Jurnal Teknologi

Full paper

Purification and Characterization of Extracellular Polymeric Substances (EPS) with Antimicrobial Properties from s Marine Epibiotic *Pseudoalteromonas* sp.

Ainul Masliana Ismail[a], Wan Nur Atirah Wan Azemin[a], Azmi Rani[b], Mohd Shahir Shamsir[a*]

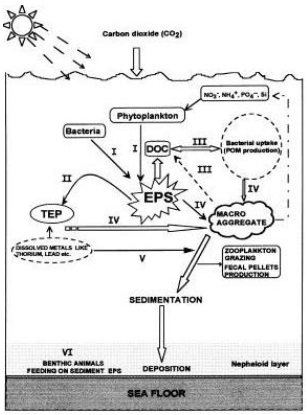
[a]*Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia*
[b]*Brackishwater Culture Research Centre, Department of Fisheries, Ministry of Agriculture and Agro-based Industry, Malaysia*

*Corresponding author: shahir@fbb.utm.my

Article history

Received :1 August 2012
Received in revised form :7 Sept. 2012
Accepted :1 October 2012

Graphical abstract



Abstract

We present a study to purify and characterize the extracellular polymeric substances (EPS) produced by *Pseudoalteromonas sp.* isolated from fish epidermal surface. The purified EPS was recovered from the culture supernatant by using cold ethanol precipitation and dialysis method. Colorimetric analysis of EPS revealed that it was primarily composed of proteins, 0.0251g/L, carbohydrates, 0.0561g/L and uronic acid, 0.0217 g/L. The EPS also exhibited high emulsification activity when being tested with hydrophobic substances such as paraffin oil, (94.5 % and 79.4%), kerosene, (56.1% and 37.2%) and Tween 80 (46.6% and 32.9%). In addition, the EPS demonstrated discernible antimicrobial activity against Gram-negative bacteria like *E.coli*, *P.aeruginosa* and *E.aerogenes*. The SDS-PAGE profile revealed up to eight types of unknown proteins were detected within the EPS, with the molecular weight ranging from 15.486 kDa to 113.058 kDa. The results of the study would contribute to the elucidation of antimicrobial components and its mechanism in *Pseudoalteromonas sp*.

Keywords: Extracellular polymeric substances; pseudoalteromonas; fish mucus; colorimetric analysis; antimicrobial activity; antimicrobial components

Abstrak

Kami membentangkan kajian penulenan dan pencirian bahan-bahan polimer luaran yang dihasilkan oleh bakteria *Pseudoalteromonas sp.* yang didapati dari mukus ikan. EPS yang dimurnikan telah dirawat dari supernatan kultur dengan menggunakan kaedah pemendakan ethanol sejuk dan juga dialysis. Melalui analisis colorimetric, dapat diketahui bahawa EPS ini mengandungi 0.0251g/L protein, 0.0561g/L karbohidrat dan 0.0217 g/L asid uronik. EPS ini juga menunjukkan aktiviti pengemulsi yang tinggi apabila diuji dengan bahan-bahan hydrophobic seperti minyak paraffin, (94.5 % and 79.4%), kerosene, (56.1% dan 37.2%) dan Tween 80 (46.6% dan 32.9%). Tambahan pula, dari ujian yang dilakukan dapat dilihat bahawa EPS ini menunjukkan aktiviti anti-bakteria terhadap bakteria jenis Gram-negative seperti *E.coli*, *P.aeruginosa* dan *E.aerogenes*. Dari profil SDS-PAGE pula, lapan jenis protein yang tidak diketahui telah dikesan dari EPS ini di mana berat molekulnya adalah dari 15.486 kDa ke 113.058 kDa. Keputusan-keputusan dari kajian ini boleh menyumbang kepada penjelasan terhadap komponen-komponen anti-mikrob dan juga mekanismanya dalam *Pseudoalteromonas sp.*

Kata kunci: Bahan polimer luar sel; pseudoalteromonas; lendir ikan; analisis colorimetric; aktiviti sntimicrob; bahan antimikrob

© 2012 Penerbit UTM Press. All rights reserved.

