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Biofilm inhibitory potential of *Chlamydomonas* sp. extract against *Pseudomonas aeruginosa*

Chari Nithya[1], Felix LewisOscar[1], Selvaraj Kanaga[2], Renganathan Kavitha[2], Dhamodharan Bakkiyaraj[1], Manivel Arunkumar[1], Naiyf S. Alharbi[3], Arunachalam Chinnathambi[3], Sulaiman Ali Alharbi[3], Nooruddin Thajuddin[1]*

[1]Division of Microbial Biodiversity and Bioenergy, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India.
[2] Department of Microbiology, AVS college of Arts and Science, Salem, Tamil Nadu, India.
[3]Department of Botany and Microbiology, College of Science, King Saud university, Riyadh- 11451, Kingdom of Saudi Arabia.
[*]Author for correspondence: E-mail: nthaju2002@yahoo.com, Tel: +91 431 2407082, Fax: +91 431 2407045.

Abstract:
Dominance of *Pseudomonas aeruginosa* over human health has been drastically increasing due to their biofilm forming ability. Increased resistance of bacteria over antimicrobial agents has also been a major cause for these issues. Biofilm forming bacteria have developed multidrug resistance and to overcome these issues researcher turned towards natural product which could eliminate the biofilm of *P. aeruginosa*. Among the microalgae *Chlamydomonas* sp. is considered as potential organism. The study had been focused on exploring the biofilm inhibitory potential of microalgae *Chlamydomonas* sp. against *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145. Biofilm quantification assay, Light microscopic and confocal microscopic images were taken, further quantification of cell surface hydrophobicity(CSH), Extracellular polymeric substances (EPS) and Pyocyanin was performed to determine biofilm inhibition by *Chlamydomonas* sp. methanolic extract (CME). The results showed the ability of CME to inhibit pathogenicity of *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145. CME inhibited 30% and 51% of biofilm, also significant reduction was observed in CSH (95% and 67%), EPS (28% and 48%) and Pyocyanin (68 % and 66%) against *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145 respectively. The study reveals the potential of *Chlamydomonas* sp. as biofilm inhibitory agent.

Keyword: Biofilm; *P. aeruginosa*; CSH; EPS; Pyocyanin; *Chlamydomonas* sp.

Introduction:

Biofilm is strong structural architecture of bacteria composed of self-developed polymeric matrix (Patterson et al. 1991). Bacteria adhere to any surface to form the complex structure of biofilm. The adhesion of biofilm to living and non-living surfaces occurs in a sequential process, they are (i) transport of microbes to a surface; (ii) initial attachment; (iii) formation of micro colonies; and (iv) biofilm maturation (Costerton et al., 1999; Tolker-Neilsen et al., 2000; Sauer et al., 2002). Initially bacteria are found as free floating planktonic cells and later form a firm structure by means of communal interaction (Potera 1999). Biofilm efficiently infects catheter, medical devices and contact lens (Yao et al., 2007). Biofilm forming bacteria are 100- 1000 fold resistant to antimicrobial compounds (Kociolek 2009).*Pseudomonas aeruginosa* is an increasingly prevalent opportunistic human pathogenic, Gram-negative bacterium causing nosocomial and life-threatening infections of immunocompromised patients (Van Delden and Iglewski 1998). *P. aeruginosa* is found in an estimated 10–20% of all hospital-acquired infections (Ikeno et al., 2007). Patients with cystic fibrosis are especially infected by *P. aeruginosa* leading to high rates of morbidity and mortality (Høiby and Frederiksen 2000; Lyczak et al., 2002). Biofilm formation has ruined antimicrobial treatment and resistance of bacterial cell to killing was also proved by *in vitro* susceptibility test (Macé et al., 2008). Low susceptibility of *P. aeruginosa* is attributed by the action of multidrug efflux pump (for example, *mexAB-oprM, mexXY*, etc.) responsible for the intrinsic resistance and low permeability of bacterial cellular envelope (Poole 1994; Li et al., 1995). Certainly new approaches have to be developed, which should not kill the bacteria instead inhibit the pathogenicity of *P. aeruginosa*.

