Development of Antibiofilm Biosurfactants from Marine Bacteria Against Shrimp *Vibrio* pathogens

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Abstract

Vibrio disease is being described as a major bacterial disease obviously known as penaeid bacterial septicaemia, penaeid Vibriosis, luminescent Vibriosis or red leg diseases. Signs of *Vibrio* disease include lethargy, tissue and appendage necrosis, slow growth, slow larval metamorphosis, body malformation, bioluminescence in shrimp particularly produced in floc systems, muscle opacity, melanization, empty midgut and anorexia. In Asia, *V. alginolyticus* and *V. harveyi* were considered as the most significant pathogens in the grow-out ponds of giant black tiger shrimp *Penaeus monodon*. Survival and pathogenicity of *Vibrio* was associated with the biofilm formation and quorum sensing. Therefore, disruption of biofilm formation and/or quorum sensing would be an effective management strategy in aquatic systems instead of killing the pathogens which obviously leads to the development of resistant strains. Biosurfactants are surface active smart biomolecules showed strong antibiofilm activity against *Vibrio* pathogens. In this report, biofilm producing *Vibrio* pathogens include *V. harveyi VB1*, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V. parahaemolyticus* VB5 and *Photobacterium damselae* VB6 were isolated from the moribund shrimp samples collected from farms located southeast coast of India. Based on their surface-active properties, we hypothesized that biosurfactants could disrupt biofilms of *Vibrio* pathogens. To test the hypothesis, we examined the effects of the lipopeptides extracted from marine bacteria MSI-A 07 and MSI-A 08, on the biofilm-forming capacity of biofilm infection causing pathogenic *Vibrio* spp. (*V. harveyi* VB1, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V. parahaemolyticus* VB5 and *Photobacterium damselae* VB6). The both lipopeptide biosurfactants potentially disrupted biofilm formation under dynamic conditions. The biofilm disruption potential of the lipopeptide biosurfactants was consistent against all shrimp pathogens. Based on this finding, biosurfactant incorporated feed can be formulated to contain *Vibrio* outbreaks in shrimp aquaculture.

Key words: Biosurfactants, vibriosis, shrimp aquaculture, biofilm disruption

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Introduction

Aquaculture is the quickest developing sustenance part all around and it was quickly venturing into multibillion dollar industry. Be that as it may, by and by, the real inconvenience confronted by the aquaculture business worldwide is infections caused because of different biological and non-biological operators. The biological agents bacteria, virus and fungi were accepted to be the reason of extreme monetary misfortune in the incubation centers and develop out lakes in all aquaculture creating nations (Ruangpan and Kitao, 1991). Among the gatherings of microorganisms that reason serious misfortunes in shrimp culture, the best known are bacteria as a result of the stunning financial impacts they have on influenced ranches (Lightner, 1996; Karunasagar *et al.* 1994).

Pathogenic Vibrios are one of the significant wellsprings of shrimp sickness as a result of their nearby relationship with low survival rates in hatcheries and develop out lakes. In many shrimp cultivating areas, infections credited to Vibrio spp. are viewed as the most regular and vital irresistible issues (Ruangpan & Kitao, 1991; Sung *et al*. 2001). Vibrios can ability to grow as biofilm with resistance to disinfectants and antibiotics that cause a variety of shrimp disease in hatcheries and grow- out ponds (Karunasagar., 1994, 1996; Alvarez *et al*. 1998). In 1996, Karunasagar *et al*. revealed the antibiotic resistant Vibrio harveyi held on in the larval tanks of a shrimp hatchery, most presumably as biofilm bacteria and consequently not effectively expelled by sanitizer treatment.

Vibrio biofilm

In most environmental specialties, Vibrios are developed on regular or artificial surfaces as single or multispecies groups known as biofilms. A biofilm is a sessile microbial group comprising of cells that are irreversibly joined to a substratum and installed in an extracellular polymeric framework (Donlan & Costerton, 2002). The vast majority of the investigations show that biofilms are a steady point in a natural cycle that starts with the vehicle and connection of the bacterium to surfaces. After the underlying connection, colonization of a surface is interceded by the development and development of appended microbes. Surface colonization at that point prompts

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the development of microcolonies, which are frequently encompassed by extrapolymeric substances. Assist development of bacteria and proceeded with creation of exopolysaccharide prompt the advancement of develop biofilm structures portrayed by columns and channels. It has been demonstrated that advancement of these structures relies upon biomass development rate, twitching motility, signalling molecules, and production of exopolysaccharide (O'Toole *et al*. 1998: Costerton *et al*. 1995; and Parsek & Fuqua, 2004). As per the O'Toole. (1998) the physiology, cell surfaces, imperviousness to natural put-down, and different properties of biofilm cells are notably not the same as their planktonic partners. Biofilm development rises as on essential component for microbial survival in nature. Vibrios are ubiquitous in situations mostly amphibian environments. The biofilm framing limit of *V. cholerae* is all around archived, both in common environments and under research center conditions (Faruque *et al*. 2006; Watnick and Kolter, 1999; Yildiz & Schoolnik, 1999). A few research discoveries uncover the significance of biofilms in survival, harmfulness, and stress resistance components of Vibrio spp. (Watnick & Kolter 1999; Watnick *et al.* 2001; Zhu & MeKalanos, 2003; Faruque *et al.* 2006; You *et al.* 2007; Yildiz &Visick., 2009). With this standpoint the present investigation was intended to assess the biofilm framing capability of pathogenic Vibrio spp., related with shrimp malady.