1.0 INTRODUCTION

Bacterial extracellular polymeric substances (EPS) are important in the interaction between bacteria and their environment. The mucus are chemically diverse with a wide range of chemical structures of homopolymeric or heteropolymeric type, made up of sugar and non-sugar components, is possible and the range of monosaccharide combinations, together with non-carbohydrate constituents and varied linkage types, makes the exopolymer an excellent emulsifying agent and attributes diversity in bacteria (Flemming and Wingender 2001). The bacterial exopolymers are usually acidic heteropolysaccharides possessing the functional groups (e.g., hydroxyl, carboxyl, and phosphoric acid), which exhibit high affinity towards certain metal ions. Many interesting physical and chemical properties of microbial exopolysacharides, have found a wide range of applications in the field and can be

used as stabilizing, suspending, thickening, gelling, coagulating, film-forming, and water retention capabilities. They may be useful in detergents, textiles, adhesives, paper, paint, food and beverage industries, oil recovery, heavy metal polluted soils and water, mining industry and petroleum industries, replacement of chlorinated solvents used in cleaning-up of oil-contaminated pipes, and the formation of stable oil-in-water emulsions for the food. Exopolysaccharides like xanthan, dextran, and gels produced by bacteria are now widely accepted products of biotechnology (Flemming and Wingender 2001).

Microbial EPS are widely distributed in marine environments and are found in free dissolved form, colloids, and discreet particles like TEP or associated with particulate matter, including cell aggregates, detritus, biofilms, microbial mats, etc. The chemical composition of EPS is influenced by various factors, including nutrients, temperature, pH, physiology and age of the culture. Microbial EPS perform various functions, and are involved in diverse marine processes. The unique gelling properties of microbial EPS are considered important in the transport and transformation of organic matter, complexion of dissolved metals and biogeochemical cycling of elements. EPS are rich in organic carbon and therefore are an important source of carbon for different organisms in the food chain. The production of dissolved EPS during the bacterial growth, production of particulate organic carbon from the dissolved EPS and enzymatic breakdown and conversion of particulate EPS into dissolved organic matter within the microbial loop, form an important alternate route of organic carbon cycling in the marine trophic web (Figure 1). The marine environment is a dynamic one, wherein microorganisms experience changes in salinity, pressure, and nutrient levels which can influence EPS production in microorganisms (Bhaskar and Bhosle 2005).

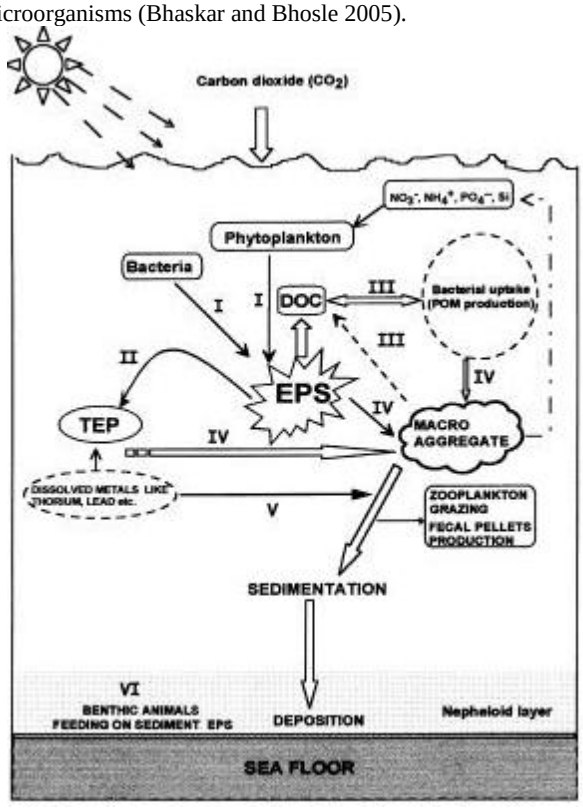


Figure 1 Schematic representation of the various roles of EPS in marine environment (Bhaskar and Bhosle 2005).

Chemically, EPS are rich in high molecular weight polysaccharides (10 to 30 kDa) and generally have heteropolymeric composition. The polysaccharide chain might be unbranched or branched with side chains of other compounds attached to the polymeric chain. Generally, the polysaccharides are made of monosaccharide, with hexoses and pentose forming the bulk of EPS. However, the contribution of different monomers to the total polysaccharide varies with the source and such variations in the polysaccharide chain composition can alter its physicochemical properties. For example, bacterial EPS are generally rich in hexoses like glucose and galactose, whereas phytoplankton EPS have relatively higher content of sugars like rhamnose, xylose and mannose. Such variations in monomer composition can alter the properties of EPS. The presence of sugars like arabinose in EPS helps in cell aggregation in bacteria, whereas deoxy sugars like fucos and rhamnose found in diatom EPS help in foaming and flocculation. Apart from polysaccharides, EPS also contain fair amounts of proteins, non-sugar moieties like uronic acid, pyruvates, hexosamines, acetates, sulphate esters and generally small amounts of lipids and nucleic acid. Although these non-sugar components are present in relatively smaller quantities, they are generally attached to the sugar residues and are important in imparting unique characteristics to the EPS (Bhaskar and Bhosle 2005).

