INVESTIGATING THE ANTIMICROBIAL, ANTI-BIOFILM AND ANTI-QUORUM SENSING POTENTIAL OF SOUTH AFRICAN SEAWEED-ASSOCIATED BACTERIA

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Signature: _____ Date: _____

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ABSTRACT

Marine substrata are colonized by a variety of marine microorganisms, which are capable of producing novel compounds due to their diverse and often extreme environmental conditions as well as interactions with their associated eukaryotic host organisms. Seaweed serve as an important host for bacteria that synthesize bioactive natural substances. Seaweed-associated bacteria represent an inexhaustible reservoir of bioactives with potential application in pharmaceutical, medicine and food industries. The antibacterial, anti-biofilm and anti-quorum sensing potential of bacteria associated with ten South African seaweeds was therefore assessed.

Cultivable seaweed-associated bacteria (n=96) were screened for antibacterial activity against five resistant clinical and six aquaculture indicator bacteria utilizing the primary cross streak screening assay. Following shake flask fermentations and ethyl acetate extractions extracts, from 30 selected isolates were screened using the agar-well diffusion assay and minimum inhibitory concentrations (MIC) were determined for 14 extracts. Selected bacteria were identified by 16S rRNA gene amplification and sequencing. Active crude extract of MAB24-SW1 was then analysed using nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). Extracts capable of inhibiting initial adhesion and detachment of mature biofilm of clinical and aquaculture pathogens were identified using the crystal violet microtitre plate assay. The anti-quorum sensing inhibitory potential was assessed utilizing *Staphylococcus aureus agr* and *Enterococcus faecalis fsr* inhibition assays.

Primary screening indicated that 38% (37/96) and 73% (71/96) of isolates displayed varying antimicrobial activity against clinical and aquaculture indicator strains, respectively. Isolate MAB24-SW1 was found to be active against clinical and aquaculture indicators with MICs as low as 0.39 mg/ml. The isolate identified as *Bacillus velezensis*, produced a potential analogue of surfactin, which was identified through NMR following partial purification of the crude extract. Crude extracts also demonstrated anti-biofilm potential. Inhibition of adhesion was most effective at 5 mg/ml for *Staphylococcus aureus, Yersinia ruckeri, Vibrio parahaemolyticus, Pseudomonas aeruginosa* and *Aeromonas hydrophila.* Extracts were most effective in the dispersal of mature biofilms of clinical and aquaculture strains, with the best results been observed against *S. aureus, V. parahaemolyticus A. hydrophila, P. aeruginosa,* and *Edwardsiella tarda*. The total range of biofilm inhibition by extracts was between 0.08% - 113.84%. Variation was observed in the efficacy of extracts against initial adherence of indicator strains, with ≥ 90% reduction potential being demonstrated by MAB10B-SW1

(*Bacillus* sp.), MAB24-SW1 (*B. velezensis*), AB4-SW2 and AB3-SW6 against *Pseudomonas aeruginosa*. Anti-quorum sensing inhibitory potential against *Staphylococcus aureus* was assessed utilizing luminescence and green protein fluorescence (GFP) reduction in the *Staphylococcus agr* assay, crude extracts of MAB6-SW1 (*Rhodococcus fascians*), MAB10B-SW1 (*Bacillus* sp.), MAB24-SW1 (*B. velezensis*), AB7-SW8 (*Streptomyces* sp.), AB1-SW9 (*Microbacterium maritypicum*) and AB4-SW10 (*Strepyomyces labedae*) were capable of quorum sensing inhibition without causing cell death, with some isolates demonstrating $\geq 50\%$ inhibition of GFP. When the anti- quorum sensing inhibitory potential of extracts were tested against *Enterococcus* virulence factor under the control of the *fsr* quorum sensing system (gelatinase production), isolate AB2-SW8 (*Streptomyces* sp.) demonstrated inhibitory potential against both *fsr* and *agr* systems without causing cell death.

Decreased efficiency and resistance of pathogens to antibiotics has necessitated the development of new therapeutic alternatives. Based on their anti-biofilm and anti-quorum sensing potential, South African seaweed-associated bacteria with their secondary metabolites may provide viable alternatives to antimicrobial therapy in the form of anti-virulence compounds.