Several factors influence *P. aeruginosa* biofilm formation are Quorum sensing (QS), Cell surface hydrophobicity (CSH) and Extracellular polymeric substances (EPS). Along with these three factors, pyocyanin production also plays a major role in pathogenicity of *P. aeruginosa*. QS is a process of cell to cell communication, which controls bacterial behavior in response to bacterial density and leads to the formation of tightly packed biofilm structure, along with the production of virulence factor (Karatan et al., 2009; Miller and Bassler 2001). CSH, EPS and Pyocyanin production is also controlled by QS (Marketon et al., 2003). CSH provides surface interaction between the host and pathogen (Swiatlo et al., 2002). EPS is responsible for the strong structure of the bacterial biofilm preventing it from external environment and antibacterial compounds (Sutherland 1972; Zhang and Miller 1992). Pyocyanin is the major phenazine pigment produced by *P. aeruginosa* and has been shown to contribute to its pathogenicity (Parsons 2007). Therefore targeting these factors will reduce the pathogenic potential of *P. aeruginosa* leading to biofilm inhibition. Search of novel compound is necessary to replace antibacterial agents. More number of works has been done using synthetic compound but the cost and side effects of synthetic compounds is the major issue (Kong et al., 2009). View of researchers has turned towards exploring natural compounds which are presumed to be safe and non-toxic is under consideration and could be the remedy against *P. aeruginosa* biofilm (Koo and Jeon 2009).

Among the natural resources work has been done on plant extract (Proksch et al., 1982; Bakkiyaraj et al., 2013), bacteria (Nithya et al., 2010), fungus (Aly et al. 2008), sponges and cyanobacteria (Cardellina et al., 1978; Marquez et al., 1998). But usage of microalgae for antibiofilm property have not been explored. Microalgae are diverse group of unicellular, eukaryotic and prokaryotic group of photosynthetic organism. Microalgae are potential organisms utilized for food, feed, cosmetics, fuel etc. Microalgae are rich source of Phycobiliproteins, carotenoids (Furuki et al., 2003), Polyunsaturated fatty acids (PUFAs) (Cardozo et al., 2007; Valencia et al., 2007; Bigogno et al., 2002) and Vitamins (Baker et al., 1981). Among the various microalgae *Chlamydomonas* is one of the most potential green algae. *Chlamydomonas* is a unicellular, flagellated green algae found in marine and fresh water habitat. Previous studies proved that *Chlamydomonas reinhardtii* secretes substance similar to Acyl homoserine lactone (AHL) stimulating bioluminescence in *Vibrio harveyi* and also found to affect quorum sensing controlled expression in wild type strain of *Sinorhizobium meliloti* (Teplitski et al., 2004). Based on these reports, the present study deals with exploring the marine strain of *Chlamydomonas* sp. for inhibiting *P. aeruginosa* biofilm. Methanolic extract of *Chlamydomonas* sp. was used to inhibit the factors like QS, CSH, EPS and pyocyanin which responsible for the biofilm and pathogenicity of *P. aeruginosa*.

Materials and methods:

Bacterial strains and culture condition:
P. aeruginosa PA14 and *P. aeruginosa* ATCC 10145 was provided by Dr. S. Karuthapandian, Department of Biotechnology, Alagappa University, Karaikudi, India. The bacterial culture were grown and maintained in LB agar plates at 37ºC.

Algal strains and culture condition:
The *Chlamydomonas* sp. was obtained from germplasm culture collection, Division of Microbial Biodiversity and Bioenergy, Department of Microbiology, Bharathidasan University, Tiruchirappalli, India. The algae culture was grown in MN+ medium and maintained at 25ºC.

Preparation of *Chlamydomonas* sp. methanolic extract (CME):
Biomass of *Chlamydomonas* sp. obtained was harvested, lyophilized and dried. 1 g of lyophilized culture of *Chlamydomonas* sp. was weighed and grinded with mortar and pestle into fine powder with lumps. The grinded sample was dissolved in 1 ml methanol, filter sterilized and allow to air dry. From the air dried sample 10mg of the sample were taken and diluted with 1:9 v/v of methanol: water (MilliQ). The final stock was used for further studies.