The importance of microbial EPS has been highlighted by recent publications covering the chemistry, structure and function of EPS, their role in microbial ecology, medicine, dairy industry, biofilms and corrosion, and their applications in the field of biotechnology. In particular, polysaccharides from prokaryotes offer a number of novel properties and commercial opportunities. For example, some species from *Pseudoalteromonas sp.* is said able to produce antibacterial, antifungal, antiviral and algicidal molecules which may obtain from the EPS that is a possible novel alternative antibiotic against dangerous microorganisms. In fact, these molecules are an important resource and are being used increasingly in the biotechnology and biopharmaceutical industries. In this study, we aim to purify, characterise and determine the antimicrobial activity of the EPS produced by *Pseudoalteromonas sp*.

2.0 MATERIALS AND METHODS

Microorganism used in this study was stock culture *Pseudoalteromonas sp.* And preliminary screening with Marine Nutrient (MN) was performed in order to determine whether *Pseudoalteromonas sp.* is EPS producer based on its morphology. Initially, steps were taken for refresh bacteria on MN and this was followed by the preparation of bacterial stock cultures. Bacteria growth in batch fermentation was then carried out for production of EPS. Batch culture was performed by using MN broth as the media. Temperature and pH was maintained at 28±2^{o}C and 7.5 respectively as these are optimal condition for bacterial growth. Two 250ml conical flasks containing 100ml of the above media was inoculated with 10ml of the exponentially growing batch cultures of the bacterial isolate. Cultures then were harvested after 24 h. The harvesting process included separation; precipitation and purification of bacterial EPS were conducted. The purified EPS then, undergo characterization analyses. For colorimetric analyses, metahydroxydiphenyl analyses, Lowry protein assay, and phenol-sulphuric acid method were conducted. We also conducted emulsification activity test, gel electrophoresis for determination of molecular weight of protein and finished by an antimicrobial activity test. Marine Nutrient (MN) agar and broth was used for growing marine bacteria (Bernadsky and Rosenberg

1992). Colonies morphology was observed and studied after 24 h bacterial growth.

To separate supernatant containing EPS from biomass, culture broth was centrifuged at 4000 x g for 90 min at 4°C. The pellet then was freeze-dry and weighed. The supernatant was filtrated through 0.45µm pore size cellulose nitrate filter membrane by using filter housing. To precipitate the EPS, cold ethanol was added, (3 vol of 95% ethanol) and was leaved at 4 °C overnight. Then, the precipitate was collected by centrifugation at 4000 x g at 4 °C, for 30 min. Ethanol decanted was done and precipitate was dissolved in distilled water. Dissolved precipitate was dialyzed extensively against distilled water for 2-3 days to completely remove the remaining ethanol. Lastly, the EPS obtained was lyophilized under freeze-dryer and was weighed. The EPS was store at room temperature for further analysis.

Several characterization methods were performed to characterise the various components in EPS. We utilised the Lowry protein assay to determine protein content, the Phenol-sulphuric acid method to determine carbohydrate content and the Meta-hydroxydiphenyl analyses to determine the uronic acid content. We measured the emulsifying activity of EPS was assayed by modifying method described by The SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to determine the molecular mass of protein in the EPS. The anti-microbial protein are then purified and tested for antibacterial activity using the disk diffusion method. As a comparison, different gram-negative and gram-positive bacteria strains belonging to different genera or species were used for their sensitivity to bacterial EPS. 8 types of target bacteria which are *Escherichia coli, Profeus vulgaris, Pseudomonas aeruginosa, Entrobacter aerogenes, Micrococcus luteus, Streptococcus feacalis, Staphylococcus aureus* and *Bacillus cereus* were grown on the agar plates by spread plate technique. Target bacteria were grown in duplicate. Further, sterile disk papers were dipped in chloramphenicol (as a positive control), non-purified EPS, and purified EPS and placed on each target bacterial plates (Figure 1). All the plates undergo pre-diffusion (stored in refrigerator overnight). After incubated at 37°C overnight, the antibacterial activity was observed. The antibacterial activity was evaluated by quantifying the clear zone of inhibition in the producer lawn around the filter paper disk.

