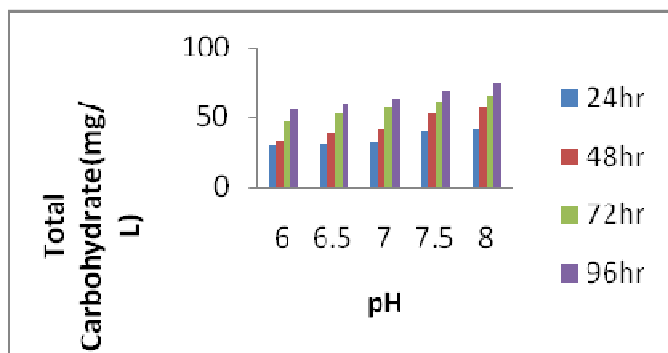
DRY EPS Total Protein in 37°C



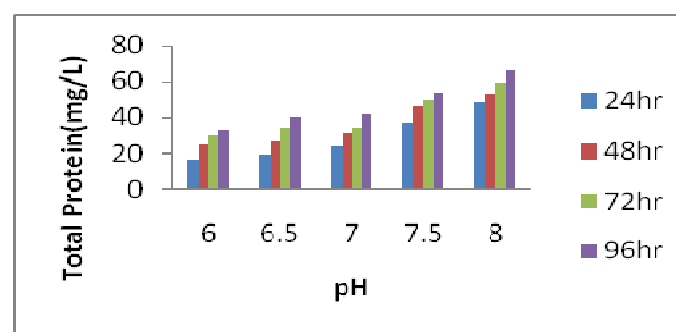
CRUDE EPS -Total Carbohydrate in 27°C



CRUDE EPS Total Protein in 27°C



DRY EPS -Total Carbohydrate in 27°C



DRY EPS Total Protein in 27°C

Figure-1

Total Carbohydrate Estimation

Figure-2

Total Protein Estimation

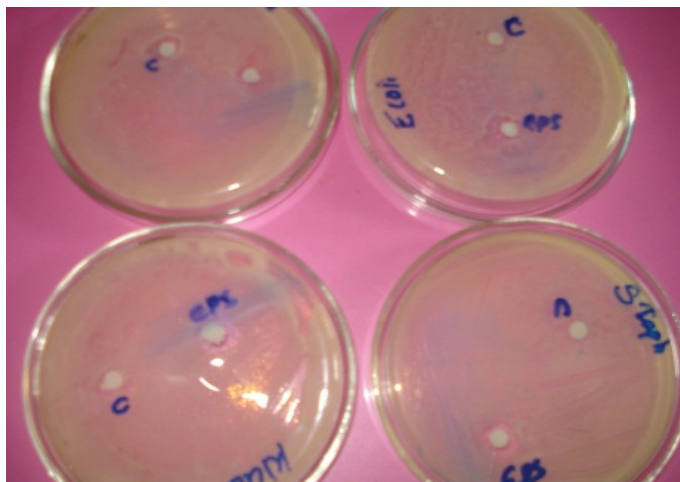


Figure-3
Antimicrobial Activity of Crude EPS

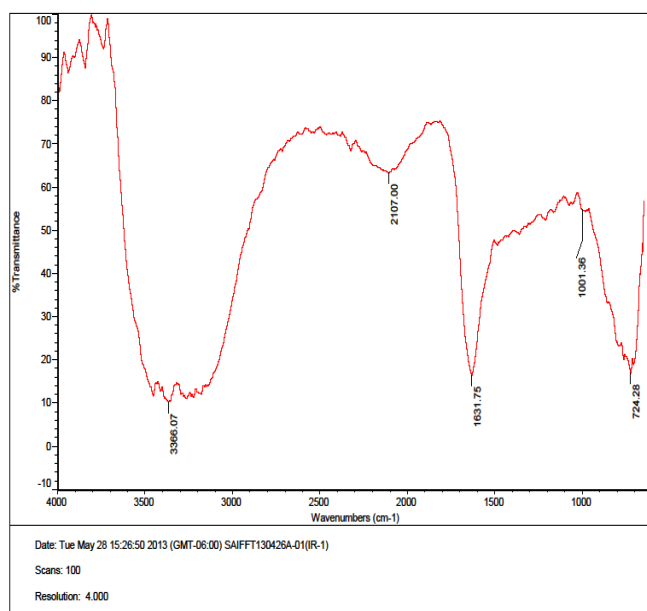


Figure-4
FTIR of Crude EPS

Conclusion

Halomonassp (MP) isolated from 'Arabian Sea' can capable to adhere on surfaces and produce biofilm. *Halomonassphas* a potent producer of exopolysaccharide and Crude EPS isolated from MP hasan antimicrobial activity. The optimum conditions for higher EPS production from MP was at 96hr, 27°C and pH 8. FT-IR analysis of crude EPS indicates the presence of cell wall protein, 1°, 2°amines and alkynes and C-H groups. EPS production was increased in the log phase and maximum at stationary phase that was in 96hr of incubation. This is the primary research work on *Halomonassp* (MP)in EPS optimization and expecting the presence of bioactive compound.

Thus further studies are needed to evaluate the potential application of the biofilm exopolysaccharides and identification of bioactive compound.

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