Effect of CME on *P. aeruginosa* biofilm:
Biofilm inhibition assay was performed in 24- well polystyrene plate using CME *against P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145. CME at 50 µg mL^{-1} concentration was used against *P. aeruginosa* PA14 and the effect of CME was tested against the biofilm of *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145. Sterile glass piece was added in each well, and the well was supplemented with 1ml LB broth and inoculated with 1% of *P. aeruginosa* overnight culture (10^7CFU/ml) along with CME at concentration of 50 µg $mL^{-1}$and incubated at 37˚C for 24 hours. After 24 hours of incubation the media along with the planktonic cells were discarded and the well was thoroughly rinsed twice with distilled water. Then 400 µl of 0.4% crystal violet solution (v/v) was added and allowed for 10 min. The dye was discarded and 1ml of absolute ethanol was added before solubilization of the dye and optical density was determined at 570 nm (Limsuwan et al., 2008). The glass slide were stained with 0.4% of crystal violet, excess dye was removed by washing with distilled water and visualized under light microscope at 40X magnification (You et al., 2007).

Antibacterial effect of CME:

The effects CME at 50 µg mL^{-1}concentrations were tested for antibacterial activity against the growth of the bacterial cultures used. Before the crystal violet assay the wells containing bacterial suspension all concentrations were analyzed spectrophotometrically at 660 nm. To check whether CME have antibacterial effect against the pathogens used, well diffusion method was used. In LB agar plates, bacterial cultures (O/N) were swabbed and wells were cut. CME at 50 µg mL^{-1} concentrations were loaded in well. LB broth alone was used as control. The plates were incubated at 37ºC overnight and observed for zone of clearance.

Effect of CME on Cell Surface hydrophobicity (CSH):

BATH assay was used to determine the cell surface hydrophobicity (CSH) of *P. aeruginosa* PA14and *P. aeruginosa* ATCC 10145 at 50 µg mL^{-1} concentrations. Bacterial culture inoculated with and without CME was placed in glass tube (OD_{600} = 0.8), 1 ml of treated and untreated bacterial culture were placed into glass tube. To this, 1ml of toluene was added and vortex for 2 min. the aqueous phase were examined for their OD. The percent hydrophobicity was calculated by the formula: 5%Hydrophobicity = [1-(OD_{600} after vortexing/OD_{600} before vortexing)] x100 (Thenmozhi et al., 2009).

Effect of CME on Extracellular polymeric substances (EPS):

EPS quantification was performed according to the method described by Favre-Bonté et al., (2003). Overnight culture of *P. aeruginosa* PA14and *P. aeruginosa* ATCC 10145 was added to 10 ml LB broth supplemented with and without CME extract and incubated for 24 hrs at 37º C. After 24 hrs of incubation 10 % of Trichloroacetic acid and equal volume of acetone were added. Incubated at 4º C for overnight, after incubation again it was centrifuged at 10,000 rpm for 10 min and pellet was weighed.

Effect of CME on Pyocyanin:

Overnight cultures of *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145 were grown in LB medium at 30ºC with shaking. The 10 µl of bacterial culture was inoculated in 1 ml of LB broth, to this 50 µg mL^{-1} of CME was added in the treated and negative control was left without adding the extract. Before that the treated and untreated culture were taken in the test tubes add 1ml of chloroform and vortex for 1 min. Then the organic phase were separate (it contain chloroform) and add 1ml of 0.2N HCl. Again vortex for 1 min, and the aqueous phase were taken and it quantified at 520 nm using UV Vis spectrophotometer (Essar et al., 1990).