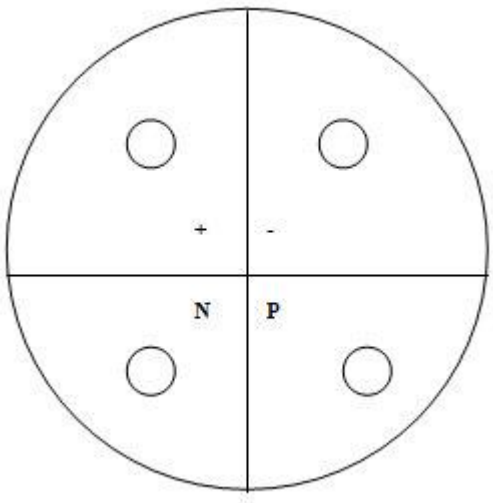


Figure 2 Schematic representation of the arrangement of the disk paper. (N) Disk paper dipped in non-purified EPS. (P) Disk paper dipped in purified EPS. (+) disk paper in chloramphenicol. (-) sterile disk paper

3.0 RESULTS AND DISCUSSION

Preliminary identification has confirmed that the strain is an earlier isolated *Pseudoalteromonas sp.* as the morphological studies showed that the bacterium is a Gram-negative rod with a growth profile at 28±2°C on MN agar. The colonies formed were creamy in colour and showed mucoid phenotype. Comparison between productions of EPS at 24 h and 48 h in batch culture showed that the total purified EPS obtained was higher at 24h fermentation (14.5mg) compared at 48h culture (3.7mg). This is because *Pseudoalteromonas sp* is a fast growing bacterium and was able to produce more EPS during the first 24 hours. Therefore, a series of repeated batch cultures were completed where the cells were harvested after 24 h fermentation to ensure that optimum EPS production is obtained.

The colorimetric result showed that the carbohydrate content in the EPS was higher, (54.5%) compared to protein and uronic acid content which present only 24.4% and 21.1% (Table 1).The higher carbohydrate content compared to uronic acids and proteins is a characteristic observed in other bacterial EPSs (Bramhachari, Kishor *et al*. 2007). The occurrence of uronic acids indicates the acidic nature of the EPS and play an important role in emulsification activity (Table 2). Fazio and co-workers have previously shown that exopolymer from a marine bacterium contains high quantities of galacturonic acid (Fazio, Uhlinger *et al*. 1982).

Table 1 Comparison of Components in EPS

	Carbohydrate	Protein	Uronic acid
Content, g/L	0.0561	0.0251	0.0217
Percent, %	54.5	24.4	21.1

Table 2 Percentage (%) of Emulsification Activity at min 30 and min 60

Hydrocarbon	A_0	A_{30}	A_{60}	t_{30}	t_{60}
Kerosene	0.401	0.225	0.149	56.1%	37.2%
Tween 80	0.502	0.234	0.165	46.6%	32.9%
Paraffin oil	0.364	0.344	0.289	94.5%	79.4%
n-hexadecane (control)	0.846	0.34	0.165	40.19%	19.5%

The emulsification activity of the exopolysaccharide was determined by its strength in retaining the emulsion breaks rapidly within an initial incubation of 30 min. The absorbance reading after 30 and 60 min gives a fairly good indication of the stability of the exopolymer emulsion (Bramhachari, Kishor *et al*. 2007). The emulsifying activity of *Pseudoalteromonas sp*. exopolysaccharide was tested on hydrophobic substances such as paraffin oil, kerosene and Tween 80. Paraffin oil was the substrate most effectively emulsified, followed by kerosene and Tween 80 (Table 2). The dialyzed fraction of the exopolymer produced by *Pseudoalteromonas* strain retained 94.5% and 79.4% in paraffin oil, 56.1% and 37.2% in kerosene and 46.6% and 32.9% in Tween 80 of the emulsification activity after 30 min and 60 min respectively while the n-hexadecane was used as a control. The functional groups in the molecular chains of the polymer are considered as important determinants for emulsification activity

and the *Pseudoalteromonas sp* EPS was comparable to other EPS producing bacterial isolates. Presence of relatively high protein content could be due to a high percentage of hydrophobic amino acids in the protein moiety were favourable for emulsification (Bramhachari, Kishor *et al.* 2007).