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INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The increased incidence of infectious diseases worldwide together with the utilization of antimicrobial agents to treat infections has created a global epidemic, with the emergence of multidrug-resistant (MDR) bacteria (*Aeromonas*, *Enterococcus*, *Escherichia*, *Klebsiella, Pseudomonas*, *Staphylococcus*, and *Vibrio* spp.) being observed in clinical and aquaculture settings (Defoirdt, 2016; Tan *et al*., 2016). Given this global antimicrobial resistance problem, the discovery of novel antimicrobial agents and investigation of their mechanisms of action as potential therapeutic compound leads are essential issues for modern pharmaceutical research. The increasing prevalence of resistant bacteria is a limiting factor in the efficacy of current drugs, however, antimicrobial drug discovery is highly unattractive to pharmaceutical companies due to short antibacterial drug life cycles and the acute, rather than chronic, nature of antibacterial therapy (Gillings, 2013).

Since the marine environment covers more than 70% of the world's surface and remains relatively unexplored (Zozaya-Valdes *et al*., 2015), it is an important source for the discovery of novel bioactive compounds as most of the Earth's microbial communities are located within this source. The oceans are an untapped resource for discovery of new compounds since only a fraction the biodiversity of marine microbes have been identified and the biological and chemical diversity of their bioactive metabolites has not been fully explored (Wahl et *al*., 2012). The marine environment is thus the focus for natural product discovery as it is a prolific source of not only plants and animals but the microorganisms associated with them, due to their adaptation to this unique environmental habitat and production of a wide variety of primary and secondary metabolites that have demonstrated significant biological activities (Kiuru *et al*., 2014). It thus represents a source of novel chemical compounds with potential applications as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals, and agrichemicals (de Oliveira *et al*., 2012; Penesyan *et al*., 2013a).

Marine biodiversity exploration has included an intensive focus on marine macroalgae or seaweeds for the discovery of novel treatment options (Lee *et al*, 2014). Marine macroalgae, or seaweeds, have been utilized as sea vegetables, medicines, and fertilizers for centuries. Seaweed surfaces are home to a variety of microorganisms, spores and invertebrate larvae that attempt to settle on the thallus surface in which some share a beneficial relationship with their host. They employ a variety of mechanisms to protect their host which includes the synthesis

of bioactive compounds (anti-biofouling, antimicrobial agents, etc), which can be adapted for human use, with the discovery and development of useful metabolites and potentially novel technologies (Wahl *et al*., 2012; Singh *et al*., 2015).

Seaweed-associated bacteria are potential sources of pharmaceutical interest due to their production of structurally diverse compounds exhibiting broad-spectrum biological activities (Subramani and Aalbersberg, 2013; Singh *et al*., 2015). Approximately 100 novel compounds (e.g., polyketides, alkaloids, fatty acids, peptides and terpenes) are isolated from marine bacteria per year (Kiuru *et al*., 2014). Since seaweed-associated bacteria are effective in preventing colonization and fouling by competitor, often pathogenic bacteria (Egan *et al*., 2013; Egan *et al*., 2014; Singh *et al*., 2015), they can be explored as a source of biofilm and/or quorum sensing inhibitory compounds. These would combat bacterial virulence instead of targeting bacterial survival, and potentially reduce the risk of evolution of drug resistant microbes (Padmavathi *et al*., 2014).

1.2 The problem of multidrug-resistant bacteria

1.2.1 Multi-drug resistance in the clinical environment

The emergence of MDR bacteria a result of the use, as well as misuse of antibiotics (Ibrahim and Saber-Ayad, 2012). The World Health Organization (WHO) has reported high rates of resistance in bacteria such as *Klebsiella pneumoniae* against cephalosporin and carbapenems, *Staphylococcus aureus* against methicillin, and *Mycobacterium tuberculosis* against rapamycin, isoniazid and fluoroquinolone (WHO, 2014). With an increase in the number of immuno-compromised patients and longer periods spent in hospital settings, these pathogens have become a serious threat to vulnerable patients. Among these opportunistic pathogens are staphylococci, *Pseudomonas aeruginosa* and *Enterococcus faecalis,* which cause potentially lethal infections (Nair *et al*., 2014). These pathogens are widely distributed in hospitals and are increasingly being isolated from community-associated infections (Pop-Vicas and Opal, 2014). Many studies are now focused at finding new antimicrobial agents, however, new families of antibiotics have a short life expectancy (Ibrahim and Saber-Ayad, 2012).

The burden of MDR bacteria has substantially increased world-wide, these organisms survive for a prolonged time in hospital settings and can easily be transferred between patients through the hands of health-care workers. In studies of *S. aureus*, blood stream mortalities ranged from 13 - 39% in immuno-compromised individuals (Pop-Vicas and Opal, 2014). Of the MDR organisms, *P. aeruginosa* requires special mention as this organism is resistant to all currently available antimicrobial agents which leaves limited options for treatment.

