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Antibiofilm Activity of Crude Cell Free Extract from *Bacillus subtilis* S01 against *E. coli*

S. M. A. Sayem [1*], A. J. M. T. Chowdhury[1], M. Z. Alam[1], P. K. Sarker[2]

[1]Department of of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet, Bangladesh

[2]Microbial Biotechnology Division, National Institute of Biotechnology, Savar, Dhaka, Bangladesh

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Abstract

Antibiofilm phenomenon has become a novel area of research for removing deleterious biofilm. In the present study, strains from different environmental sources were tested for screening antibiofilm compounds. Crude extracts from various microorganisms were evaluated for antibiofilm phenomenon through crystal violet assay and growth curve analysis. Characterization of antibiofilm compound was performed by pre-coating microtiter plate and Cell Surface Hydrophobicity experiment. Among the organisms, cell free extracts (5% v/v) from *Bacillus subtilis* S01 inhibited the development of *E. coli* PHL628 biofilm by 63%. The cell free extracts possessed no amylase activity and had no effect on the planktonic growth of biofilm forming bacteria. Moreover, no competition with quorum sensing analogues was found with the extract. Biofilm formation was more inhibited (76%) in the *B. subtilis* S01 extract pre-coated wells than uncoated wells (62%). However, no effect on preformed biofilm was observed with the extracts of *B. subtilis* S01. The extract also reduced the cell surface hydrophobicity (69%) of the biofilm forming bacteria. The present study indicated that the crude extracts of *B. subtilis* S01 from soil origin has anti-adherence properties towards biotic and abiotic surfaces and thus can be a potential candidate in preventing the development of biofilm.

Keywords: *Bacillus subtilis*; Biofilm; Pre-coating; Antibiofilm; Anti-adherence.

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1. Introduction

Microorganisms have the natural tendency to attach to biotic and abiotic surfaces, to multiply and to embed themselves in a three-dimensional gelatinous slimy matrix of self-produced extracellular polymeric substances (EPS) comprising of polysaccharides, proteins, DNA and other substances [1,2]. Today biofilm is considered as the most prevalent mode of microbial growth. However, the ability of microbes to exist within

* *Corresponding author:* asayem08@yahoo.com

biofilm allows them to withstand harsh environmental conditions and antimicrobial agents [3]. Biofilms have been reported to show increased resistance to antimicrobial agents including antibiotics compared to free-floating cells [4,5]. When antimicrobial agents or biocides are used to kill or inhibit microorganisms within biofilm, the sensitive cells die off rapidly but the resistant cells grow in a competitor free environment, thereby creating strong selection pressure for resistance against that antimicrobial or biocide [6]. Therefore, the emergence of bacterial resistance against available antibiotics is a common phenomenon which creates demand for the discovery of new environmental friendly non-antimicrobial approaches.

In recent years, a series of different approaches have been developed targeting biofilm that have the potential to suppress resistance. Most studies seek to prevent biofilm formation while others aim to disrupt the polymeric ties that bind the biofilms together [7-12]. Although currently a lot of research is going on for isolation of antibiofilm compounds involving many natural compounds ranging from plant extracts to bacterial metabolites, the focus is mainly on preventing the initial adhesion of bacteria to a surface or lowering the force of adhesion between bacteria and a surface to facilitate removal [13]. Several anti-adhesion strategies have been proposed, including the development of receptor blocks, pre-conditioning of the surfaces with biosurfactants, enzymes, polysaccharides or other bioactive compounds or alteration of the physicochemical properties of the outermost layer of biofilm forming cells for interfering cell-to-surface and/or cell-to-cell communication [14-19]. Such approaches might be able to disarm the pathogenic biofilm forming bacteria, establishing "evolution-proof" solution with no selection for resistance. Although many researchers have been striving to minimize or avert the initial adhesion during biofilm development, very few compounds from microbial origin have been found to be potential against biofilm development.

The purpose of the present research was to screen compounds from various microbial sources that inhibit the initial stage of biofilm development without having any antimicrobial effect and to evaluate the mechanism of the potential crude extracts for inhibiting cell-surface interaction and cell-cell attachment during biofilm development.