The SDS-PAGE result showed eight different peptide sizes. All of these bands are not identical to any protein type of the marker. The molecular weight of these unknown proteins ranged from 15.486 kDa to 113.058 kDa. The sixth band has MW around 20.310 kDa which closely with MW of Soybean Trypsin Inhibitor (21.5 kDa) whereas the fourth band has MW around 30.5 kDa which nearly to MW of Carbonic Anhydrase (31 kDa). Generally, antimicrobial proteins with low molecular weight, (<10kDa) display a broad spectrum microcidal activity. These antibacterial proteins are assumed to form ion channels in bacterial membrane and kill both Gram-positive and Gram-negative bacteria. We did not determine the amino acid sequence would require extraction of individual bands and sequencing it.

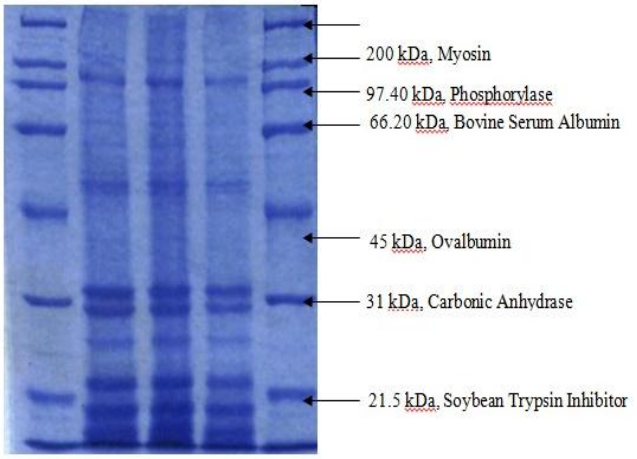


Figure 3 Representative lanes on gel SDS-PAGE. (1,5) Marker with type of proteins and their MW , (2, 3and 4) EPS from *Pseudoalteromonas* sp.

The antibacterial activity was measured by the disk diffusion method assay against eight types of target bacteria: *Entrobacter aerogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus feacalis* and *Profeus vulgaris*. Among eight target bacteria tested, only four types of target bacteria which are *E.aerogenes*, *E.coli*, *P.aeruginosa*, and *M.luteus* showed discernible sensitivity against EPS produces by *Pseudoalteromonas sp*. Antibacterial activity of the EPS was markedly stronger against Gram negative bacteria compared to Gram-positive bacteria.

The result showed that only weak activity was observed around disks that have been dipped with non-purified EPS compared to purified EPS. This is because of possibility the presence of low concentration of antibacterial protein in the supernatant, non-purified EPS. Previous study has demonstrated that the antimicrobial activity was highest in the pellet compared to supernatant. In addition, further studies can be carried out to investigate method to maintain and stabilize antibacterial protein within the EPS using buffer solution (Longeon, Peduzzi *et al.* 2004).

Table 3 Result of the Antibacterial Activity test using the disc diffusion test. The '-' represents no observed inhibitory zone, G –ve : gram negative bacteria and G +ve : gram positive bacteria

Target bacteria	Inhibition zone (in mm)		
	Control	Purified EPS	Non-purified EPS
Escherichia coli, (G -ve)	42	8	1
	42	8	1
Profeus vulgaris, (G -ve)	43	-	-
	40	-	-
Pseudomonas aeruginosa, (G -ve)	42	14	1
	50	14	2
Entrobacter aerogenes, (G -ve)	40	16	2
	40	14	1
Micrococcus luteus, (G +ve)	> 50	8	2
	50	8	2
Streptococcus feacalis, (G +ve)	50	-	-
	50	-	-
Bacillus cereus, (G +ve)	40	-	-
	42	-	-
Staphylococcus aureus, (G +ve)	50	-	-
	50	-	-

