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Reduction of *Bacillus cereus sensulato* Biofilms on Stainless Steel Surfaces by *Pestalotiopsis* sp. Culture Crude Extract

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Abstract: ***The biofilm formation of fourBacillus cereus sensulato isolates was induced on milk pre-soiled stainless steel coupons (20 ×5 mm) under nutrient-depleted broth medium for a total of 48 h. Biofilms on these coupons were immersed for 15 min in 25, 50, or 100 mg/mL of Pestalotiopsis sp. (P-03) culture crude extract at 25 or 50°C. Results showed that the biofilm cells were significantly reduced ($p < 0.05$) as compare to nontreated biofilms after immersion in 50 and 100 mg/mL of P-03 crude extract, which the most significant was found at 100 mg/mL with 2-Log reduction (99%). The crude secondary metabolites obtained from P-03 fermentation broth cultures were detected through thin layer chromatography. Pestalotiopsis spp. hasfound to produce novel secondary metabolites with a wide range of bioactivities. Additionally, fluorescence micrographs were supplemented and showed decreasing light intensities emitted by the fluorescent dye ethidium bromide at 605 nm. The interactive 3-D surface plots, which generated using ImageJ 1.52a software, indicated that the extracellular matrix was disrupted. This study concluded that the unknown secondary metabolites from P-03 crude extract have antibiofilm activity against B. cereus s.l. biofilms on stainless steel surfaces and could be an alternative method for biofilm reduction.***

Keywords: antibiofilm activity, biofilm reduction, microbiology, secondary metabolites

1. Introduction

On soiled substrata in food industries (*e.g.*stainless steel surfaces), biofilms are able to form if cleaning and sanitization programs are conducted inappropriately.When biofilm cells come together, they produce extracellular matrix (ECM) thatprotects them from harsh environmental factors [1]. ECM is primarily made of complex exopolysaccharides (EPS) and proteins, as well as some trace elements. Because of intricate structure and functions, biofilms can tolerate chemicals and disinfectants that are used for cleaning and sanitization of surfaces in food production [2, 3]. Therefore, biofilms are frequent sources of cross-contamination resulting to product spoilages and foodborne illnesses. An example of food pathogen is the *Bacillus cereussensulato* (*s.l.*) that is frequently isolated in dairy production and has the ability to cause food poisoning by producing diarrheal or emetic type of toxins. The spores of *B. cereus s.l.* have the ability to attach on stainless steel surfaces because of appendages and hydrophobic characteristics.

Due tobiocidal resistance, researchers have led to the exploration of alternative antibiofilm agents for biofilm control and mitigation. For instance, authors have reviewed the effectiveness of various enzymes and its possible application in food industry to mitigate biofilms [4]. The use of bacteriophages, as phage cocktails, showed promising effects in reducing biofilms of *Salmonella* strains and *Listeria monocytogenes*on hard surfaces [5, 6]. Also, the biologically active compounds from several fungal species such as microporenic acids [7] and arvoredols [8] have showed biofilm inhibiting factors. On the other hand, the Genus *Pestalotiopsis*is a group of ascomycete fungi that have been reported and documented the many novel secondary metabolites produced by various species of this genus [9, 10], which demonstrating *Pestalotiopsis*to be a rich source for bioprospecting. This group of ascomycete fungi is widely distributed in tropical and temperate regions of the world that can be isolated as plant endophytes.

Diverse plant fungal endophytes have been studied for their secondary metabolites that have exhibited antibiofilm activities [11, 12]. To this date, no specific studies were published about the potential of secondary metabolites produced by the *Pestalotiopsis* spp. as antibiofilm agent. Thus, the main objective of present study is to test the antibiofilm activity of crude extract from *Pestalotiopsis* sp. by reducing the*B. cereuss.l.*, as model organism, biofilms grown on milk pre-soiled stainless steel coupons (SSCs). The crude extract from *Pestalotiopsis* sp. fermentation cultures is obtained using EtOAc and theoretically contains biologically active secondary metabolites. The 48-hr old *B. cereuss.l.* biofilms on SSCs are immersed in different prepared concentrations of crude extract at 50°C, which is the lowest recommended temperature of water used during sanitization in food production, and at room temperature – 25°C – within 15 min. To support the main objective, the biofilms are subjected to fluorescence microscopy and the micrographs were gathered for image analysis using software. Consequently, the study will provide insight on the possible application of secondary metabolites fromthe *Pestalotiopsis* sp. as antibiofilm agent thus alternative method for biofilm control in food industries.