Vibrio disease is depicted as Vibriosis or bacterial infection, penaeid bacterial septicaemia, penaeid Vibriosis, luminescent Vibriosis or red leg maladies and is globaly circulated. Indications of Vibrio ailment incorporate laziness, tissue and extremity putrefaction, moderate development, moderate larval transformation, body deformity, bolitas negricans, bioluminescence, muscle mistiness, melanization, purge midgut and anorexia (Karunasager *et al*. 1994; Lightner and Redman, 1994; Smith, 2000). In Asia, among the pathogenic *Vibrio* gathering, 11 species were accounted for from the shrimp culture frameworks (Lavilla-Pitogo, 1995). Of these, *V. alginolyticus and V. harveyi* are considered as the most huge ones in the develop out lakes of giant black tiger shrimp *Penaeus monodon* in India (Karunasagar *et al.* 1997; Selvin and Lipton, 2003; Manilal *et al.* 2010). The pathogenecity of microbial intruders in the scavanger haemocoel at last lies in the capacity of the life forms to avoid or evade the host resistance systems.

In this examination, hopeless shrimp isolates were morphologically and biochemically portrayed in to six gatherings. Among the six gatherings, the most dynamic biofilm makers were screened. They were named as *V. harveyi VB1, V. alginolyticus VB2, V. vulnificus VB3, V. fischeri VB4, V.*

parahaemolyticus VB5 and Photobacterium damselae VB6. With respect to antibiotics resistance, all isolates were impervious to choloramphenicol taken after by oxytetracycline. The antibiotic resistance pathogens and amassing of deposits in the shrimp tissue have turned out to be normal in Indian Shrimp ranches (Selvin and Lipton, 2003). Antibiotic resistant *V. harveyi* from tainted larvae showed bring down LD_{50} esteems for post larval P. monodon than *V. harveyi* secludes acquired from sea water (Karunasagar *et al.* 1997). In thinks about by Karunasagar *et al.* (1994, 1996), antibiotic resistant *Vibrio harveyi* persevered in the larval tanks of a shrimp hatchery, most likely as biofilm bacteria and in this way not effortlessly disposed of by sanitizer treatment. These discoveries proposed the potential peril of standard utilization of anti-infection agents in aquaculture and show that they may build the Vibrio spp. harmfulness, for example, biofilm development.

All incurable shrimp isolates (*V. harveyi VB1, V. alginolyticus VB2, V. vulnificus VB3, V. fischeri VB4, V. para haemolytic us VB5 and Photobacterium damselae VB6*) on CRA plate, created black colonies. Slime production assume an essential part in the pathogenesis of contaminations caused by various microorganisms (Alcaráz, 2003; Abdallah *et al. 2008*), and is thought to be a noteworthy harmfulness factor for some Vibrio alginolyticus and *Vibrio parahaemolyticus* (Abdallah *et al. 2008*).

Biofilm forming capacity of *Vibrio* pathogens

Quorum sensing assume a key part in the formation of biofilm. Davies et al. (1998) distributed the principal ponder that demonstrated a part for majority detecting in the biofilm formation, and propelled a time of dynamic research of cell-to-cell communication in biofilms. McLean et al. (1997) have demonstrated that acyl HSL autoinducers are distinguishable in normally happening biofilms, proposing that biofilm groups in nature contain populaces that can experience cell density dependent regulation. In light of the investigation the presence of cell to cell signaling molecule were seen in just 3 biofilm producers (*VB3, VB4 and VB5*) among the 6 selected isolates (VB1-VB6). In light of the above investigation material and research finding the *Vibrio spp*. detached from the moribund shrimps are recognized as potential biofilm producers *V. harveyi VB1, V.*

alginolyticus VB2, V. vulnificus VB3, V. fischeri VB4, V. parahaemolyticus VB5 and Photobacterium damselae VB6.

The stereozoom microscope principally used to watch the biofilm amassing in strong surfaces. The biofilm was created on to the microtiter polystyrene plates and it was seen under stereozoom magnifying instrument. The biofilm accumulation on the strong surface was watched and photograph was taken. The accumulation biofilm makers in microtiter plate is fluctuated in light of their surface connection nature. The photograph was shown in Figure 1. The *Vibrio spp.* were delivered distinctive measure of biofilm in the polystyrene microtiter plates.

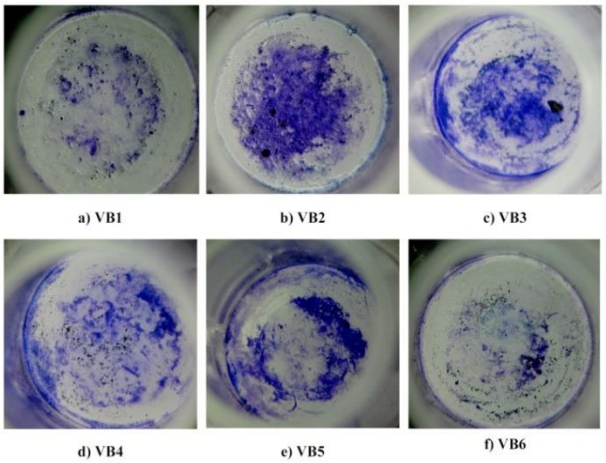

Figure 1. Stereozoom microscope images demonstrating the biofilm forming potential of *Vibrio* spp. (VB1 to VB6).