Result and Discussion:

The work focuses on inhibition of *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145 biofilms using the microalgae *Chlamydomonas* sp. the effect of CME on *P. aeruginosa* biofilm was quantified and observed that 50µg mL^{-1}concentration of CME inhibited 30% and 51% of *P. aeruginosa* biofilm respectively **(Fig.1).**

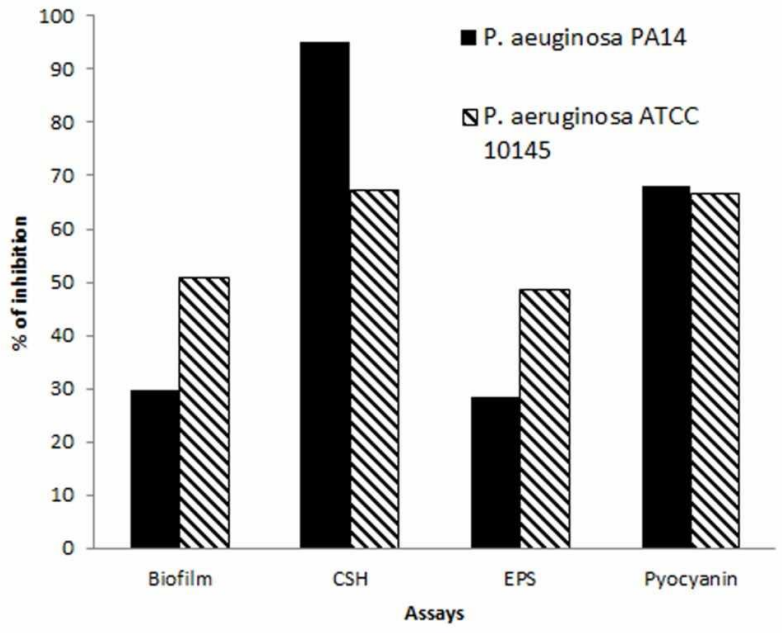

Fig.1: Effect CME on Biofilm, EPS, CSH and Pyocyanin of *P. aeruginosa* PA14 and *P. aeruginosa* 10145 at a concentration of 50µg mL^{-1}.

CME partially reduced the biofilm of the opportunistic pathogen. The AHL inhibiting property of *Chlamydomonas* sp. is known through earlier studies by Teplitski et al., (2004), but there is no information on the antibiofilm property of *Chlamydomonas* sp. Considering this fact CME at 50 µg mL^{-1}concentration was used to determine the antibiofilm property against *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145. Initially bacteria are free floating planktonic cells and later they tend to attach to any biotic or abiotic surface to form a strong structure of biofilm which further develops to a mature biofilm (Hoiby et al., 2001; Marshall 1994)**.** Mature biofilms are difficult to eradicate and more resistant to antimicrobials, thus forming a basis for the need to study the antibiofilm potential of novel metabolites (Davies et al., 1998; Brown et al., 1990)**.** Furanones showed reduction in biofilm morphology and reduction in thickness of biofilm, but it has not been quantified (Hentzer et al., 2003; Rasmussen et al. 2005). Only few works has been focused on quantification of bacterial biofilm (Bakkiyaraj et al., 2013)**.** Since biofilm is quorum sensing mediated process, there could be suppression in the biofilm inducing gene. Therefore utilization of *Chlamydomonas* sp. methanolic extract against *P. aeruginosa* biofilm could be a useful and new report.

Supporting results were obtained by microscopic visualization of the biofilm grown on glass surfaces. Light microscopic images showed reduction is biofilm colonized surface area **(Fig.2)** and the CLSM images showed the reduction in the thickness of *P. aeruginosa* biofilms upon treatment with CME **(Fig.3)**.