2. Methodology

2.1. Bacterial and fungal test isolates

The four*B. cereus s.l.* test isolates (B-01, B-06, B-03, B-10) were used for biofilm formation on SSCs. The *Pestalotiopsis* sp. (P-03), which represents an undescribed species, was isolated from a *Mangiferaindica* leaf by the surface sterilization method [13] using quarter-strength potato dextrose agar (PDA). The fungal colony was identified according to its conidial morphology and culture characteristics. All test isolates were deposited at the University of Santo Tomas Collection of Microbial Strains (UST-CMS).

2.2. Fungal culture crude extraction and thin layer chromatography-bioautography

A total of 15 L fermentation broth cultures of P-03 was harvested after three weeks incubation at 28°C with constant shaking at 150 rpm/min (Model 420 Series, Forma Scientific Orbital Shaker, Thermo Scientific, U.S.) [14]. The collected filtrates from the cultures were extracted three times with EtOAc (1:1 [v/v]). The solvent phase was collected and evaporated using rotary evaporator (Eyela N-1200A, Tokyo Rikakikai Co. Ltd.) under reduced pressure at 40°C. The residues (crude extract) stored at 4°C for further analysis. The thin layer chromatography (TLC) was conducted using mobile phases: (**1**) 9:1 chloroform/methanol, (**2**) 6:3 chloroform/acetonitrile, (**3**) 9:1 chloroform/EtOAc, and (**4**) 3:1 EtOAc/hexane. The EtOAc crude extract was spotted on TLC plates (3 × 10 cm) (TLC Silica Gel 60 F_{254}, Merck) and the developed spots were viewed under UV lights (MinUVIS; Desaga, Heidelberg, Germany) at 254 and 366 nm. The chromatograms were cut and placed upside-down on 24-hr old lawns of *B. cereus s.l.* isolates on trypticase soy agar (TSA) agar plates to determine the bioactivity.

2.3. Formation of *B. cereus s.l.* biofilms on SSCs

Spore suspensions of *B. cereus s.l.* isolates were prepared [15]. The milk pre-soiled SSCs were made [16] then immersed in *B. cereus s.l.* spore suspensions for 5 sec then transferred into sterile vials with 1 mL diluted TSB (1:20 [v/v]) and incubated statically at 30°C for 24 hr. After incubation, the SSCs were gently removed from the vials, rinsed with sterile water then transferred into new sterile vials with 1 mL full-strength TSB and incubated again under the same conditions as described earlier. Test controls were vials containing SSCs and broth only. After incubation, the SSCs were used for immersion in different concentrations of P-03 culture crude extract.

2.4. Immersion of *B. cereus s.l.* biofilms on SSCs in P-03 culture crude extract

The *B. cereus s.l.* biofilms on SSCs were immersed for 15 min in 100, 50, or 25 mg/mL of P-03 culture crude extract at 25°C (Set 1) or 50°C (Set 2). Each biofilm was tested in triplicate while the nontreatedbiofilms were immersed in sterile distilled water only. After immersion, all SSCs were carefully rinsed with sterile water, transferred into centrifuge tubes with sterile PBS, pH 7.2, and vigorously shaken. Serial dilutions were prepared with 0.1% peptone water and plated on Petri plates with molten TSA to enumerate the remaining biofilm cells. After 48 hr of incubation at 30°C, the bacterial colonies were counted, then the colony forming units (CFUs) were calculated and expressed in logarithmic form (Log CFU/coupon) [17]. The differences of before and after the treatment were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test ($p< 0.05$).

