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ISOLATION AND CHARACTERIZATION OF BIOFILM PRODUCING BACTERIA FROM PERIYAR RIVER, KERALA

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INTRODUCTION

Biofilms are densely packed communities of microbial cells that grow on surfaces and surround themselves with secreted polymers (Nadell *et al*., 2009) and can adhere to natural or artificial surface and form sessile multicellular communities as biofilms (Dalton and March., 1998). Bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming state (Costerton *et al*., 1999; Donlan and Costerton 2002; Dunner, 2002).

The bacteria in most biofilms are embedded in extracellular polymeric substances (Lawrence *et al.,* 1991) these exocellular polymers comprise the capsular polysaccharides, which form a cohesive layer or capsule covalently linked to the cell surface, and the exopolysaccharides (EPS), which form a slime layer loosely attached to the cell surface or secreted into the environment (Madigan *et al*., 1997). During the process of colonization on particular surfaces, bacteria produces extracellular polymeric substance (Geesey and White, 1990), which construct the biofilm matrix. Capsule EPS are produced mainly during the log phase of bacterial growth and slime EPS produced during the stationary phase (Plante and Shriver. 1998).

Bacterial colonization on abiotic materials such as suspended particles, metal surfaces and concrete or on biotic surfaces was thought to be one of the microbial survival strategies because it provides microorganisms with important advantages like increased access to nutrients, protection against toxins and antibiotics, maintenance of extracellular enzyme activities and shelter from predation (Dang and Lovell, 2000)

The rates of the biofilm formation processes vary widely depending on the type of microorganisms and environmental conditions like, the characteristics of the suspending medium, such as pH, ionic strength and temperature, are also considered as important factors in altering the physicochemical properties of a bacterial surface and the microbial adhesion to the substrate (Hamadi *et al*., 2004). The effect of pH levels of the suspending medium has received comparatively little attention.

In this study bacteria were isolated from Periyar River and screened for the biofilm synthesis. SY was a potent strain which is used for further studies and identified as *Micrococcus luteus* based on Bergeys manual determinative bacteriology and 16SrRNA sequencing. Yellow pigment was isolated from SY and determine the antibiotic sensitivity test. EPS production was assessed and quantified at pH 6, 7 & temperature at 27°C& 37°C and at incubation time such as 24hr, 48hr, 72hr &96 hr.

MATERIALS AND METHODS

Isolation of bacteria

Water samples were collected from Periyar River 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 (Medpoint-USA) as surfaces. The surfaces were washed with acetone , immense in a detergent for 1hr,washed with distilled water and dried for 1hr. at 160°C.Surfaces were separately

immersed in a conical flask containing 100 ml of YMG media (glucose 10g, yeast extract 3g, malt extract 3g, peptone 5g, distilled water 1000 ml) and inoculated with 12 selected organisms. After 7days 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 inoder to achieve the biofilm formation. Superior biofilm producer in both surfaces was used for further studies and named as SY.

Identification

SY was identified based on morphological, biochemical and physiological characters according to Bergey's manual of determinative bacteriology.

16S rRNA sequencing

The 16SrRNA gene sequences are used to study bacterial phylogeny and taxonomy. The sequences were submitted to the ADVANCED BLAST search program of the NCBI to identify whether they aligned with closely related organisms.

Extraction of pigment from SY

The bacterial strain was inoculated in nutrient broth and incubated at 120 rpm for 3days. The cultured media was centrifuged at 7500 rpm for 20 min. The supernatant and pellets were extracted using 95% methanol or 99.5% acetone in the ratio of 1:5. Remove the pellets and takeout the supernatant. Extracted pigment with methanol was used as a pigment source for the following studies.

Antimicrobial activity of Crude Pigment-Kirby Baeur method

The antimicrobial activity of a pigment was determined by using 4 test organisms such as *Escherichia coli, Klebsiella spp., Salmonella typhi,* and *Staphylococcus sp.* Filter paper discs which were dipped in the pigment extract before placed on MHA(Mueller hinton Infusion Agar) plates. After 24 hr incubation at 37°C, zone of inhibition was measured in millimeter. Control was made with methanol and antimicrobial test was done in triplicate.

