Characterization of Extracellular Polymeric Substances (EPS) produced by *Pseudomonas aeruginosa*

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Abstract

Recently many investigations have been carried out in microbial biofilms. Biofilms produced by microbial species has fascinated application in industries. In this present study, *Pseudomonas aeruginosa* isolated from sputum and urinary catheter and screened for biofilm formation. The extracellular polymeric substances (EPS) produced by microbes are composed of biopolymers involving polysaccharides, nucleic acids, lipids, proteins and humic substances. Morphology of the *Pseudomonas aeruginosa* EPS was characterized by HR-TEM and FTIR. Antibacterial activirty of EPS produced from *Pseudomonas aeruginosa* was examined by disc diffusion method against *Staphylococcus aureus and Acenatobacter baumannii*. Environmental factors influencing EPS production such as temperature (22 ºC and 31 ºC), pH (5.5, 7.0 and 8.5) and incubation time (24hrs, 48hrs and 72hrs) were monitored. The potent EPS production by *Pseudomonas aeruginosa* was observed at temperature 31 ºC, pH-8.5 and incubation period of 72 hrs. Crude EPS and dry EPS produced in optimum conditions were quantified by total protein and total carbohydrate concentration.

Keywords: *Pseudomonas aeruginosa*, EPS, characterization, antibacterial activity

Introduction

It has long recognised that bacteria can switch from planktonic unicellular organisms to sessile multicellular communities known as biofilms (1, 2). Microbial biofilms pertain its integrity and is protected by a matrix composed of microbes isolated from polymeric compounds called extracellular polymeric substances (EPS) (3). The biosynthesis of EPS is believed to play a crucial role in enhancing the initial attachment of cells to solid surfaces, synthesis and maintenance of microcolonies, structure of mature biofilm and increased biofilm resistance to disinfectants and environmental stress (4, 5, 6). EPS compounds belong to different classes of macromolecules such as polysaccharides, proteins, nucleic acids, glycoproteins and phospholipids (7, 8). EPS is known to be the efficient factor enhancing the biofilm formation in microbes. Recent studies evaluated that mostly exo-polysaccharides, extracellular proteins and extracellular DNA are responsible for the morphology and structure of the biofilm matrix (8, 9, 10). From a medical perspective, it was observed that the attached bacteria on catheters, lenses or implants are of the major concern which causes serious infections (11). In this present study, we investigate the extraction of EPS from *Pseudomonas aeruginosa*, its morphology characterization by HR-TEM and optimization at temperature (22 ºC and 31 ºC), pH (5.5, 7.0 and 8.5) and incubation period (24hrs, 48hrs and 72hrs). Antibacterial activity of crude EPS was evaluated and presence of bioactive compounds were analysed by FT-IR spectroscopy.

Materials and methods:

Isolation of bacteria:

Sputum, urinary catheters and hospital swab were collected from Pondicherry hospital. The isolation of organisms was performed by using serial dilution method.

Screening of biofilm bacteria:

Potent biofilm producer were screened by adherent assay using glass, rubber and plastic chips as surfaces (12). The surfaces were rinsed with acetone, placed in a detergent for 1 hr, washed with distilled water and dried for 1hr at 160 ºC. The chips were aseptically immersed into each of the sterile conical containing selected culture. The flasks were incubated at ambient temperature of 28 ºC for 24 hrs. At the end of incubation chips were removed and subjected for biofilm quantification using crystal violet binding assay (13). Efficient biofilm producer found in all the 3 surfaces was taken for further studies.

Identification:

Identification of potent biofilm producing *Pseudomonas aeruginosa* was determined based on morphological, biochemical and 16s rRNA sequencing. The selected strain was cultured in LB broth for 24 h at 37 ºC and genomic DNA were amplified by polymerase chain reaction (PCR) employing universal primers forward primer- 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG- 3' and reverse primer- 5'-CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT- 3'. PCR was performed in a thermal cycler using an initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min and final extension at 72°C for 10 min.