2.5. Fluorescence microscopy and biofilm imaging

The treated and nontreatedbiofilms on SSCs were stained with 1% ethidium bromide (EtBr) for 10 min. The SSCs were rinsed carefully with sterile water then air dried and viewed using high power objective (HPO) under a fluorescence microscope (Olympus BX43) with an attached camera (Olympus DP25) and UV illumination at 605 nm. The fluorescence micrographs were analyzed using ImageJ (Image Processing and Analysis in Java) 1.52a software (http://imagej.nih.gov.ij). The micrographs used for ImageJ analysis were approximately 131 × 131 pixels in size. The software generated interactive 3-D surface plots, where the z-axis indicated the recorded intensities of each coordinate within the 131 × 131 pixels (the x- and y- axes).

3. Results

Results showed that among the four mobile phases used for TLC, the highest number of crude secondary metabolites (9) was found in mobile phase**2**and followed by the mobile phase **4** (7) on varying polarities. The bioautographic procedure revealed that these crude secondary metabolites have no antibacterial properties against the 24-hr old *B. cereus s.l.* isolates on TSA plates due to low concentrations of the metabolites present in chromatograms. The collected crude extract was still used for antibiofilm assay against the *B. cereus s.l.* biofilms on SSCs.

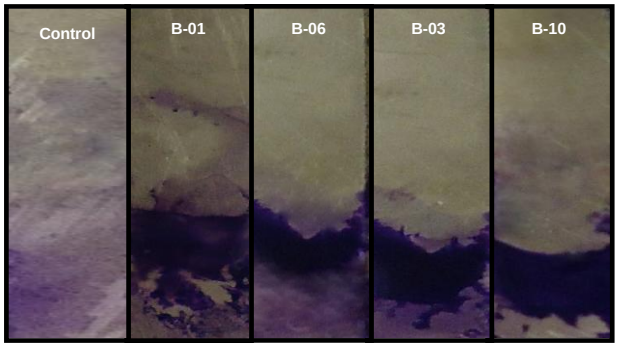

Figure 1: Stained *B. cereuss.l.* biofilms on SSCs.

The spore suspensions of selected isolates inoculated on the milk pre-soiled SSCs with nutrient-depleted broth medium (diluted TSB) in its first 24 hr of incubation, the biofilms were observed and formed preferentially at the air-liquid interface (ALI). The formation continued by developing submerged biofilms, which was below the ALI, after the next 24 hr in nutrient-rich broth (Fig.1).

The biofilms on SSCs were immersed in 25, 50 or 100 mg/mL of P-03 culture crude extract for 15 min. The initial quantity of biofilm cells before the treatment was ca. 5.6 Log

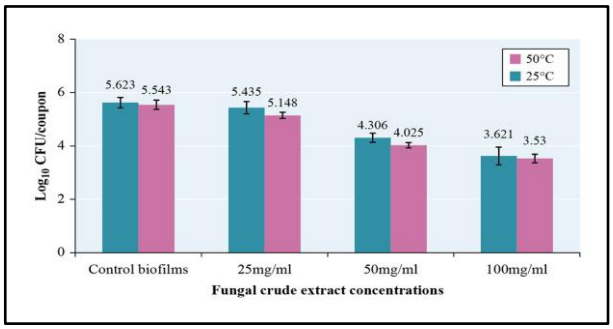

Figure 2: Reduction of *B. cereuss.l.* biofilms on milk pre-soiled SSCs after 15 min exposure to different concentrations of P-03culture crude extract at 25°C or 50°C. Error bars indicate the standard deviation from the mean.