2. Materials and Method

2.1. *Microorganism*

The reference bacterial culture *E. coli* PHL628 was obtained from Microbiology laboratory of the Department of Structural and Functional Biology, University of Naples Federico II, Italy. The other bacterial cultures were obtained from Environmental Microbiology laboratory of the Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet, Bangladesh. The cultures were maintained on Tryptone Yeast extract (TY) agar medium. The organisms were stored at 4°C in refrigerator for routine laboratory use. For the long term preservation, the log growth phase of the bacteria was maintained in 15% glycerol broth at -20 °C.

2.2. *Extract collection*

Seven bacterial species (*Staphylococcus epidermis, Staphylococcus aureus, Pseudomonas fluorescens, Pseudomonas aeruginosa, Bacillus pumilus, Bacillus cereus, and Bacillus subtilis* S01) were sub-cultured on TY agar plate media and incubated overnight at 37 °C. Single colony from each plate was inoculated into TY broth, incubated in shaker at 37 °C for 48 h at 120 rpm. 10 mL from each culture was poured in falcon tube and centrifuged at 6000 × g for 10 min. Extracts were transferred into different test tubes respectively and pellets were discarded. The extracts were filtered through 0.2 μm pore size filter (Cellulose Nitrate Filter, Sartorius Stedim Biotech GmbH 37070 Goettingen Germany) membrane. 100 μL extract was spread on TY agar plate and incubated overnight to ensure that no cells were present in filtrates.

2.3. *Screening for compounds for biofilm inhibition*

Filtered extracts were used to perform the assay for biofilm formation. The method used was a modified version of that described by Djordjevic, 2002 [20]. Overnight cultures of *E. coli* PHL628 strain grown at 37 °C in TY broth was refreshed in TY broth and incubated again at 37 °C for 5-6 h. 200 μL of inoculum was introduced in the 96 well polystyrene microtiter plate with an initial turbidity of 0.05 at 600 nm in presence of the filtered extracts at a concentration of 5% (v/v). The microtiter plate was then left at 30°C for 48 h in static condition. To assay the biofilm formation, the remaining medium in the incubated microtiter plate was removed and the wells were washed two times with sterile distilled water to remove loosely associated bacteria. Plates were air-dried for 10 min and each well was stained with 200 μL of 0.01% crystal violet solution for 45 min. After staining, plates were washed with sterile distilled water four times. The quantitative analysis of biofilm production was performed by adding 200 μL of ethanol-acetone solution (4:1) to de-stain the wells. The level (OD) of the crystal violet present in the de-staining solution was measured at 570 nm after 5 min.

2.4. *Growth curve analysis*

The extract was added at a concentration of 5% (v/v) to a conical flask containing 50 mL of TY broth, to which 1% inoculum from the overnight culture of *E. coli* PHL628 was added. The flask was then incubated at 37 °C and 120 rpm. Growth medium with the addition of bacterial inoculum in the absence of the extract was used as a control. OD values at 600 nm were recorded for up to 7 h at 30 min intervals.

2.5. *Competition with quorum sensing signals*

E. coli PHL628 extract along with the potential antibiofilm extract were prepared by using the same conditions and methods used above. Equal volumes of the two extracts at a concentration of 5% (v/v) were added either in combination or alone in the micro titer

plate containing a culture of *E. coli* PHL628 at an initial turbidity of 0.05 at 600 nm and biofilm formation was measured as described above.

2.6. *Pre-coating of microtiter plate*

Wells were treated with 200 µL of the extract for 24 h and then the unabsorbed extract was withdrawn from the wells. Such pre-coated wells were inoculated with *E. coli* PHL628 cultures having an OD of 0.05 at 600 nm. In another set of wells that were not coated with the extract, the fresh culture of *E. coli* PHL628 having the same density mentioned above were added together with the extract (10 µL). In a parallel experiment, the extract (10 µL) was added in pre-formed biofilm of *E. coli* PHL628 in another set of wells. The microtiter plates were then incubated for 36 h in static conditions and biofilm formation was estimated. The control experiments were carried out in wells that were not pre-coated or initially added with the extract.