Haemolytic activity:

Blood Agar is a differential growth medium which can differentiate pathogenic bacteria from nonpathogenic one. Inoculate organisms tested on blood agar that is enriched with 5 % human blood and the plate was incubated at room temperature for 24 h. The plate was observed for the ability of red blood cells lysis. Zone of inhibition determine alpha, beta and gamma. (14,15).

Production of EPS:

The pre inoculum was made in Yeast Malt Glucose broth (YMG) by incubation at 37 ºC for 24 hrs. To 50 mL of YMG broth, 180µL of pre inoculated culture was added and incubated at room temperature, 120rpm for 5 days. The culture broth was centrifuged at 1000rpm for 20min. After discarding the pellet, 3 volumes of ethanol was mixed with supernatant and kept overnight at 4 ºC for precipitation. Precipitated EPS was dried at 80 ºC for 24 hrs. Extraction of EPS was performed according to the methods followed by (16, 17).

Characterization of EPS:

Morphology of the EPS was analysed by HR-TEM (IIT, Mumbai).

Quantification of EPS synthesis at various growth parameters:

Estimation of total carbohydrate in EPS:

Quantification of carbohydrate content of crude EPS and dried EPS were estimated at temperatures 22 ºC and 31 ºC, pH-5.5, 7.0 and 8.5 and at incubation period of 24 hrs, 48 hrs and 72 hrs by (PSA) phenol sulphuric acid method employing glucose as standard (18).

Estimation of total carbohydrate in EPS:

Quantification of total protein concentration were analysed by Lowry *et al* method using BSA as standard (19). The estimation was performed at temperatures 22 ºC and 31 ºC, pH-5.5, 7.0 and 8.5 and at incubation period of 24 hrs, 48 hrs and 72 hrs .

Fourier Transform Infrared Spectroscopy:

To assess the functional group present in the copper oxide nanoparticles FTIR spectrum analysis was performed. FTIR spectrum was recorded using KBr pellet technique in the range between 4500 and 400 cm-1.

Antimicrobial activity of crude EPS:

The cultured supernatant collected before ethanol treatment (centrifuged after 3 days of incubation) was employed as a crude EPS sample to examine the antibacterial activity of test organisms such as *Staphylococcus aureus and Acenatobacter baumannii*. After 24 hrs of incubation period at 37 ºC zone of inhibition on Muller Hinton (MHI) agar plate was measured in mm.

Results:

Identification of clinical isolate:

Gram staining and biochemical analysis of 16 clinical isolates of *Pseudomonas Sp.* reveals gram negative, catalase positive , oxidase positive, indole negative and motile bacteria. The selected potent biofilm forming *Pseudomonas Sp.* genomic DNA was isolated and PCR amplified with universal primer. The amplified product was sequenced and 1309 base pairs DNA fragment was compared to the sequences available in GenBank, NCBI (AE004091). Partial sequencing of 16S rRNA was performed by Eurofins, Bangalore. BLAST analysis of the sequence data revealed that the isolate belonged to the genus *Pseudomonas* with the closest species *P. aeruginosa* most identity with *P. aeruginosa* (GenBank accession number AE004091). Thus, the bacteria isolated from the sputum was confirmed up to species level and had a maximum of 99% similarity with existing *P. aeruginosa* strains. The partially complete (*1309 bp) 16S rDNA sequences of *P.aeruginosa* strain SRC 123 isolate have been deposited in the GenBank database under accession number KX881765.

Blood Agar Haemolysis:

The types of hemolysis are alpha, beta and gamma. Alpha hemolysis represents bacteria that can partially break down the blood cells, indicating green or brown discoloration of the agar around the colony. Beta hemolysis determine bacteria which completely lyse the blood cells forming a clear zone around the colony. Gamma hemolysis record bacteria that cannot lyse the cells, thus

causing no effect in the agar. Out of 16 *Pseudomonas Sp.* isolates 7 were determined a clear β hemolysis zone formation, which records the presence of bacterial toxin and 5 isoltes indicate α hemolysis and the rest 4 isolates record γ haemolysis.