Biofilm formation is perceived as an imperative destructiveness factor for both opportunistic and true pathogens (O'Toole *et al.* 2000). Bacterial biofilms have a basically intricate and dynamic design and form on numerous abiotic surfaces (plastic, glass, metal and minerals) and biotic (plants, creatures and people) surfaces (Stoodley *et al.* 2002; Hall-Stoodley *et al*. 2004) as single- or various species groups. Biofilm arrangement is a critical component for microbial survival in the earth. Biofilm development is an imperative component for microbial survival in the earth. Biofilm-framing microorganisms are less susceptible to numerous antimicrobial compounds and different biocides. Biofilm formation on medical devices assumes a vital part in the issue of numerous nosocomial and wellbeing related disease and also the development of biofilm favors survival and steadiness of *Vibrio* spp. in the aquatic environment and furthermore inside the host. In light of the above reasons, novel antibiofilm agents are required for the avoidance/control of

pathogenic microbial biofilms on the surfaces and hosts. It was set up that the vast majority of the marine organisms have advanced effective techniques to battle epibiosis. Particularly, marine sponges create particular obstacles to avert biofilm-forming microorganisms (Selvin *et al.* 2010). In any case, it has been estimated that these poisonous hindrances may be delivered by the related microorganisms rather than the host sponge.

Biosurfactants from marine bacteria

Biosurfactants are a heterogeneous group of bioactive amphiphilic particles produced on microbial cell surfaces or extracellularly (Karanth *et al.* 1999). The most potential advantage of microbial surfactants is biodegradability and nontoxicity to common habitats (Banat, 1993). The biomedical significance of biosurfactants was built up because of their antibacterial, antifungal and antiviral properties; hindrance of fibrin clump arrangement; and their anti-biofilm ability against few pathogenic microorganisms (Meylheuc *et al.* 2001, 2006; Singh and Cameotra, 2004; Rodrigues *et al.* 2006). Sponge related marine microorganisms are rising as a potential wellspring of novel biosurfactants (Gandhimathi *et al*. 2009; Kiran *et al.* 2009, 2010). It has been speculated that the antimicrobial fouling process speaks to a substance safeguard of host wipes intervened by the related microscopic organisms. Consequently, the biosurfactants created by the sponge related marine actinobacteria were assessed for the control of pathogenic Vibrio spp. biofilms, separated from moribund shrimps.

Biofilm inhibition potential of lipopeptide biosurfactants

In light of their surface-dynamic properties and writing confirm (Kiran *et al.* 2010; Dusane *et al.* 2010), we estimated that glycolipids could influence biofilm arrangement. To test the speculation, we inspected the impacts of the lipopeptide separated from MSI-A 07 and MSI-A 08, on the biofilm-framing limit of biofilm contamination causing pathogenic Vibrio spp. (*V. harveyi VB1, V. alginolyticus VB2, V. vulnificus VB3, V. fischeri VB4, V' VB5 and Photobacterium damselae VB6*). The both lipopeptide biosurfactants possibly disturbed biofilm development under powerful conditions. The biofilm interruption capability of the lipopeptide biosurfactants was reliable

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against all shrimp pathogens. The lipopeptide biosurfactants, specifically MSI-A 07 and MSI-A 08 demonstrated magnificent hindrance against the biofilms of shrimp pathogens (*VB1 to VB6*). The lyophilized lipopeptide biosurfactant were utilized to quantify biofilm inhibitory fixation. To decide the BIC of these two lipopeptide biosurfactants on shrimp Vibrio spp., extricates with changed scopes of focuses (10– 50 µg/mL) were utilized. Fixation subordinate reduction in biofilm arrangement of test pathogens was gotten upon treatment with the lipopeptide biosurfactants. The biosurfactants got from MSI-A 07 indicated most extreme inhibition of biofilm of 75-80 % at a grouping of 30 µg/mL (Figure 2.1) and the lipopeptide biosurfactant isolated from MSI-A 08 repressed the biofilm development of shrimp pathogens up to 70-75 %, at a focus 40 µg/mL (Figure 2.2). Thus, 30 µg/mL and 40 µg/mL were fixed as the BIC for MSI-A 07 and MSI-A 08 lipopeptides separately and additionally measures were done at this extract concentration.