Production of EPS from SY

The pre inoculum was prepared in YMG broth by incubating at 25°C for 24 hours and 200µl of this culture broth was inoculated into 50ml of YMG broth .After incubation at room temperature for 5 days at 120 rpm, the culture broth was centrifuged at 10,000 rpm for 20 min ,after removing the pellet, supernatant was 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. The weight of the precipitated EPS was measured after drying 80°C for 24 hour. EPS was extracted according to the method followed by Ohno *et al.* (2000).

Quantification of EPS Production in different growth parameters

Estimation of Total Carbohydrate in EPS

The total carbohydrate content of crude EPS and dried EPS were determined at pH 6,7&8, different time of incubation 24,48,72 & 96 hours and different temperature 27°C ,37°C by Anthrone method (Gaudy, 1962) by using glucose as the standard.

Estimation of Total Protein in EPS

The total content of protein crude EPS and dried EPS were estimated by Lowry's method (Lowry,S,1951) by using Bovine serum albumin as the standard. The estimation was carried out at pH6, 7 & 8, different incubation time 24, 48, 72 & 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 incubation at 37°C.

Characterization of Pigment extract and Crude EPS

Pigment extract was subjected to UV-Vis spectrophotometry and Crude EPS was

characterized by FT-IR spectroscopy. (Both were Analyzed at STIC-Cochin University)

RESULTS AND DISCUSSION

The process of biofilm formation by microorganisms is influenced by various factors including nutrients level, pH, temperature, incubation period, ionic strength, culture concentration, etc., but the bacterial cell surface appendages (fimbriae, flagella, curli, exopolysaccharides, outer membrane proteins) and the contact surface characteristics are the most important among all of them as its formation begins when bacterial cells encounters a suitable surface and its outer surface adheres to the substratum. Interactions between bacterial cells and inorganic surfaces are different for adhesion onto hydrophobic or hydrophilic surfaces (Sommer *et al*., 1999).

Table 1. Characteristics of SY

No	Biochemical test	Result
1	Grams reaction	+
2	Shape	Cocci, Tetrads and occasionally clustered.
3.	Aerobic growth	+
4.	Growth at 55°C	-
5.	Mac conkey growth	+, NLF colonies.
6.	Motility	-
7.	Endospore	-
8.	Capsule	+
9.	Starch hydrolysis	+
10.	Gelatin hydrolysis	-
11.	Glucose fermentation	-
12.	Lactose fermentation	-
13.	Sucrose fermentation	-
14.	Mannitol fermentation	-
15.	H_2S production	+
16.	Indole test	-
17.	Methyl red	-
18.	VP	-
19.	Citrate	+
20.	Catalase	+
21	Oxidase	+
22.	Urease	+
23.	6.5% NaCl	+ slight growth
24.	Pigmentation	Yellow color
	Identify(genus)	*Micrococcus sp*

In this work, the biofilm producing bacterial strain isolated from PERIYAR RIVER. Biofilm production ability of the isolated bacteria was checked, adherence on two solid surfaces such as glass rod and lancet. Based on the attachment of the organisms both on two surfaces, selected for further biofilm studies. This result is in agreement with the report of Sinde and Carballo. (2000), Donlan, (2000) who reported that glass and stainless steel are surfaces that provide a greater bacterial adherence (Djordjevic *et al.,* 1979), Fletcher and Loeb (2002).

More attachment on both surfaces showed by SY and identified as *Micrococcus luteus* (Table 1) and which has yellow pigmented bacteria. The extracted yellow pigment has an antibacterial activity against *Escherichia coli, Klebsiella spp.,Salmonella typhi and Staphylococcus sp* (Table 2). Yellow pigment isolated from bacteriahas an antimicrobial activity against *Bacillus cereus* and *E. coli, reported by* Ahmed *et al*

Table 2. Antimicrobial Activity of Crude Pigment

Sample	**Test Organisms**	**Zone of Inhibition(mm)**
CRUDE PIGMENT	*Escherichia coli*	10
	Klebsiella spp.	18
	Salmonella typhi	14
	Staphylococcus sp.	10

Table 3. Antimicrobial Activity of Crude EPS

Sample	Test Organisms	Zone of Inhibition(mm)
CRUDE EPS	*Escherichia coli*	8
	Klebsiella spp.	10
	Salmonella typhi	12
	Staphylococcus sp.	12

(2012). Antibacterial activity of crude EPS was checked with *Escherichia coli, Klebsiella spp., Salmonella typhi* and *Staphylococcus sp* (Table 3) Pigment and Crude EPS produced zone of inhibition against almost all tested organisms.