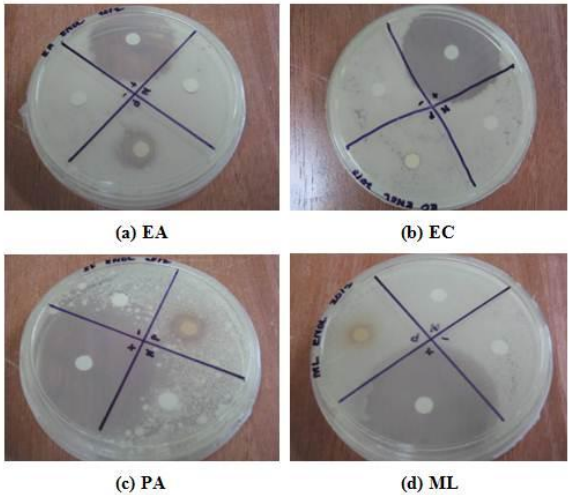


Figure 4 Representative inhibitory zone on the agar plates. (a) *Entrobacter aerogenes*, (b) *Escherichia coli*, (c) *Pseudomonas aeruginosa* and (d) *Micrococcus luteus*

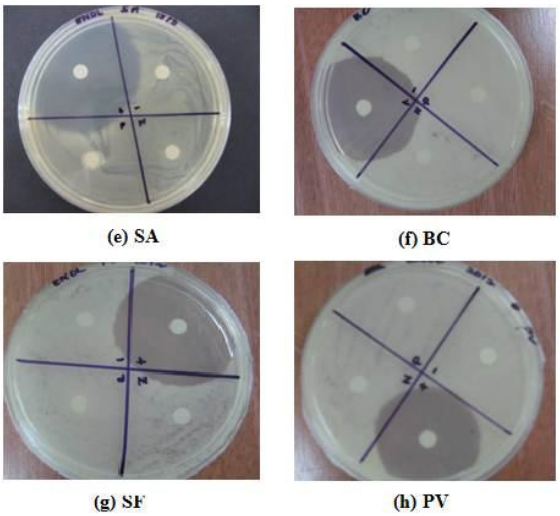


Figure 5 Representative inhibitory zone on the agar plates. (e) *Staphylococcus aureus*, (f) *Bacillus aureus*, (g) *Streptococcus feacalis* and (h) *Profeus vulgaris*

4.0 CONCLUSION

The *Pseudoalteromonas sp.* is shown to produce the highest amount of EPS during the first 24 hours of culture. Colorimetric analyses revealed that the purified EPS has a higher content of carbohydrate compared to protein and uronic acid content respectively with significant emulsification ability. There are eight bands of unknown proteins which molecular weight ranging from 15.486 kDa to 113.058 kDa in the EPS with discernible antibacterial activity against Gram-negative bacteria like *E.coli*, *P.aeruginosa* and *E.aerogenes*. We believe that improvements can be made to enhance purification of EPS by *Pseudoalteromonas sp*. alternatively; other purification methods can be explored and experimented in order to find out the best purification scheme for obtaining EPS possessing both high yield and purity. Analyses of the components in EPS can be achieved using size exclusion chromatography. The antibacterial activity can also be tested with more additional gram-positive and negative "challenge bacteria" to measure specific antibacterial activity.

References

[1] Bernadsky, G. and E. Rosenberg. 1992. Drag-Reducing Properties of Bacteria from the Skin Mucus of the Cornetfish (Fistularia commersonii. *Microbial Ecology*. 24(1): 63–76.

[2] Bhaskar, P. V. and N. B. Bhosle. 2005. Microbial Extracellular Polymeric Substances in Marine Biogeochemical Processes. C*urrent Science.* 88(1): 45–53.

[3] Bramhachari, P. V., P. B. Kishor, *et al*. 2007. Isolation and Characterization of Mucous Exopolysaccharide (EPS) Produced by Vibrio Furnissii Strain VB0S3. *Journal of Microbiology and Biotechnology*. 17(1): 44–51.

[4] Fazio, S. A., D. J. Uhlinger, *et al.* 1982. Estimations of Uronic Acids as Quantitative Measures of Extracellular and Cell Wall Polysaccharide Polymers from Environmental Samples. *Applied Environmental Microbiology*. 43(5): 1151–1159.

[5] Flemming, H. C. and J. Wingender. 2001. Relevance of Microbial Extracellular Polymeric Substances (Epss)--Part II: Technical Aspects. *Water Science and Technology.* 43(6): 9–16.

[6] Longeon, A., J. Peduzzi, *et al.* 2004. Purification and Partial Identification of Novel Antimicrobial Protein from Marine Bacterium Pseudoalteromonas Species Strain X153. *Marine Biotechnology*. 6(6): 633–641.