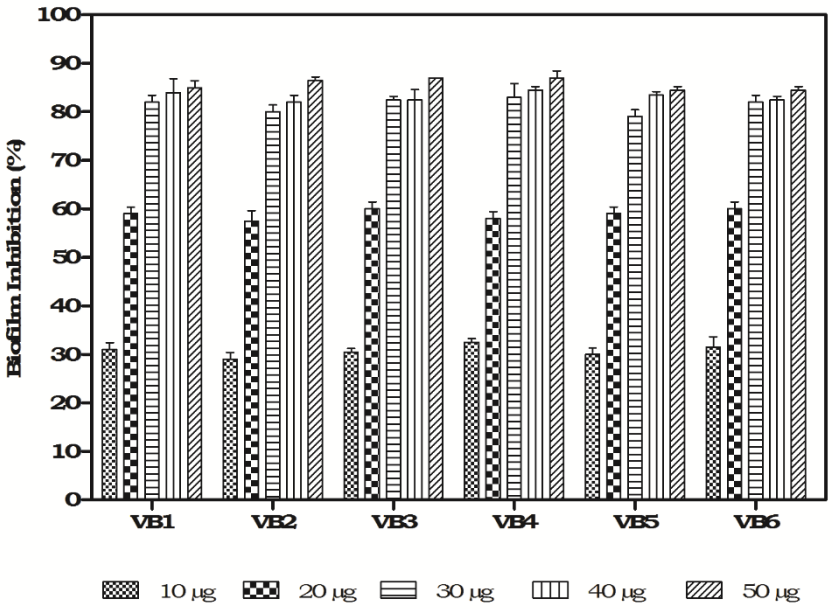

Figure 2. 1. Efficacy of the lipopeptide biosurfactant (MSI-A 07) in the biofilm forming potential of shrimp pathogens.

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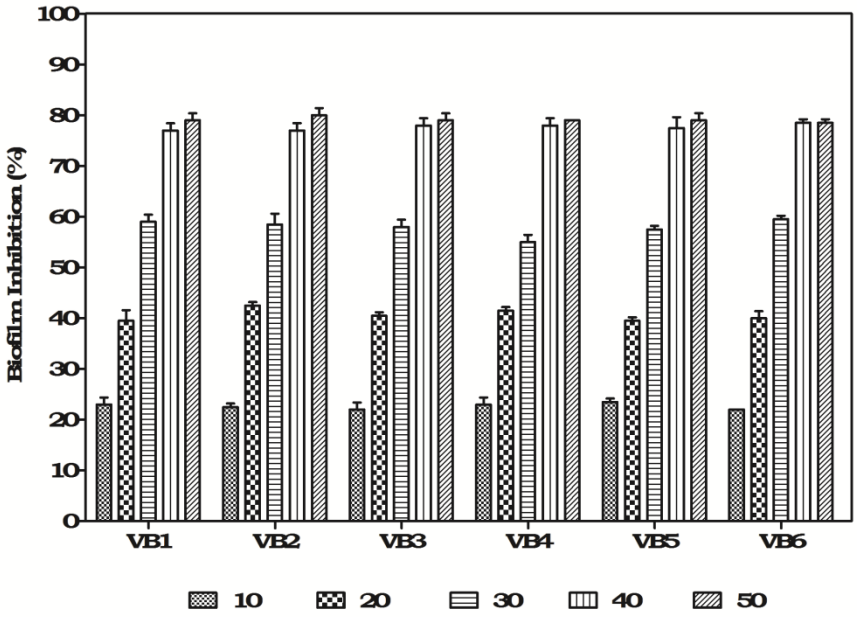

Figure 2. 2. Efficacy of the lipopeptide biosurfactant (MSI-A 08) in the biofilm forming potential of shrimp pathogens.

Biofilm disruption potential of lipopeptide biosurfactants

The pictures got from the stage phase contrast microscope uncovered that the lipopeptide biosurfactants extracted from MSIA-07 and MSI-A 08 had potential biofilm disruption. In the cover slip examine, the biofilm disruption was clear and demonstrated a disrupted biofilm under phase contrast microscope (Plate 3.1). These outcomes legibly demonstrate that the lipopeptide biosurfactants disrupts the initial attachment to the surface.one of the important feature of biofilm is the initial attachment. Along these lines, counteractive action of biofilm connection prompts the biofilm disruption. The pictures acquired from light microscope showed that the control slides portrayed all around well-formed biofilm of test pathogens, while, the test pathogens upon treatment with lipopeptide biosurfactants formed poor biofilm development than the control test (Plate 3.3). To decide the outcomes acquired in light microscopy (i.e., breaking down of biofilm structures by biosurfactants), we utilized confocal laser scanning microscopy (CLSM) to additionally illustrate the antibiofilm capability of lipopeptides against biofilms of pathogenic shrimp *Vibrio spp*. (*V. harveyi VB1, V. alginolyticus VB2, V. vulnificus VB3, V. fischeri VB4, V.*

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parahaemolyticus VB5 and Photobacterium damselae VB6) (Plate 3.4). CLSM demonstrated strong adhering capacity of shrimp pathogens, which prompt the improvement of thick biofilm development on glass slide of control samples, while treated samples showed the antibiofilm capability of MSI-A 07 and MSI-A 08 by crumbling the refractory biofilm design of tried pathogens upon treatment. The microtiter plate test likewise demonstrated biofilm disruption capability of MSI-07 and MSI-A 08 lipopeptide biosurfactant on microtiter plates under stereozoom microscopic examination (Plate 3.5).