Biofilm production (Dry EPS) and the yield at different incubation period (24hr, 48hr, 72hr and 96hr) and temperature (37°C) and at pH 7 were quantified (Fig. 1).The total protein and total carbohydrate of Crude EPS and Dry EPS were estimated at different incubation period (24hr, 48hr, 72hr and 96hr) and temperature (27C, 37°C) and at pH 6, 7&8 (Fig. 2). *Bacillus polymyxa* had produced EPS in the presence of sucrose (61g /g of sugar) in 31 hr of cultivation (Kim GJ *et al*., 1997). From the results, higher production of EPS (dry weight) was at 72hrs to 96 hrs, 49gm/L in 96 hrs of incubation time at 37°C. From the quantification results of Crude EPS supernatant, higher carbohydrate content was at 96 hrs and at pH 7, 149 mg/L and nearest values were in 72hr incubation, 144mg/L. The higher content of total protein in Crude EPS supernatant was 68mg/L at 96 hrs and at pH 7 while 62 mg/L at 72hr of incubation time. Total carbohydrates and total protein contents of dry EPS were comparatively lower than crude EPS and both were maximum at incubation temperature 37°C and 96hrs of incubation time and pH 7. Total carbohydrates and total protein contents of crude and dry EPS were lower at all other parameters studied.

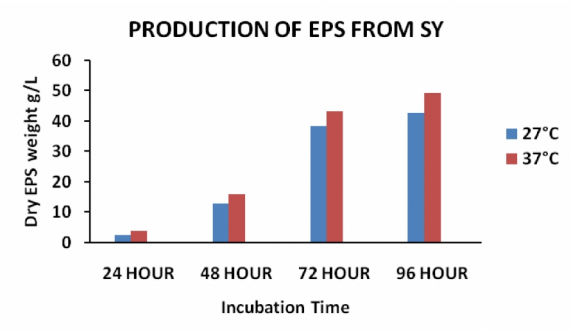


Fig. 1. Dry EPS (gm/L)