CFU/coupon. In Set 1, the quantity of remaining biofilm cells was significantly reduced after immersion in crude extract with 0.2 – 2 Log reduction as compared to the control biofilms ($p< 0.05$) (Fig. 2). Also, when the quantity of biofilm's residual cells in Set 2 was assessed, a pronounced cell reduction ($p< 0.05$) was also obtained with 0.3 – 2.1 Log reduction (Fig. 2). However, the 25 mg/ml in both sets showedinsignificant reduction ($p< 0.05$) after the treatment. The nontreated biofilm cellshad averages of 5.6 and 5.5 Log CFU/coupon at 25°C and 50°C, respectively. Also, the results showed that there was no difference between the log reductions of nontreated biofilms in both sets in terms of exposure in temperatures thus the set temperatures have might no effect in reducing biofilm. Apparently, the 100 mg/ml was effective with 2.0- Log reduction (99%).

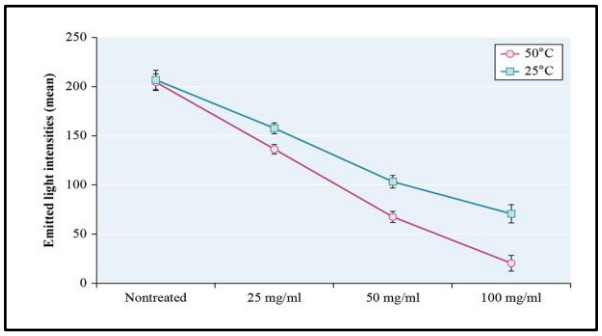

Figure 3: The emitted light intensities from fluorescence micrographs of B-10 biofilms on SSCs. Error bars indicate the standard deviation from the mean.

The fluorescence micrographs of nontreatedbiofilms and treated biofilms were collected and analyzed for their EtBr-emitted light intensities (red-green-blue, or RGB values) using the ImageJ 1.52a software. The mean recorded RGB values from the micrographs that measured and expressed as arbitrary unit (a.u.) were decreased drastically (nontreateda.u. > treateda.u.) inwhich the registered values are from 207 > 71a.u. for Set 1 and 204 > 20a.u. for Set 2 suggesting the ECMwere disrupted (Fig. 3). To further illustrate this trend, the3-D surface plots of B-10 biofilms were illustrated (Fig. 4).

4. Discussion

For the past years, novel bioactive secondary metabolites produced by the *Pestalotiopsis* spp. Have been documented. and many of these exhibited antimicrobial activities. Some of these were identified as terpenes [18], polyketide derivatives [19], meroterpenoids, isocoumarin, phenol derivatives [20] and others.

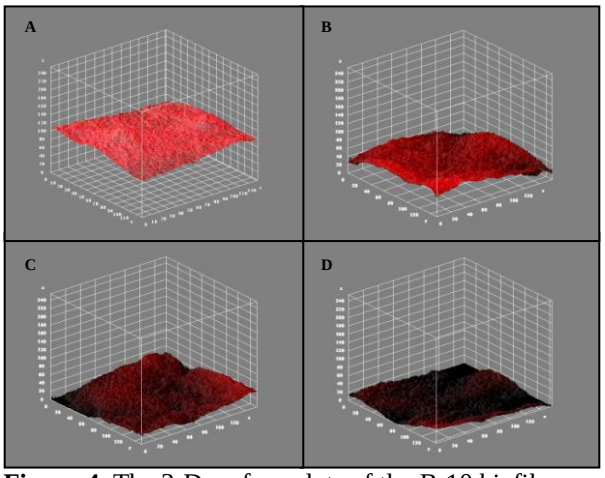

Figure 4. The 3-D surface plots of the B-10 biofilms on SSCs. Control biofilm (**A**), and biofilms after immersion in (**B**) 25 mg/mL, (**C**) 50 mg/mL, and (**D**) 100 mg/mL P-03 crude extract.

The antibiofilm activities of bioactive compounds coming from various microbial species have been conducted previously and showed encouraging findings on biofilm elimination. The results from this study are providing insights on antibiofilm activity of bioactive compounds extracted from *Pestalotiopsis* sp. fermentation cultures against the *B. cereus s.l.* biofilms on stainless steel surfaces.