2.7. *Microbial cell surface hydrophobicity (CSH) assay*

Hydrophobicity of the culture of *E. coli* PHL628 was determined by using MATH (microbial adhesion to hydrocarbons) assay as a measure of their adherence to the hydrophobic hydrocarbon (toluene) following the procedure described by Courtney, 2009 [21]. Briefly, 1 mL of bacterial culture (OD530 nm = 1.0) was placed into glass tubes and 100 µL of toluene along with the extract (5% v/v) was added. The mixtures were vigorously vortexed for 2 min and incubated 10 min at room temperature to allow phase separation, then the OD530 nm of the lower, aqueous phase was recorded. Controls consisted of cells alone incubated with toluene. The percentage of hydrophobicity was calculated according to the formula:

$$\% \text{ Hydrophobicity} = (1 - (\text{OD530 nm after vortexing} / \text{OD530 nm before vortexing})) \times 100$$

2.8. Statistical analyses

Statistical analysis was performed using SPSS. *P* values less than 0.05 were considered significant. The data are presented as mean ± standard deviation values of independent replicates.

3. Results

3.1. *Antibiofilm effect of cell free extracts of B. subtilis S01*

In order to investigate bacterial strains having antibiofilm compounds against biofilm forming bacteria *E. coli* PHL628, crude extracts from different species of *Staphylococcus*, *Pseudomonas* and *Bacillus* from different environmental sources were screened. Out of the cell free extracts, extracts from *P. aeruginosa, P. fluorescens* and *B. subtilis* S01 reduced the biofilm development of *E. coli* PHL628 by 56, 56 and 63% respectively (Fig.

1). As the biofilm inhibition with *B. subtilis* S01 of soil origin was more than that of *Pseudomonas* spp., *B. subtilis* S01 was chosen for further studies.

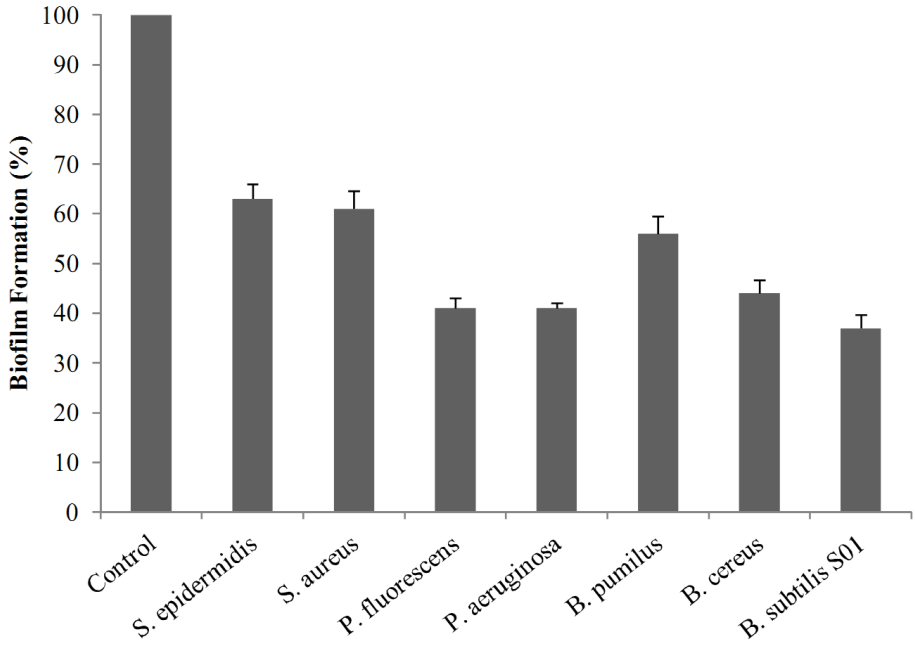

Fig. 1. Screening cell free extracts from different bacteria for antibiofilm activity. The absorbance was used to calculate the "biofilm formation" on the Y axis. X axis represents cell free extracts from different strains. The 100% is represented by *E. coli* PHL628 produced biofilm. Bars represent means ± standard errors for six replicates. Statistical analysis demonstrates significant difference between the tests and the control ($P < 0.05$).