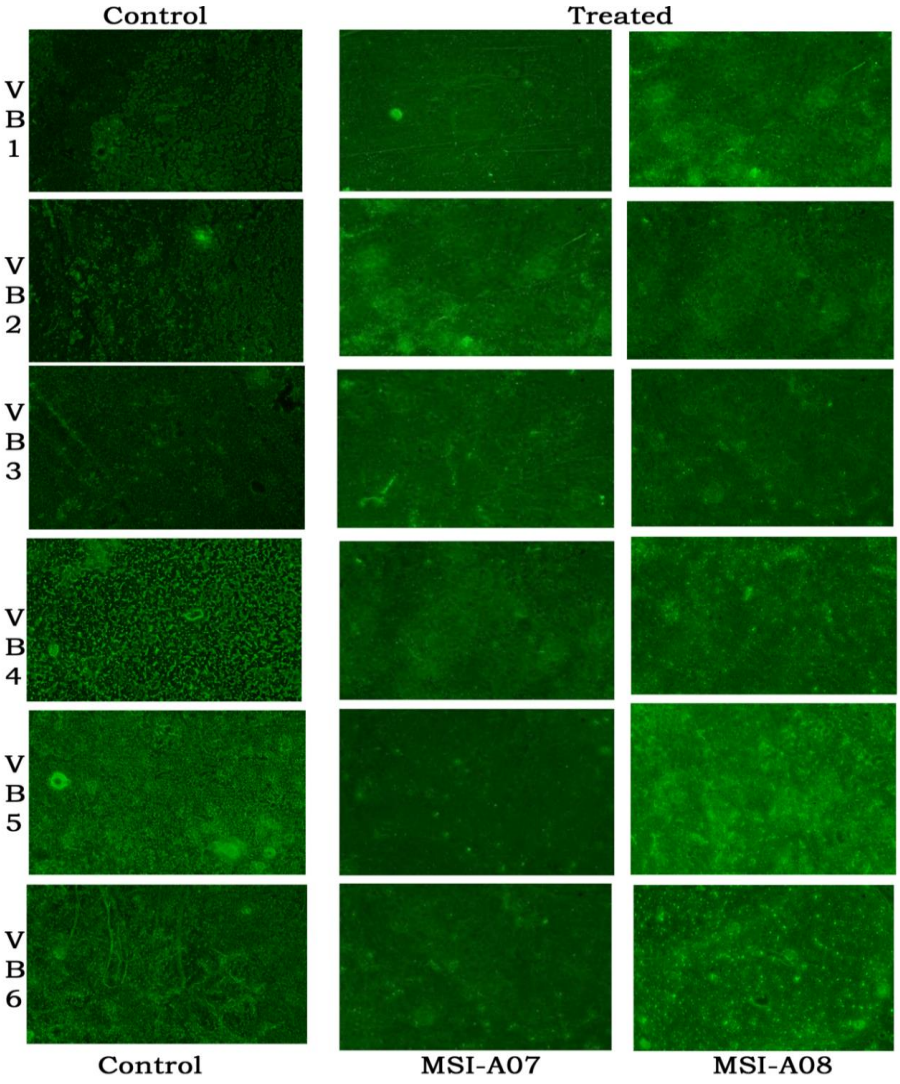

Plate 3.1. Phase contrast microscope images demonstrating the biofilm disruption potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

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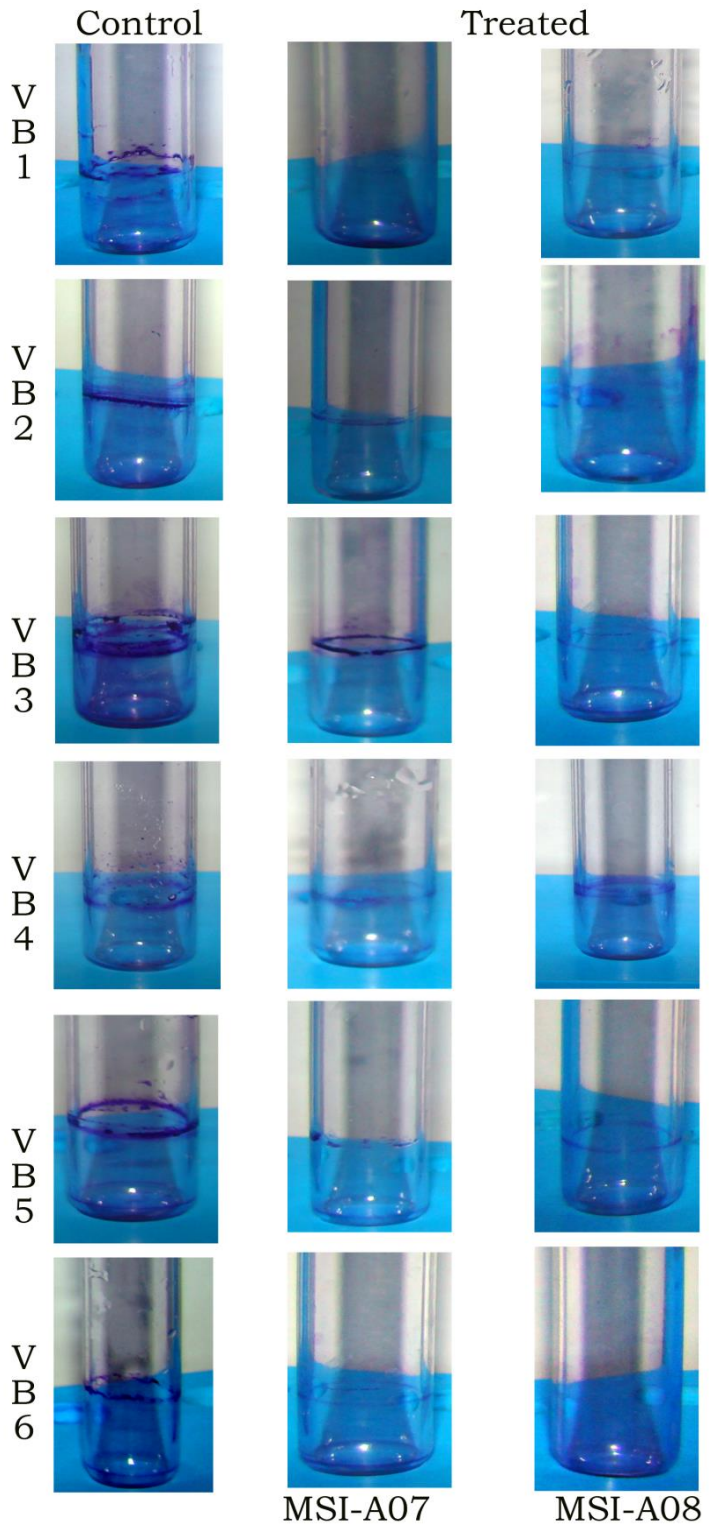