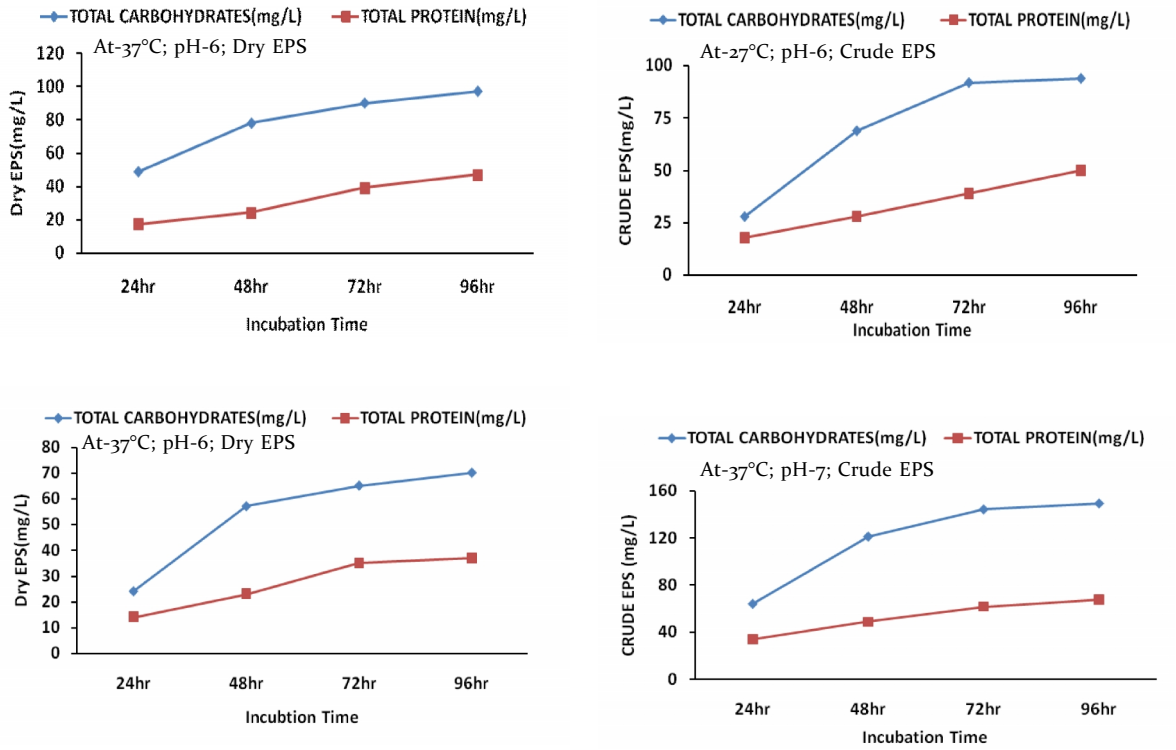


Fig. 2. Quantification of EPS in different growth parameters

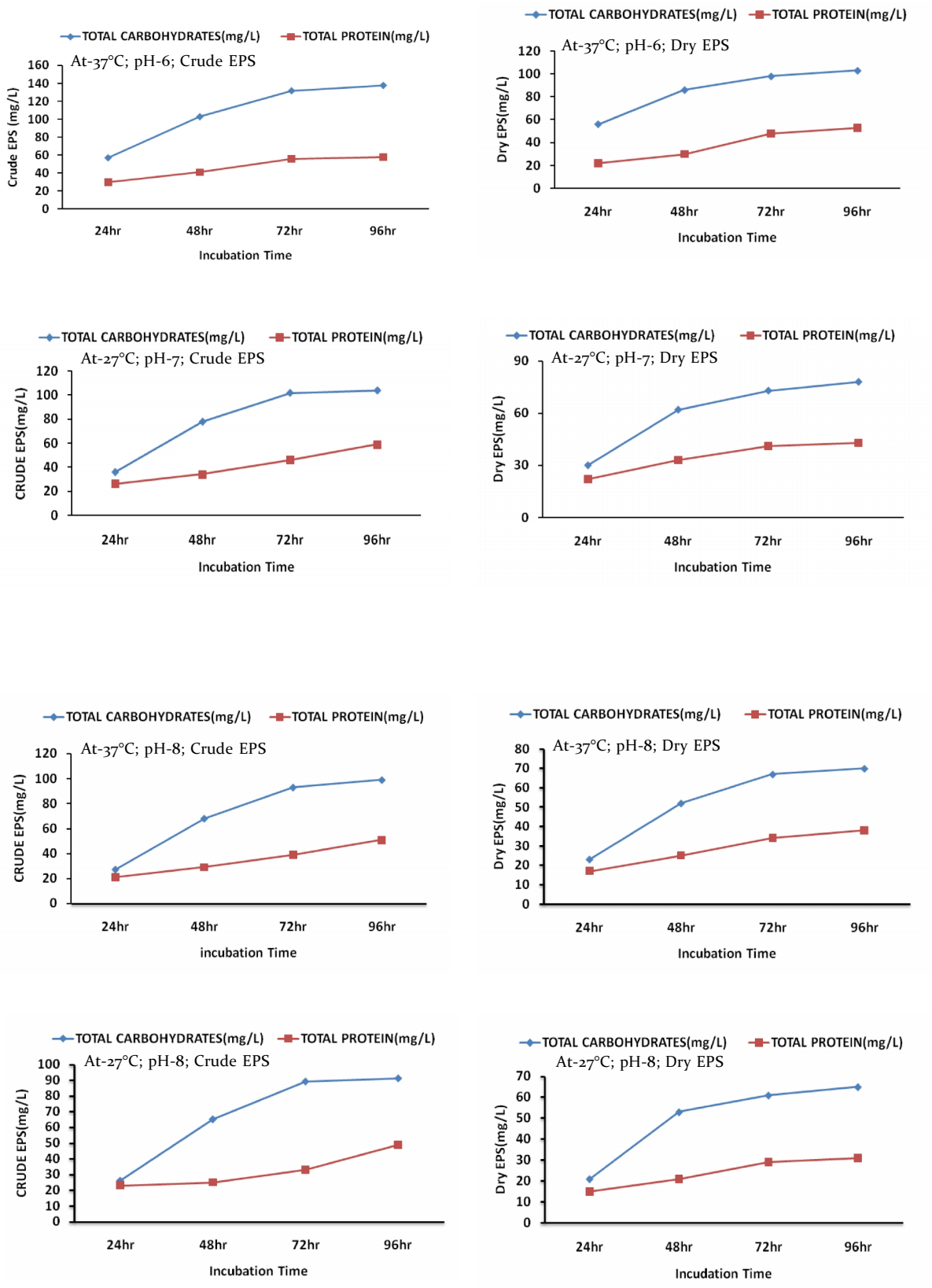


Fig. 2 (Conti....). Quantification of EPS in different growth parameters

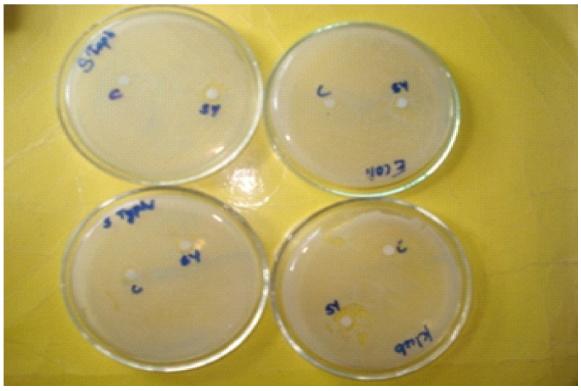


Fig. 3. Antibacterial activity of Pigment

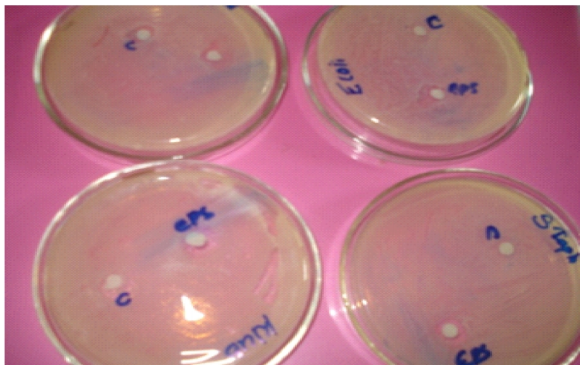
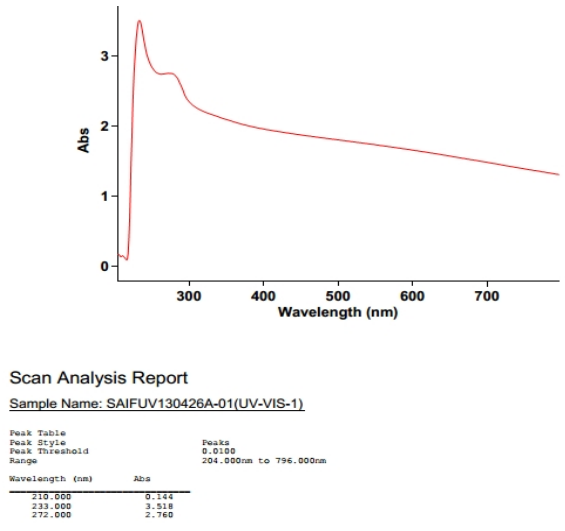


Fig. 4. Antibacterial activity of Crude EPS



Scan Analysis Report

Sample Name: SAIFUV130426A-01(UV-VIS-1)

Peak Table
Peak Style: Peaks
Peak Threshold: 0.0100
Range: 204.000nm to 796.000nm

Wavelength (nm)	Abs
210.000	0.144
233.000	3.518
272.000	2.760

Fig. 5. UV Vis analysis of Crude Pigment

UV Vis absorption of pigment extract was at 210-272nm in methanol and the peaks were at 210nm, 233nm and 272nm. It is reported that the most of peptide antibiotic exhibit maximum absorbance at 210-230 nm and 270-280 nm Kumar, Saini, and Shrivastava, 2009. An absorbance at 220-230 nm is corresponding to characteristic absorption of peptide bonds (Kumar *et al.*, 2009).

The FTIR spectra of the Crude EPS exhibited bands at various levels are obtained. The value 3344.59 cm^{-1} indicates cell proteins delivered several amide related bands 1°, 2° amines, 2260–2100 cm^{-1} alkynes 0.1633 cm^{-1} indicate 1° amines/ amide, 1329.40 and 1275.37 cm^{-1} indicate aromatic amines, 900–675 cm^{-1} indicates C–H aromatics. Further purification methods and analysis are needed to identify the compounds present in pigment and crude EPS.

EPS production was increased in the log phase and maximum at stationary phase that was in 96hr of incubation time .The optimum conditions for EPS production was at incubation temperature 37°C and 96hrs of incubation time and pH 7.