Adherence assay:

The major biofilm forming pathogenic *Pseudomonas Sp.* isolates assessed for adherence analysis predicted *Pseudomonas aeruginosa* as an efficient biofilm forming bacteria as it recorded the significant adherence capability on all the tested surfaces such as plastic, glass and rubber employing crystal violet binding assay.

Characterization of EPS by TEM analysis:

The presence of EPS surrounding biofilm was clearly noticed by HR-TEM analysis (Fig 2a, 2b).

Quantification of EPS production on different Growth parameters:

Estimation of total carbohydrate and total protein concentration of crude EPS and dry EPS, were determined at different incubation period (24hrs, 48hrs and 72hrs), pH (5.5, 7.0 and 8.5) and temperatures (22 ºC and 31 ºC). From the estimation results, it was predicted that higher concentrations of carbohydrate and protein observed in crude EPS at 72hrs, pH 8.5 and incubation temperature of 31 ºC and it were 140 mg/L and 121 mg/L (from standard graph). Similarly, carbohydrate and protein concentration found to be maximum in dry EPS at 72hrs, pH 8.5 and incubation temperature of 31 ºC and it were 110mg/L and 79 mg/L.

Fourier Transform Infrared Spectroscopy:

FT-IR spectrum of crude EPS is shown in fig 3 , the spectrum showed strong absorption band at 3429.43 cm-1 determine cell proteins indicating amide related bands 1º, 2º amines, 2980.02 cm-1 indicate –C≡C- stretch alkynes, 1647.21 cm-1 reveal 1º amines or amides, band at 1063.79 cm-1 indicate C-O stretch and 675.09 cm-1 corresponds to C-H aromatics.

Antibacterial activity of EPS:

Crude EPS of *Pseudomonas aerugnosa* displayed antibacterial activity with zone of inhibition (in mm) against the both tested organisms *Staphylococcus aureus and Acenatobacter baumannii* (fig 4). It

Discussion:

Pseudomonas aeruginosa cells form enormous quantity of extracellular DNA by the process that is independent of cellular lysis. *Pseudomonas aeruginosa*, a ubiquitous environmental gram-negative bacterium, is one of the most studied biofilm- forming organisms and has emerged as model organism in the study of surface- and biofilm-induced gene expression (11). With increasing biofilm biomass, there is increase in the concentration of EPS over the period of immersion (20). During the bacterial growth the production of EPS observed to be an efficient factor for a bacterium entangling a surface as it may stimulate the inscrease in adhesive bond strength and construct a protective glycocalyx (21). Hence, growth and EPS production is more potent under attached effects for some bacteria and adherence on solid surfaces also induces polysaccharide synthesis (22). In this present research work, the experimental samples isolated from clinical source were screened under adherence assay. Potent biofilm producer was assessed based on the adherence ability on solid surfaces such as plastic, glass and rubber. Superior biofilm producer was employed for quantification studies, this result is related with the reports (23, 24, 25).

Though following factors pH, incubation period, temperature, nutrient level, culture concentration, ionic strength influences biofilm formation the bacterial contact surface characteristics and cell surface appendages are the major effective factors among all as the microbial biofilm formation starts when bacterial cells identify a suitable surface and its adherence to the substratum. The interaction between microbes and surfaces they adhere varies with an attachment on the hydrophobic or hydrophilic surfaces. Efficient attachment of *Pseudomonas aeruginosa* was on all the three surfaces (plastic, rubber and glass). TEM image recorded the EPS production during biofilm formation.

Quantification study of EPS production in *pseudomonas aeruginosa* predicted that maximum concentrations of total carbohydrate (140mg/L) and total protein (121mg/L) observed in crude EPS at, pH 8.5, 72hrs and incubation temperature of 31 ºC. Quantification study assessed that total protein and carbohydrate concentration of dry EPS were comparatively lower than crude EPS and both of them shows maximum production at the similar pH-8.5, incubation period 72hrs and temperature 31 ºC. The result is related with the evaluations (25). It was clearly predicted that the optimum cultivation factors such as pH, temperature and incubation period of EPS production from *Pseudomonas aeruginosa* monitored at pH 8.5, temperature 31 ºC and incubation time 72 hrs .