Plate 5.2. Direct observation demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

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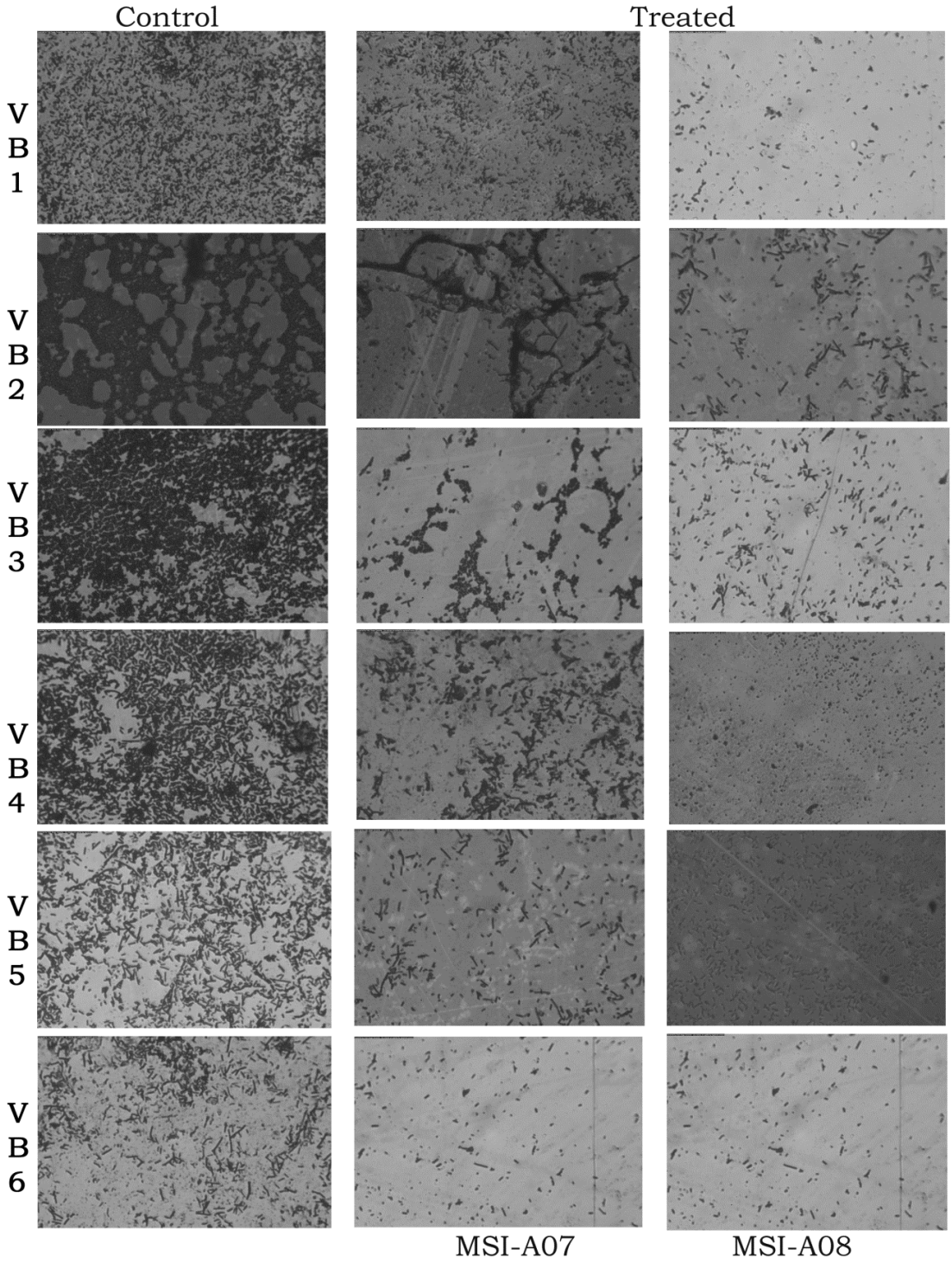

Plate 5.3. Phase contrast microscope images demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