3.2. *Bactericidal effect*

Experiments revealed that the growth of *E. coli* PHL628 was not affected with crude extracts of *B. subtilis* S01 (data not shown) which was confirmed by growth curve analysis. Growth curves of *E. coli* PHL628 were measured in both presence and absence of the cell free extract of *B. subtilis* S01. The resulting growth curves of *E. coli* PHL628 in both conditions almost overlapped each other (Fig. 2) which indicated the neutral effect of the extract on the growth of biofilm-producing *E. coli* PHL628.

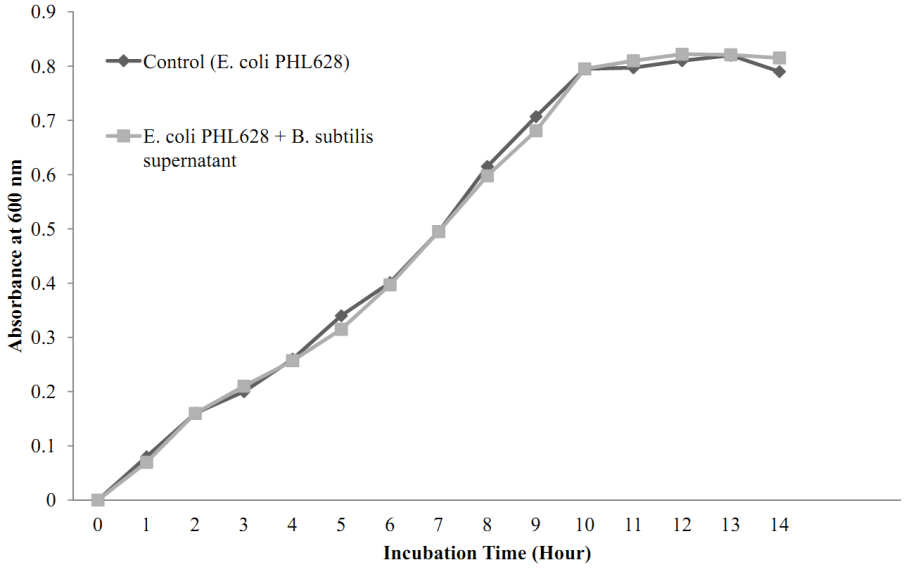

Fig. 2. Effect of *B. subtilis* S01 extract on the growth curve of *E. coli* PHL628.

3.3. *Quorum sensing competition*

In the present study, in search of quorum sensing analogues, extracts from *B. subtilis* S01 and *E. coli* PHL628 were concurrently and separately tested for identifying the presence of quorum sensing analogues assuming that quorum sensing signals presumably present in extracts of the target biofilm forming bacteria. No effect was observed either with the concurrent use of the two extracts or with the use of extract alone (Fig. 3).

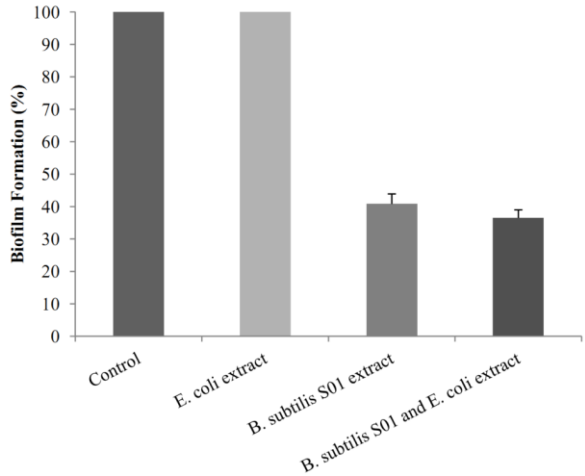

Fig. 3. Competition between quorum sensing compounds and *B. subtilis* extract. Bars represent means ± standard errors for six replicates. Statistical analysis demonstrates significant difference between the tests and the control (P<0.05).