Hemolysins are one of the potent virulence factors which suggest the presence of bacterial toxin (26). β hemolysin was recorded in *P.aeruginosa*. As EPS has adhesive property it might participate in the virulence activity of pathogenic bacteria. FTIR analysis showed the presence of toxic compounds, Hence, we can suggest that EPS has its significant role in pathogenesis (27). The presence of aromatic and carboxylic compounds that are recorded from stretch peaks in FTIR could determine antibacterial activity of EPS (28). Crude EPS indicate the zone of inhibition against both test organisms *Staphylococcus aureus and Acenatobacter baumannii*.

Conclusion:

Pseudomonas aeruginosa isolated from sputum is believed to adhere more on tested surfaces and enhance biofilm formation. The optimum condition for potent EPS from *Pseudomonas aeruginosa* was recorded at pH- 8.5, 72 hrs and incubation temperature at 31 ºC. The FT-IR spectrum analysis of crude EPS determines the presence of cell wall protein 1º, 2º amines, alkynes and C-H groups. The present study is the preliminary research work in optimization of EPS production from *Pseudomonas aeruginosa* and the presence of bioactive compounds in crude EPS is monitored employing FT-IR.

References

1. Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T Nickel, J.C., Dasgupta, M and Marrie, J.T., (1987). Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol*. 41: 435-464.
2. Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin- Scott, H.M. (1995). Microbial biofilms. *Annu. Rev. Microbiol*. 49: 711-745.
3. Yotova, L. and Marinkova, D. (2012). Investigation of the formation, structure and application of biofilms (review). *J. Chem. Technol. Metall.* 47: 243-250.
4. Flemming, H.C. and Wingender, J. (2001). Relevance of microbial extracellular polymeric substances (EPSs)- Part-1. Structural and ecological aspects. *Water Sci. Technol.* 43.
5. Dunne, W.M. (2002). Bacterial adhesion: seen any good biofilms lately? *Clin. Microbiol. Rev.* 15: 155.
6. Czaezyk, K. and Myszka, K. (2007). Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Polish J. of Environ. Study.* 16: 799-806.
7. Sutherland, I.W. (2001). Microbial polysaccharides from Gram-negative bacteria. *Int. Dairy J.* 11: 663.
8. Branda, S.S., Vik, A., Friedman, L. and Kolter, R. (2005). Biofilm: the matrix revisited. *Trends Microbiol*. 13: 20.
9. Langille, S.E., Geesey, G.G. and Weiner, R.M. (2000). Polysaccharide- specific probes inhibit adhesion of *hyphomonas rosenbergii* strain VP-6 to hydrophilic surfaces. *J. Ind. Microbiol. Biotechnol. 25: 81*.
10. Mattos- Guaraldi, A., Formiga, L.C.D., Periera, G.A. (2000). Cell surface components and adhesion in *Corynebacterium diphtheria. Microb. Inf*. 2: 1507.
11. Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science*. 284: 1318.
12. Stepnovic, S., Cirkovianin, I., Ranin, L. and Svabic-Vlahovic, M. (2004). Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett Appl. Microbiol.*, 38:428-432.
13. Asha, P., Shini, Z. and Jyothis, M. (2013). Biofilm formation in Enterococci from different sources. *Int. J. Biopharm.* 4:140-144.
14. Fadden., M. 1980. Biochemical tests for identification of Medical bacteria. Williams and Wilkins, Baltimore, USA: 51-5**.**
15. Lesmana, M., Subekti, D., Simanjuntak, C. H., Tjaniadi, P., Campbell, J. R. & Oyofo, B. A. 2001. *Vibrio parahaemolyticus* associated with cholera-like diarrhea among patients in North Jakarta, Indonesia. *Diagnostic microbiology and infectious disease*, 39: 71-75.
16. Ohno, N., Miura, N., Nakajima, M. and Yadomae, T. (2000). Antitumor 1-3-glucan from cultured fruit body of Sparasiscrispa. *Boil. Pharm. Bul.* 23: 866-872.
17. Gong, S.A., Bolster, H.C., Magda, B. and Walker, L.S. (2009). Extraction and analysis of extracellular polymeric substances: comparison of methods and extracellular polymeric substance levels in *Salmonella pullorum* SA 1685. *Environ. Engi. Sci*. 26: 1523-1532.
18. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28: 350.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 193: 265.
20. Bragadeeswaran, S., Jeevapriya, R., Prabhu, K., Sophia Rani, S., Priyadharsini, S., Balasubramanian, T. 2011. Exopolysaccharide production by *Bacillus cereus* GU812900, a fouling marine bacterium. *Afr. J. Microbiol. Res.* 5: 4124-4132.
21. Read, R.R, Costerton, J.W. 1987. Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens.* Can. J. Microbiol., 33: 1080 - 1090.
22. Vandevivere, P., Kirchman, D.L. 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl. Environ. Microbiol., 59: 3280- 3286.
23. Sinde, E. and Carballo, J. (2000). Attachment of *Salmonella spp* and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluroethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiol*. 17: 439-447.
24. Donlan, R.M. Role of biofilms in Antimicrobial Resistance. *Asian J.* 46: 547-552.
25. Nisha, P. and Thangavel, M. (2014). Isolation and characterization of biofilm producing bacteria from Arabian Sea. *Res.J.Recent.Sci.* 3:132-136.
26. Asao, T., Kinoshita, Y., Kozaki, S., Uemura, T., Sakaguchi, G. 1984. Purification and some properties of Aeromonas hydrophila hemolysin. *Infection and Immunity,* 46:122-127.
27. Anju, P., Milind, N., Santosh Kumar, D. 2010. Hemolysin, Protease, and EPS Producing Pathogenic *Aeromonas hydrophila Strain An4* Shows Antibacterial Activity against Marine Bacterial Fish Pathogens. *Journal of Marine Biology,* 10: 1-9.
28. Mohamed, O., Mugambwa, J., Fahrul, H. 2012. Characterization of Exopolysaccharides Produced by *Bacillus cereus* and *Brachybacterium* sp. Isolated from Asian Sea Bass (*Lates calcarifer*). Malaysian Journal of Microbiology, 8: 170-174.