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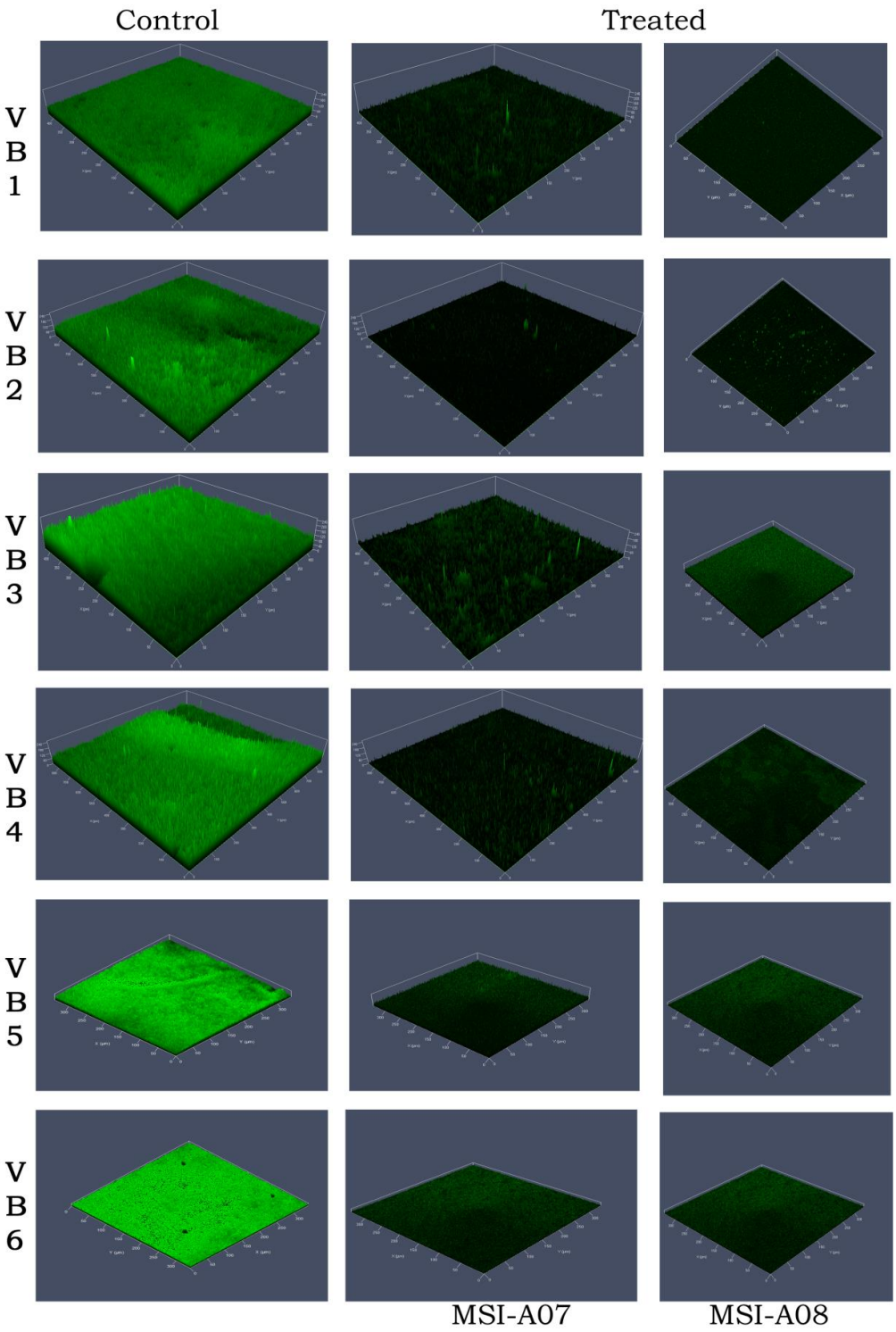