The species under *B. cereus* groups, specifically the *B. cereus sensustricto*, have been reported as causative agents in food poisoning and foodborne outbreaks recorded worldwide. It produces spores that are resistant to disinfectants and high temperatures enabling them to survive. Previous researchers reported the high adhesion rate of *B. cereus* spores on stainless steel surfaces soiled with milk residues, which associated to biofilm formation and leads to foodborne illness [21]. The present study produces biofilms at ALI, an area where biofilms have both access in liquid and gaseous phases, implicated to growth of biofilms in industrial storage systems and pipelines that are partly filled during the operation or has residual liquid after a production cycle. This may cause problems, especially in food industry, as several authors found that the attached biofilms are more resistant to chemicals and mechanical stresses [22, 23, 24].Once the biofilms are formed, removal becomes difficult using the chemicals or by any mechanical means (e.g. flow rate) due to its complex ECM compositions.

The quantity of biofilm cells of *B. cereus s.l.* in 50 and 100 mg/ml of P-03 culture crude extract is significantly less than the nontreatedbiofilms ($p< 0.05$) suggesting that the ECM, which holds the biofilm cells together, were disrupted and caused the biofilm cells to release. However, 90% of *B. cereus* biofilms are composed primarily of spores [16] and

supposed that the recovered biofilm cells that were cultured through pour plating method were emerged from *B. cereus s.l.* spores. The ECM of *B. cereus* biofilms is composed of polysaccharides, DNAs (such as adhesins), proteins, biosurfactants, natural sugars, phosphates and sulphates [25, 26, 27]. Aside of blocking the ECM synthesis, previous researchers have proposed the degradation of ECM as part of microbial biofilm control strategy [28] or modifying the surface properties by applying antifouling coatings [29].The study found that TLC revealed secondary metabolites are present in the P-03 crude extract implying that these have the ability to destruct ECM and thus reduce biofilms.

In cleaning-in-place (CIP) programs of most food industry, the sanitization (post-CIP sanitization) is usually follows after CIP and applies with chemicals with heat treatment, which temperature and contact time play an important role in eliminating microorganisms. It has been reported that heat shocks between 50°C and 80°C for 1–30 min could reduce biofilm cells concentrations up to 6.6–fold and are a reasonable approach for biofilm mitigation [30]. Furthermore, the application of QACs at 70°C for 10 min and water rinse in the first step of long-hot CIP procedure effectively removed the biofilm *B. cereus* cells from the milk pre-soiled stainless chips [18]. The bioactivity of the secondary metabolites in crude extract against the *B. cereus s.l.* biofilms were unaffected by the set temperatures suggesting its possible usage in varying temperatures between 25°C–50°C.As analyzed using the ImageJ software, the RGB values of the EtBr-stained biofilms of B-10 on SSCs were decreasing depicting that the ECM was degraded by the crude extract.

The P-03 culture crude extract, which found to contain a pool of secondary metabolites, strongly emphasize its possible application as antibiofilm agent in reducing *B. cereus s.l.*biofilms. The present study also suggests a further research on highlighting the possible synergistic combinations, to enhance its effectiveness,of P-03 culture crude extract with existing chemicals or disinfectantsat different conditions. Same with other published studies that the mode of action of antibiofilm agents were not fully understood, we recommend additional investigation on how these unknown secondary metabolites present in the crude extract of P-03 breakdown the ECM components. Also, identifying and elucidating these unknown secondary metabolites will help future researchers in searching antibiofilm treatments against vast microbial biofilms.

5. Conclusion

The present study concluded that the unknown secondary metabolites in the crude extract obtained fromP-03fermentation cultures demonstrated antibiofilm activity against the *B. cereus s.l.* biofilms on stainless steel surfaces by disrupting the ECM. The crude extract evidently showed antibiofilm activity at 100 mg/ml concentration within the contact time of 15 min at 50°C could be an interesting alternative biofilm control method. The results obtained here offers additional evidence that the fungal plant endophytes, such as the *Pestalotiopsis* sp., could be a good source of natural compoundsas an alternative method against microbial biofilms formed on many types of surfaces.

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