Figure (1a) Figure (1b)

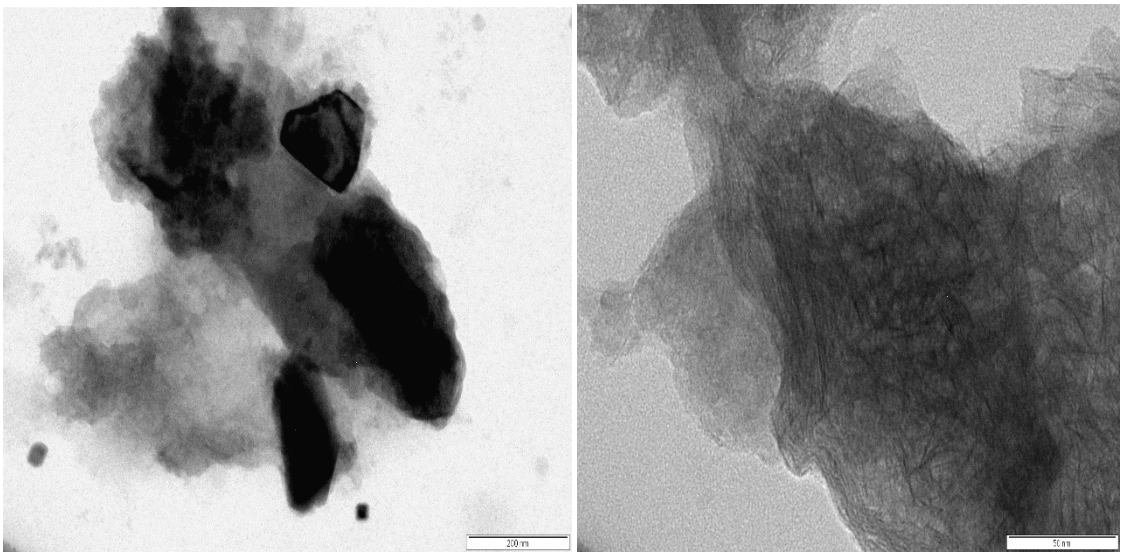
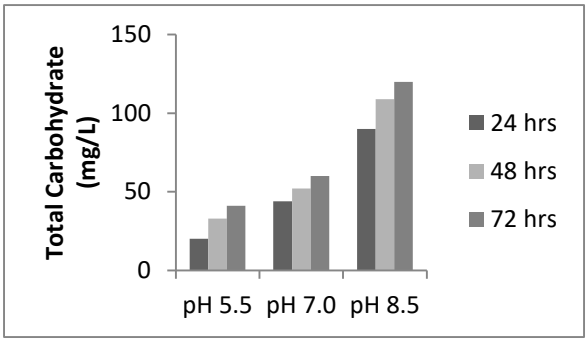
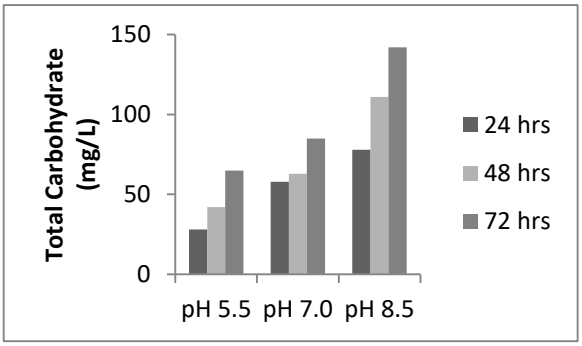