Plate 5.4. CLSM images demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

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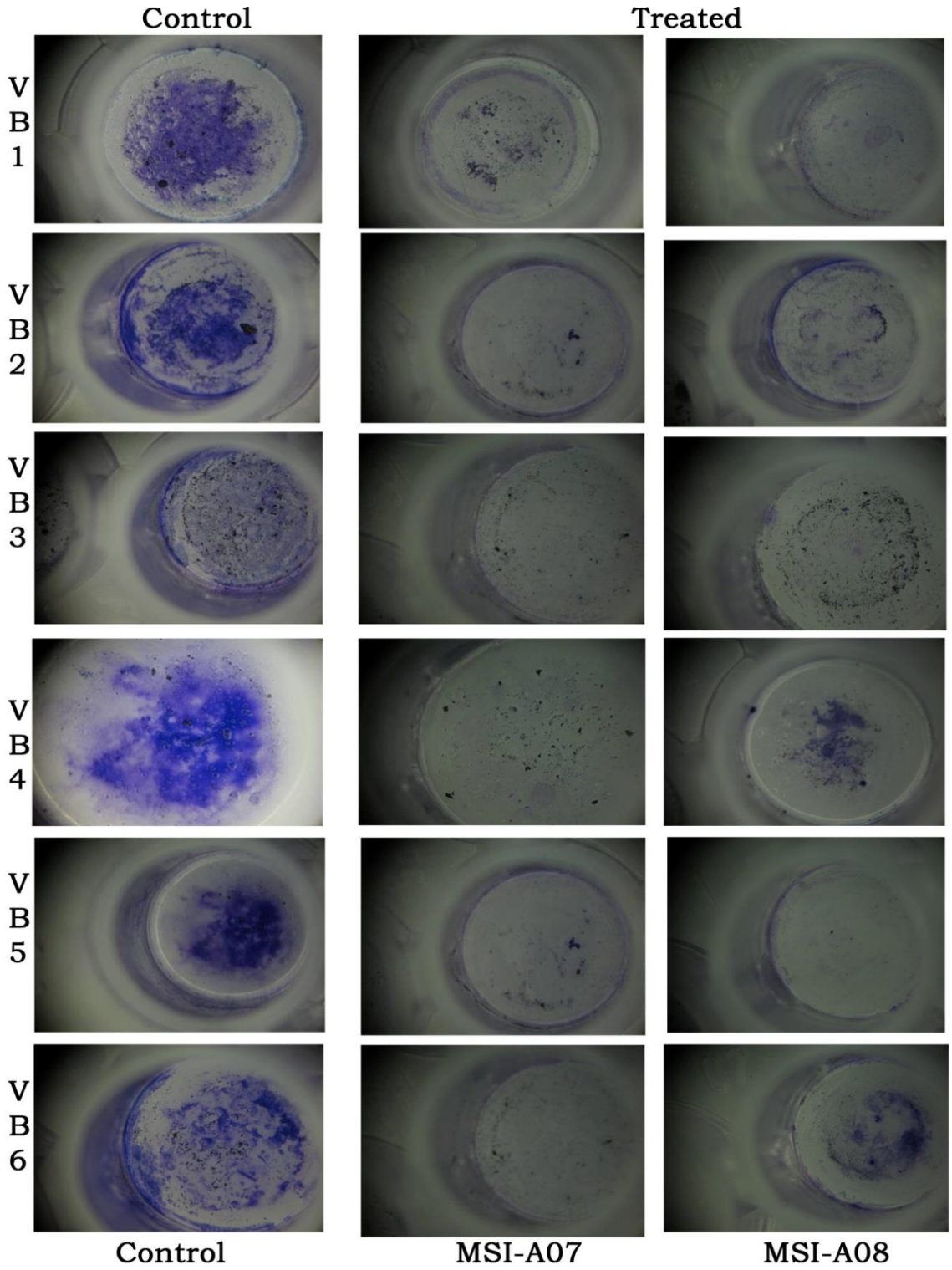

Plate 5.5. Stereozoom images demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

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Microbial surfactants or biosurfactants are surface-dynamic amphipathic particles delivered by various microorganisms. As of late, microbial surfactants have been found to have a few properties of helpful and biomedical significance, e.g. antibacterial, antifungal and antiviral properties. The antimicrobial properties of the biosurfactants have been generally detailed. One valuable property of numerous biosurfactants that has not been examined widely is their antibiofilm action. Glycolipid delivered by Brevibacterium casei (Kiran *et al*. 2010) and rhamnolipid created by Serratia *marcescens* (Dusane *et al*. 2010) have indicated high antibiofilm . As of late Quinn *et al*. (2012) detailed the antibiofilm capability of lipopeptide separated from Paenibacillus polymyxa. The capacity of surfactants to repress biofilm development is depicted for the rhamnolipid surfactant of *P. aeruginosa PAO1* (Davey *et al*. 2003) and for lipopeptides created by the Gram-positive microscopic organisms Lactobacillus, Bacillus and Streptococcus (Busscher *et al*. 1997; Velraeds *et al*. 2000; Mireles *et al*. 2001). Be that as it may, the quantity of reports on advancement of novel antibiofilm biosurfactant is negligible. In spite of the fact that there have been few reports of novel antibiofilm biosurfactants, their biofilm interruption possibilities have not been investigated in points of interest. With this standpoint, the present examination was directed to assess biofilm interruption capability of lipopeptide biosurfactants extricated from marine actinobacteria *Nocardiopsis sp*. MSI-A 07 and Streptomyces *coeruleorubidus MSI-A 08.*

Bacterial development emerges rapidly not long after its connection to a strong substratum, which is the underlying stage in biofilm formation. For beginning couple of hours of development at first glance, the attachment is reversible (Marshall, 1994, Hoiby *et al*. 2001). In this manner, the anticipation of bacterial attachment at the extremely introductory stage can essentially lessen the danger of further biofilm formation. The both lipopeptide biosurfactants hindered the biofilm arrangement at its beginning time through lessening the microcolonies framed by shrimp pathogens. The both lipopeptide biosurfactants diminished biofilm formation up to 75 and 80% at a grouping of 30 µg/mL and 40 µg/mL against shrimp pathogens, separately. Comparative outcomes was accounted for by Rodrigues *et al*. (2006), he showed that rhamnolipids repress bacterial bond over a range changing from 21% to 81%. The 96 well microtiter plate measure is the most widely utilized examine for the identification of biofilm development (Christensen,

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1985). Both inhibition of biofilm assay and microscopic observations obviously depicted that the both lipopeptide biosurfactants viably lessened and disrupted the microcolonies.

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