Pseudomonas aeruginosa makes use of various distinct quorum sensing (QS) systems to express multiple resistance mechanisms including over-expression of efflux pumps and low cell wall permeability (Nathwani *et al*., 2014). Each year, patients with hospital-acquired infections present with highly resistant strains. Amongst those is *S. aureus* an opportunistic organism that forms part of normal human skin flora (Thoendel *et al*., 2011). *Staphylococcus aureus* is the leading cause of hospital-related infections world-wide; if the epithelial barrier is compromised, *S. aureus* can cause pneumonia, bacteremia, and sepsis. Its ability to cause disease is linked to the expression of virulence factors such as adhesion molecules (biofilm formation) and toxins that affect the immune system. Quorum sensing regulates expression of genes encoding virulence factors, which include various enzymes and toxins. Toxins include superantigens, proteases, exfoliative toxins, hemolysins (alpha, beta, gamma, delta), and leukocidin (Rutherford and Bassler, 2012). These allow staphylococci to cause a myriad of infections such as subcutaneous abscesses to scalded skin syndrome, sepsis, necrotizing pneumonia (Desouky *et al*., 2013).

Persistent infections are a global problem claiming millions of lives yearly and demand costly medical expenditures. Many chronic infections in humans are associated with biofilms (Wu *et al*., 2015). Typical biofilm-associated diseases are cystic fibrosis, periodontitis, endocarditis, and chronic wounds. Chronic wounds and implants are an ideal environment for biofilm formation. The necrotic tissue and debris allow bacterial attachment, and wounds are susceptible to infection due to impaired host immune response (Kamiya *et al*. 2012). Bacteria deploy the formation of biofilms as a survival strategy, which renders them resistant to antibiotics, detergents and host immune system. As a correlation between biofilm formation and QS has been established (Fernandes *et al*., 2011; Harder *et al*., 2012), the possibility of using drugs targeting QS is a potential therapeutic approach for persistent biofilm related infections (Rodrigues *et al*., 2015). With the increasing burden of MDR pathogens, there is an urgent need for the discovery of molecules targeting virulence factors.

1.2.2 Aquaculture and the bacterial challenge

For more than 4.3 billion people, fish are a source of animal protein and aquaculture, a vital complement to global capture fisheries, is one of the fastest growing sectors in the world (Vatsos and Rebours, 2015; Tan *et al*., 2016). Aquaculture is defined as the farming of individually or corporate owned aquatic organisms such as fish, mollusks, crustaceans, and aquatic plants with interventions in the rearing process so as to enhance production (Hamza *et al*., 2015a). Some of the problems associated with the intensive commercialization of

aquaculture includes various stressors such as crowding, handling, improper water quality parameters (Defoirdt, 2013; Vatsos and Rebours, 2015; Zhao *et al*., 2015), which leads to disease outbreaks by a variety of viral, bacterial, fungal, parasitic, and other undiagnosed and emerging pathogens. Bacterial pathogens are a major cause of diseases in fish and aquaculture resulting in growth retardation and mass mortalities (Hamza *et al*., 2015a; Vatsos and Rebours, 2015).

While the control of the infectious diseases in aquaculture relies on the use of effective prophylactic as well as therapeutic measures, their use has serious impacts on the environment and increases the health risks for both humans and animals. The indiscriminate and frequent use of antimicrobial agents induces a strong selective pressure on the pathogens leading to the development of MDR bacterial strains and their isolation from aquaculture environments is associated with horizontal transfer of antibiotic resistance and virulence genes to fish and even human pathogens (Defoirdt, 2013; Vatsos and Rebours, 2015). The over-use of antimicrobial agents in aquaculture and the emergence of MDR fish pathogens has significantly reduced options for treating fish diseases (Chenia and Duma, 2016). The persistence and recurrence of these bacterial pathogens in aquaculture systems and their role as spoilage organisms and/or opportunistic human pathogens associated with fish processing can be correlated with their biofilm-forming potential. In the aquaculture environment, bacteria adhere to surfaces of submerged material, pipes, and tanks of hatcheries, form biofilms, and act as reservoirs of pathogenic bacteria (Iyapparaj *et al*., 2013; Chenia and Duma, 2016). Bacteria living in biofilms can be up to 1,000-10000 times more tolerant to antibacterial compounds than their planktonic counterparts (Penesyan *et al*., 2015).