Fig (1a) and (1b): HR-TEM analysis of EPS from *Pseudomonas aeruginosa*

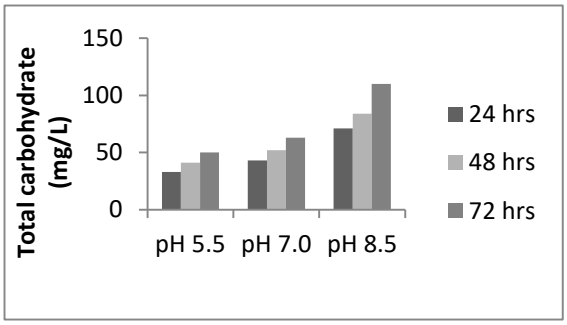
Figure 2: Quantification of EPS in various growth parameters

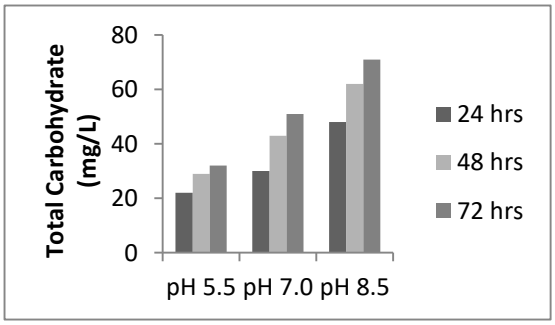


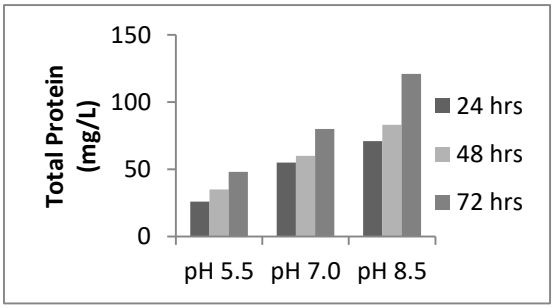


Crude EPS- Total Carbohydrate in 31 ºC Crude EPS- Total Carbohydrate in 22 ºC

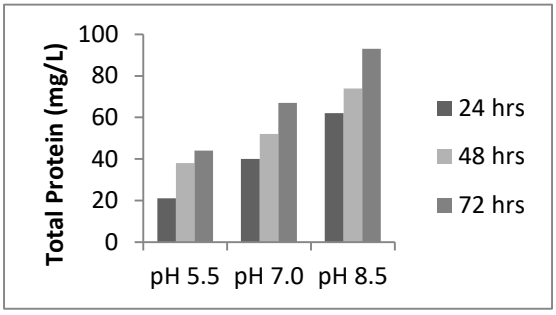
Dry EPS- Total Carbohydrate in 31 ºC Dry EPS-Total Carbohydrate in 22 ºC