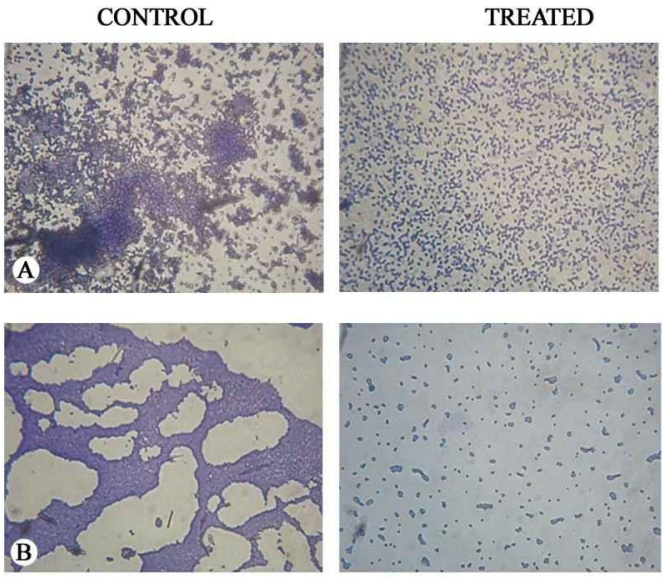

Fig.2: Light Microscopic images of biofilms formed by *P. aeruginosa* PA14 and *P. aeruginosa* 10145.

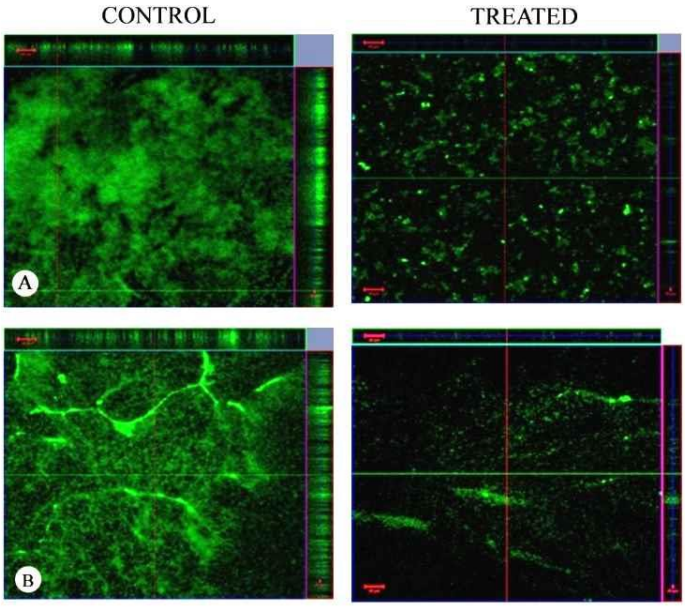

Fig.3: Confocal Laser Scanning Microscopic image of biofilms formed by (A). *P. aeruginosa* PA14 and (B). *P. aeruginosa* 10145.

Strong structural morphology of the biofilm provides resistance of the bacterial cells towards antimicrobial agent and human immune system. Since CME efficiently affects the size and thickness of the biofilm, significantly reducing the resistance of the pathogenic bacterial cells.

CME lack antibacterial activity against *P. aeruginosa*, which was confirmed by spectrophotometric quantification of bacterial growth **(Fig.4)** and standard agar well diffusion experiment **(Fig.5)**, which highlights the potential of CME as a candidate for anti-pathogenic agents which targets attenuation than growth inhibition.