Biofouling (accumulation of organisms) is a common problem on man-made objects submerged in the marine waters (such as in marine aquaculture) throughout the world (Satheesh *et al*., 2016). Like biofilm formation, biofouling growth on an aquatic environment surface is a complex process with initial biofilm formation (consisting of microbes and microalgae) followed by settlement of invertebrate larvae and algal spores. Biofouling assemblage in marine environment is composed of thousands of marine organisms such as bacteria, fungi, phytoplankton, polychaetes, barnacles, molluscs, ascidians and algae. Biofouling on submerged surfaces in the marine environment has considerable ecological and economical challenges in aquaculture with the loss of productivity well as the increased costs associated with ongoing prevention, management and control of biofouling (Satheesh *et al*., 2016).

Since aquaculture pathogens cause disease primarily through gene expression regulated by QS (Defoirdt, 2013; Zhao *et al*., 2015), it can be envisaged that disruption of this system

can be a more suitable alternative to currently available antimicrobial agents. Thus efforts are being made to identify bioactive marine natural products which are able to inhibit biofilm formation and/or quorum sensing and decrease virulence in aquaculture-related bacteria.

1.3 Seaweed as a source of bioactive compounds against microbes

Marine macroalgae are relatively simple, chlorophyllous plants, which are not differentiated into root, stem and leaf, and reproduce by spores instead of seeds. Marine seaweeds are adapted to the marine environment and usually grow either under or partly under water. Three types of seaweeds are defined according to their pigments, brown seaweeds (e.g., *Laminaria*, *Fucus*, *Sargassum*), red seaweeds (e.g., *Gelidium*, *Palmaria*, *Porphyra*), and green seaweeds (e.g., *Ulva*, *Codium*). The unique structural characteristics partly explains the unique chemical compositions observed in seaweeds, while the marine environment induces the production of unique chemicals to resist the environmental stresses the seaweeds are subjected to (Habbu *et al*., 2016).

Marine surfaces provide a habitat rich in organic material either because of the physical process of molecules adsorbing to surfaces, or due to the production of mucus or mucilage. Seaweeds release a large amount of organic carbon into the surrounding environment providing a nutrient-rich habitat for microorganisms. Marine seaweeds are, therefore, under constant colonization pressure from the millions of microorganisms within the surrounding seawater, some are epibiotic, others endophytic, and some are potential pathogens (Egan *et al*., 2014). The marine microbial communities covering seaweeds are complex and highly dynamic ecosystems, consisting of a diverse range of organisms (Goecke *et al*., 2010; Egan *et al*., 2014), with bacteria dominating among the primary colonizers of algal surfaces, followed by diatoms and fungi. While some seaweeds are heavily colonized, by contrast, other macroalgal species in the same habitat are almost epibiont-free (Goecke *et al*., 2010).

In order to defend themselves against harmful colonizers, the seaweeds, which lack a cell-based, adaptive immune response, have defense capabilities that fall into two categories. The first of these is constitutive under normal circumstances, i.e., production of antimicrobials by seaweeds or bacterial symbionts and the second involves induced defenses, which are triggered upon tissue damage and result in oxidative bursts or hypersensitive responses (Egan *et al*., 2014). The interactions of seaweed with surface microorganisms presents a constant threat to the host thus they synthesis compounds as a microbial defense mechanism (Harder *et al*., 2012). The surface of a seaweed includes secreted secondary metabolites and extracellular

exopolymers, which apart from being defense mechanisms, trigger specific interactions between seaweed and colonizers (Prabhakaran *et al*., 2012).

Many of the seaweed chemicals have been identified as fatty acids and hydroxyl unsaturated fatty acids, glycolipids, steroids, polyphenols, coumarins, terpenoids carotenoids, xanthophylls, chlorophylls, phycobilins, polysaccharides, vitamins, tocopherol and phycocyanins. Lauric acid, palmitic acid, linolenic acid, oleic acid and stearic acids are potential antibiotic or antifungal agents, while compounds halogenated with bromine, chlorine and even iodine metabolites like diterpenes, triterpenes reportedly possess diverse biological activities such as anti-bacterial, ichtyotoxic, antioxidant, anti-malarial, insecticidal and cytotoxic (Chojnacka *et al*., 2012). The extraction procedures and antimicrobial action of compounds from marine seaweeds against bacterial pathogens has been extensively reviewed recently by Perez *et al*. (2016). Majority of the seaweed antimicrobial studies have focused on the *in vitro* bactericidal or bacteriostatic properties of seaweed extracts against human bacterial pathogens, such as: *Bacillus subtilis, Clostridium* spp., *Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus* spp., *Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes* and *Vibrio cholerae* (Vatsos and Rebours, 2015).