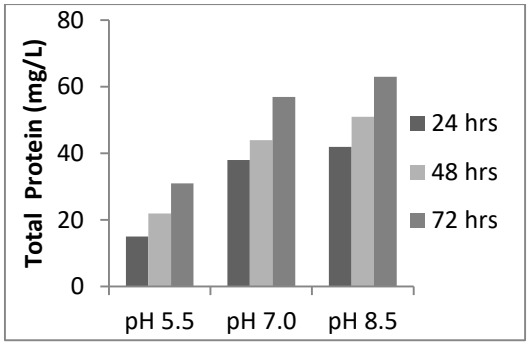




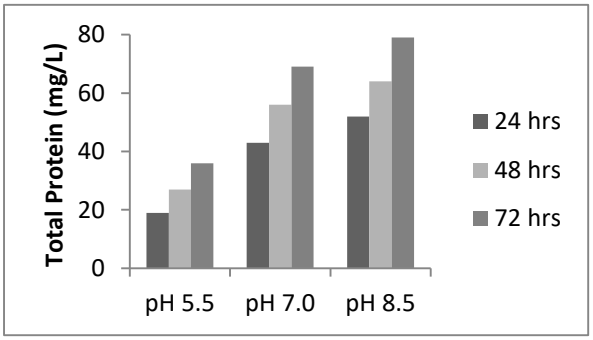
Crude EPS- Total Protein in 31 ºC



Crude EPS- Total Protein in 22 ºC



Dry EPS- Total Protein in 31 ºC



Dry EPS- Total Protein in 22 ºC

Figure 3: FTIR Spectra for Crude EPS

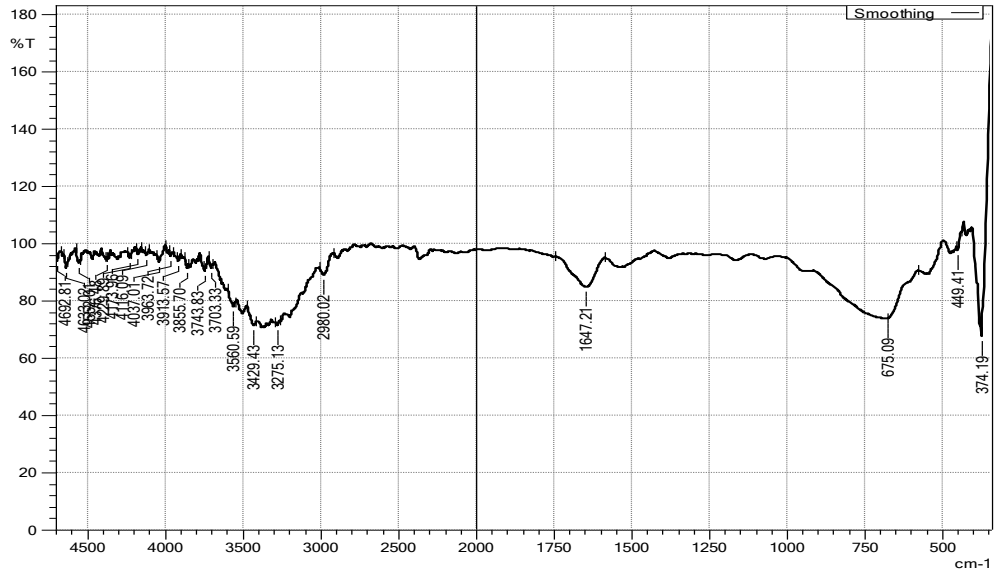


Figure 4: Antibacterial activity of Crude EPS