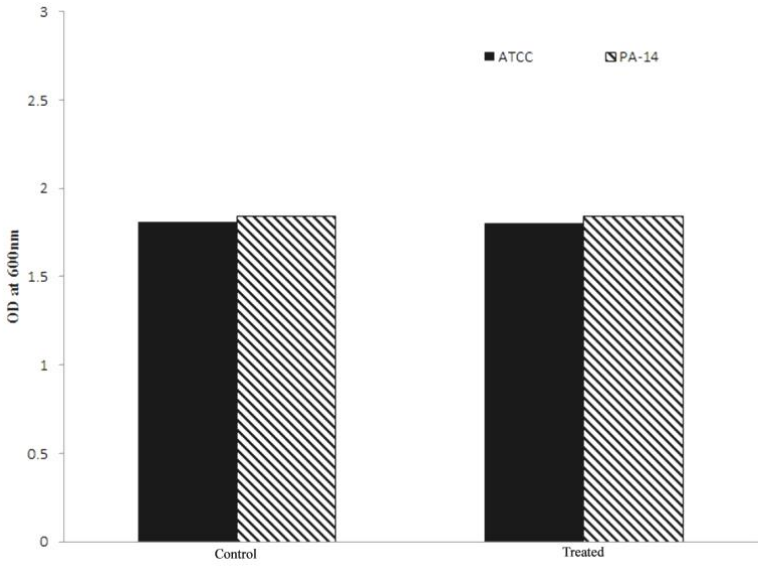

Fig.4: Effect of CME on the growth of (A). *P. aeruginosa* PA14 (B). *P. aeruginosa* 10145 at a concentration of 50µg mL^{-1}. Respective samples without bacterial extracts acted as controls. (this is for culture OD)

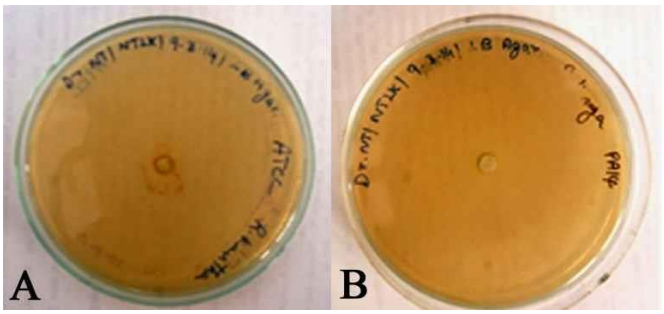

Fig.5: Antibacterial activity of CME against (A). *P. aeruginosa* PA-14 and (B).*P. aeruginosa* 10145 at 50µg mL^{-1}. (This is for well diffusion assay).

Significantly 95% and 67% of CSH inhibition was observed **(Fig.1)**, EPS reduction was about 28% and 48% in *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145 respectively **(Fig.1)** after treating with 50 µg mL^{-1} of CME. QS controls CSH and EPS in gram negative bacteria. The CSH and EPS production plays a major role in the strong adhesion and development of the rigid architecture of biofilm (Mah et al., 2003)**.** The hydrophobicity index allows the binding of several antimicrobial compounds leading to prevention of antimicrobial action (Flemming et al., 2007)**,** while EPS provides increased resistance of the bacteria towards antibiotics and harsh environmental conditions (Annuk et al., 1999)**.** Reduction in the EPS will lead to easy exposure of the bacterial cell to antimicrobial compounds and could pay way towards the reduction of bacterial biofilm. Reduced adhesion between the bacterial cells will affect the total count of bacterial population dwelling inside a biofilm matrix which will also support the inhibition of bacterial biofilm. Therefore targeting these two factors would efficiently weaken the strong bonding of the biofilm. CSH and EPS of Gram negative and Gram positive bacteria has already been targeted using plant extract (Razak et al., 2006; Kipnis et al., 2006) but no work has so far been done using CME. Since *Chlamydomonas* sp. has been so far commercialized as variable product, it could be an alternative to chemically synthesized products.

Around 68 % and 66% of pyocyanin inhibition was also observed when *P. aeruginosa* PA14 and *P. aeruginosa* ATCC 10145 was treated with 50 µg mL^{-1} of CME **(Fig.1)**. Pyocyanin is a phenanzine dye with low molecular weight, pyocyanin production is QS mediated process, considerable depletion in the nutrition level leads to pyocyanin production and it is an important pathogenic factor of *P. aeruginosa*. Inhibition of pyocyanin could lead to the reduction in the pathogenicity of *P. aeruginosa* (Lau et al. 2004)**.**

The result revealed the biofilm inhibitory potential of microalga *Chlamydomonas* sp. Further purification and characterization of the pure fragment of CME could major be a solution for the treatment of infectious disease caused by *P. aeruginosa*. Therefore reduction in the toxicity could also be reduced by utilizing CME. Thus the present study reveals that CME can efficiently inhibit the pathogenicity of *P. aeruginosa* including biofilm formation, QS, CSH, EPS and Pyocyanin.

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Conflict of Interest: Nothing to declare

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