Many seaweed species successfully prevent the heavy biofouling of marine abiotic surfaces and have evolved a number of anti-fouling strategies to combat the growth of epiphytic and/or potentially pathogenic microorganisms on their surfaces. Physical mechanisms of anti-fouling involve continuous shedding of the outer layer of cells, the mucilaginous covering and the continuous erosion of the distal ends of blades, and chemically, seaweeds secrete a rich source of bioactive secondary metabolites into the surrounding seawater to prevent fouling and grazing (Mieszkin *et al*., 2013). The anti-fouling potential has been described for the algal families Rhodophyceae, Phaeophyceae and a few Chlorophyceae and their roles in mediating seaweed–bacterial interactions have been extensively reviewed (Hollants *et al*., 2012; Egan *et al*., 2013; Mieszkin *et al*., 2013). The red alga *Delisea pulchra*, produces a range of halogenated furanones, which are localized in the central vesicle of gland cells and continuously released to the surface, that interfere with surface fouling of micro- and macro-organisms and maintain health and reproductive performance of this seaweed (Egan *et al*., 2013). The brown alga *Fucus vesiculosus* produces the pigment fucoxanthin, which prevents the attachment of bacterial isolates from co-occurring macroalgae. In contrast, bacteria isolated from the alga itself remain relatively insensitive to the effect of fucoxanthin. Fucoxanthin, unlike *D. pulchra* furanones or *B. hamifera* polyhalogenated 2-heptanones, acts as a general inhibitor of bacterial attachment,

rather than a specific inhibitor of bacterial growth which impacts on community composition (Saha *et al*., 2011). A glycerol derivative, sn-3-O-(geranylgeranyl)glycerol, isolated from the Mediterranean brown seaweeds *Taonia atomaria* and *Dictyota* spp., is associated with significant anti-adhesion effects with moderate associated toxicity against marine biofilm-associated bacteria. Yuvaraj and Arul (2014) assessed the anti-biofilm activity of crude methanol extracts from ten seaweeds and observed varying abilities to reduce biofilm formation by pathogens such as *Acinetobacter baumannii*, *Listeria monocytogenes*, *P. aeruginosa, V. anguillarum*. *V. fischeri and V. parahaemolyticus*. Saha *et al*. (2012) observed that common seaweed metabolites such as dimethylsulphopropionate and the amino acids proline and alanine inhibited surface attachment of specific bacteria (e.g. *Cytophaga* sp), while promoting the attachment of others (e.g. *Rheinheimera baltica*). Thus algal metabolites appear to influence specific members of the surface community composition and not just bacterial abundance (Saha *et al*., 2012). Alterations in bacterial community structures may thus 'select' for beneficial bacterial epibionts, while deterring potentially harmful pathogens. Undefended seaweeds have a significantly higher number of bacterial epibionts, an increased incidence of bleaching and a substantial reduction in growth compared to defended individuals (Egan *et al*., 2014). Since seaweed secondary metabolites are produced and released by specific cells, strong local effects on the bacterial epiphytes result (Egan *et al*., 2013).

In addition to growth inhibitory metabolites, seaweed metabolites interfere with bacterial communication networks and gene regulation, in particular bacterial QS communication systems. By producing QS inhibitors, seaweeds are able to interrupt communication circuits in bacteria, thus inhibiting gene expression and the resulting colonization phenotypes (Fernandes *et al*., 2011; Harder *et al*., 2012). Quorum sensing inhibitors produced by the red macroalga *D. pulchra* include 21 derivatives of halogenated furanones, that mediate a variety of ecological interactions for the alga including mimicking acylated homoserine-lactone (AHL) signals and competing with AHL for the LuxR receptor site (Harder *et al*., 2012), thereby inhibiting virulence factor production and pathogenesis in *P. aeruginosa*. The predicted mechanism of QS inhibition involves the interference of three carbon aliphatic side chains, with the binding of the smaller AHLs to their cognate receptors (Jha *et al*., 2013). Sethupathy *et al*. (2016) demonstrated the anti-biofilm and QS inhibitory potential of the brown macroalga *Padina gymnospora* against the nosocomial pathogen *Serratia marcescens*. The methanolic extract of *P. gymnospora* inhibited biofilm formation and the production of prodigiosin and protease. The predominant compound, alpha-bisabolol was

responsible for the inhibition of biofilm and QS-controlled prodigiosin, protease and swarming in *S. marcescens*, without exerting a deleterious effect on its growth and metabolic activity.