3.4. *Anti-adherence effect*

To study the mechanism of action of the extract, some wells of microtiter plate were pre-coated with the extract and were then allowed to form *E. coli* PHL628 biofilm. Biofilm formation of *E. coli* PHL628 was more inhibited (76%) in the *B. subtilis* S01 extract pre-coated wells than uncoated wells (62%) (Fig. 4). However, the extract was found to be ineffective in removing already formed biofilm of *E. coli* (Fig. 4).

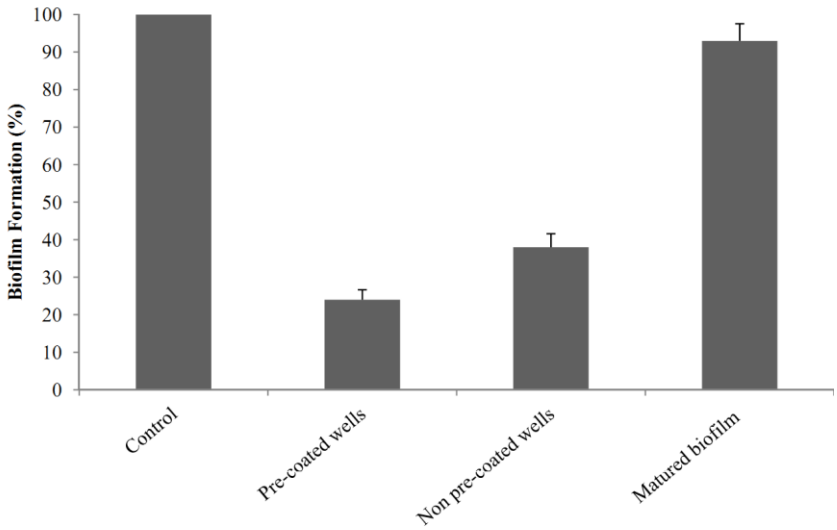

Fig. 4. Effect of pre-coating with *B. subtilis* S01 extracts on biofilm inhibition. Bars represent means ± standard errors for six replicates. Statistical analysis demonstrates significant difference between the tests and the control (P<0.05).

3.5. *Cell surface hydrophobicity*

Cell surface hydrophobicity experiment of *E. coli* PHL628 by the extract of *B. subtilis* S01 revealed that the extract decreased the cell surface hydrophobicity of *E. coli* PHL628 by 69% (Fig. 5) as compared to control.

4. Discussion

The main purpose of the present study was to identify compounds from natural sources that down regulate biofilm development ruling out the chance to develop selection pressure for resistance. Crude cell free extracts from the studied *B. subtilis* S01 was promising since their presence had no effect on planktonic growth observed during the growth curve analysis experiment (Fig. 2). Such antibiofilm effect without decreasing bacterial viability has also been reported with the extracts (exo-polysaccharide) from marine bacterium *Vibrio* sp. [22]. Similarly, coral associated bacterial extracts having

antibiofilm potential did not show any antibacterial activity [23]. Extract from a marine sponge associated *B. licheniformis* also displayed antibiofilm activity without affecting bacterial growth [24]. Almost similar results were obtained by Pihl *et al.* [25] who reported that the supernatant from *P. aeruginosa* inhibited biofilm formation and dispersed cells from preformed *S. epidermidis* biofilms.