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REFERENCES

Bizani, D., Dominguez, A.P., and Brandelli, A. 2005. Purification and partial chemical characterization of the antimicrobial peptide cerein 8A, *Lett. Appl. Microbiol.*, 41(3): 269–273.

Costerton, J.W., Z. Lewaudowski, D.E. Caldwell, D.R. Korber and Lappin-Scott, J.M. 1999. Microbial. biofilms. *Annu. Rev. Microbiol.,* 49: 711-745.

Dalton, H.M. and March, P.E. 1998, Molecular genetics of bacterial attachment and biofouling. *Curr. Opin. Biotechnol.,* 9: 252-255.

Dang, H. and Lovell, C.R. 1994, Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. *Appl. Environ. Microbiol.*, 66: 467-475.

Djordjevic, D., Wiedmann M. and Mclands borough, L.A. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.*, 68(6): 2950-2958.

Donlan, R.M., 2000. Role of biofilms in Antimicrobial Resistance. *Asaio J.*, 46: 547- 552.

Dunner, W.M. and Jr. Biofilm. 2002. Adhesion seen any good biofilm lately? *Clinical Microbiology Review,* 15(2): 155-166.

Ezra, D., Castillo, U.F., Strobel, G.A., Hess, W.M., Porter, H., Jensen, J.B., Condron, M.A., Teplow, D.B., Sears, J., Maranta, M., Hunter, M., Weber, B., and Yaver, D. *Microbiology*, 150: 785–793.

Fletcher, M. and Loeb, G.I. 1979. Influence of substratum characteristics in the attachment of marine *psuedomonad* to solid surfaces. Appl. for assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.*, 68(6): 2950-2958.

Geesey. G.G and White, D.C. 1990, Determination of bacterial growth and activity at solid-liquid interfaces. *Ann. Rev. Microbiol.*, 44: 579-602.

Goundy. 1962. Determination of extracellular carbohydrate secretion in bacteria. *J. Bacteriology*.

Hamadi. H., Latrache A., El ghmari M. Ellouali, Mabrrouki, M. and Kouider. N. 2004. Effect of pH and ionic strength on hydrophobicity and electron donor and acceptor characteristics of Escherichia coli and Staphylococcus aureus.. *Annals of Microbiology*, 54(2): 213-225.

Kumar, A, Saini, P, and Shrivastava, J.N. 2009. Production of peptide antifungal antibiotic and biocontrol activity of Bacillus subtilis., Indian J. Exp. Biol., 47(1): 57-62.

Lee, I.Y., Seo, W.T. and Kim, G.J. 1997. Optimization of fermentation conditions for production of exopolysaccharide by Bacillus polymyxa. Bioprocess., Eng., 16: 71-75.

Lowry, S. 1951. Determinants of extracellular protein secretion in Gram-negative bacteria, J. Bacteriol., 174: 3423-3430 .

Madigan, M.T., Martinko, J.M. and Parker, J. 1997. Brock Biology of Microorganisms. 8th ed. Prentice Hall International Ltd., London, UK

Nadell, C.D., Xavier, J.B. and Foster, K.R. 2009. The sociobiology of biofilms. FEMS Microbiol Rev., 33: 206-24.

Ohno, N., Miura, N. Nakajima, M. and Yadomae, T. 2000. Antitumor 1-3-glucan from cultured fruit body of Sparasis crispa. Boil. Pharm. Bul., 23: 866- 872

Plante, C.J and Shriver, 1998, Differential lysis of sedimentary bacteria by Arenicola marina L.: examination of cell wall structure and exopolymeric capsules as correlates. Jour. Exp. Mar. Biol. Ecol., 229: 35-52.

Simoes, M., Simoes, L.C. and Vieira, M.J. 2010. A review of current and emergent biofilm control strategies. LWT- Food Sci. Technol., 43: 573-583.

Sinde, E. and Carballo, J. 2000. Attachment of Salmonella spp and Listeria monocytogenes to stainless steel, rubber and polytetrafluro ethylene: the influence of free energy and the effect of commercial sanitizers. Food Microbiol., 17: 439-447.

Sommer, P., Martin-Rouas, C. and Mettler, E. 1999. Influence of the adherent population level on biofilm population, structure and resistance to chlorination. Food Microbiology, 16: 503–515