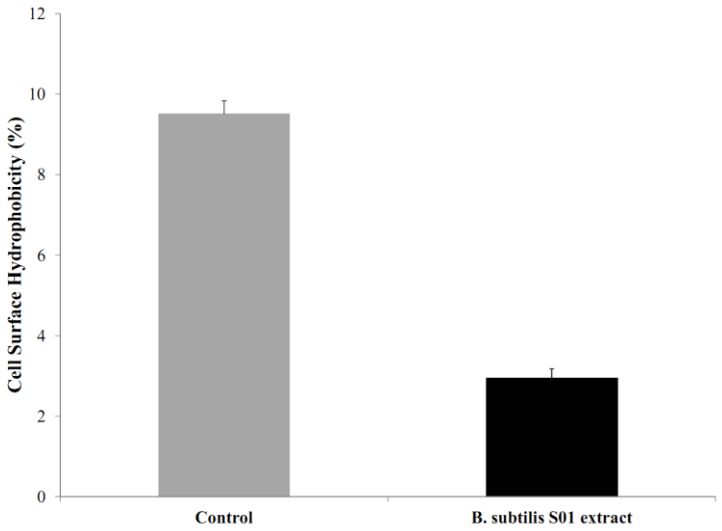

Fig. 5. Effect of *B. subtilis* S01 extract on Cell Surface Hydrophobicity (CSH) of *E. coli* PHL628. Statistical analysis demonstrates significant difference between the tests and the control ($P < 0.05$).

Since extracellular α-amylase from *Bacillus subtilis* S8-18 of marine origin was proved as an antibiofilm agent against methicillin-resistant *Staphylococcus aureus* (MRSA), *Vibrio cholera* and *Pseudomonas aeruginosa* ATCC10145 [26], the finding of antibiofilm effect of the crude extracts of *B. subtilis* S01 in the present study is not surprising. However, no clear zone was observed (data not shown) while testing amylase activity through zone clearing technique by Atlas *et al.* [27] which clearly indicates that the antibiofilm activity of the extract is not due to amylase activity as reported previously [26].

Quorum sensing inhibition also played a key role in inhibiting biofilm formation [28-31]. Most recently, Sing *et al.* [32] reported that 3-oxo-C12-HSL based quorum sensing system of *P. aeruginosa* showed inhibitory effect against *S. epidermidis* biofilm, initial attachment, and EPS production. However, in our present study no competition was observed between the two extracts from the studied *B. subtilis* and the tested *E. coli* PHL628 while down regulating biofilm development. It can be concluded that the extracts mediate antibiofilm effect through means other than the quorum sensing inhibition.

In order to find out the inhibition stage of biofilm development by the extract, pre-coated and uncoated wells as well as application of extract on preformed biofilm wells

were compared. The pre-coating experiment with the extracts indicated that the extract modified the polystyrene wells of microtiter plate in a negative way so that initial cell to surface interaction, prerequisite for biofilm formation, is blocked. However, the extract was found to be ineffective in degrading biofilm since it showed no effect on already formed biofilm of *E. coli*. Capsular polysaccharides of *Klebsiella Pneumoniae*, also exhibited nonbiocidal antibiofilm activity by modifying the initial bacteria-surface interactions rather than disrupting the bacterial interactions [15]. Similar results were found where bioactive compound can produce anti-adherence effects between microorganisms and surfaces [16,33]. The *E. coli* group II CPS and exopolysaccharides of marine *Vibrio* sp. were reported to inhibit biofilm formation not only by weakening cell surface contacts but also reducing cell-cell interactions or disrupting the interactions of cell-surfaces and cell-cell [19].

In addition to anti-adherence effects, reduced cell surface hydrophobicity of the target bacteria were observed which indicated that the extracts can alter the physicochemical characteristics of the outermost surface of biofilm forming bacteria. Our results are in good agreement with previous studies with coral and marine sponge associated bacterial extracts [23,24,34]. Fonseca *et al.* [35] also reported similar observations with some antibiotics. Reduced cell surface hydrophobicity indicates less colonization, thereby less biofilm development which augments the overall antibiofilm effect.

5. Conclusion

To our knowledge, anti-adherence nature and considerable reduction of cell surface hydrophobicity by the extracts having no α-amylase activity from *B. subtilis* of soil origin have been reported for the first time here. In conclusion, the present study revealed that the crude extracts of *B. subtilis* S01 of soil origin is a promising source for antibiofilm compounds due to several features. The extract inhibits biofilm formation without affecting planktonic growth, displays antibiofilm activity by reducing initial attachment of biofilm forming cells to surfaces and modulates the surface of the biofilm forming bacteria in such a way that there is a considerable reduction or inhibition of cell-cell or cell-surface interactions. More studies need to be carried out on the supernatant to identify the molecular structure of the active anti-biofilm compound present in the supernatant and finally to explore detailed molecular mechanism of action in inhibiting biofilm.

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