From an ecological perspective, the antimicrobial, anti-biofilm, anti-fouling and anti-QS defense mechanisms of marine seaweeds may reduce epibiosis, inhibit premature decomposition and directly provide resistance to infectious diseases, by elimination or control of the number of pathogens, epiphytes or endophytes. Seaweeds control bacterial colonization by use of QS inhibitory chemical defenses to suppress the expression of specific pathogenicity traits, while not necessarily killing the pathogen, thus averting disease without promoting resistance traits (Egan *et al*., 2014). Marine seaweeds are thus a natural source of bioactive molecules with a broad range of biological activities, such as antimicrobial, anti-viral, anti-cancer, anti-tumour, antioxidant, anti-inflammatory, anti-biofilm, anti-fouling and anti-virulence.

Aquaculture-based food products can be a source of foodborne pathogenic and spoilage bacteria. Seaweed extracts contain compounds, which have significant positive effects on the growth and immune system of aquaculture, in addition to demonstrating antibacterial properties against many fish pathogenic and opportunistic human pathogenic bacterial species that infect farmed fish (Vatsos and Rebours, 2015). Bacterial fish pathogens tested include: *Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Pseudomonas anguilliseptica, Photobacterium damselae* sbsp *piscicida, Photobacterium damselae* subsp. *damselae, Renibacterium salmoninarum, Streptococcus iniae, Vibrio alginolyticus, Vibrio anguillarum, Vibrio fischeri, Vibrio harveyi, Vibrio ordalii, Vibrio parahaemolyticus, Vibrio salmonicida, Vibrio vulnificus,* and *Yersinia ruckeri* (reviewed extensively by Vatsos and Rebours, 2015). The *in vitro* and *in vivo* antibacterial effects of aqueous extracts of bromoform and dibromoacetic acid from the red seaweed *A. taxiformis* was assessed against the fish pathogen *S. iniae* (Mata *et al*., 2013). When extracts were added into the water containing *Lates calcarifer* (barramundi) fingerlings already infected with *S. iniae*, a delay was observed in the growth of the bacterium in the water, but this did not affect significantly the mortalities caused by *S. iniae.* Addition of higher concentration of the extracts was more effective against the pathogen, but also induced mortality in the fish. However, Manilal *et al*. (2012) examined the therapeutic potential of the ethyl acetate fraction of *Asparagopsis* spp. in black tiger shrimp post-larvae challenged with lethal doses of *V. harveyi, V. alginolyticus, V. parahaemolyticus* and *P. damselae*. Shrimp fed with varying concentrations of extract exhibited significantly increased survival rate. Roohi Fatima *et al*. (2016) observed enhanced protection of farmed Mozambique tilapia fish against *V. parahaemolyticus* infection in housing tanks by addition of

an antibacterial methanol extract (1 part per trillion per litre) from the red seaweed, *Portieria hornemannii*. Cavallo *et al*. (2013) reported that *Gracilariopsis longissimi* extracts might serve as a potential source of antibacterial compounds with possible application in aquaculture plants for the treatment of fish diseases associated with *Vibrio* infections. Extracts from *Padina gymnosporawere* were effective against *Oreochromis mossambicus* infected with *P. aeruginosa* (Thanigaivel *et al*., 2015). Sivakumar *et al*. (2014) demonstrated that the antimicrobial properties of *U. fasciata* seaweed extracts against the pathogen *V. harveyi*, were due to reduced phospholipase, proteolysis, lipolysis and thermonuclease activities of treated bacteria and this significantly reduced mortality. Thanigaivel *et al*. (2014) examined the antimicrobial potential of an ethanol extract from the green seaweed *Chaetomorpha antennina* by immersing *Penaeus monodon* infected with *V. parahaemolyticus* into water containing 250 mg/l of the seaweed extract for 12–48 h. This treatment resulted in survival of 98% of the treated shrimp, and intramuscular injection of 25 µl of the extract per shrimp protected the animals when they were subsequently infected by the bacterial pathogen.

Defoirdt *et al*. (2006) examined the antibacterial effect of halogenated furanone extracted from *D. pulchra* against the shrimp bacterial pathogens *Vibrio campbellii, V. harveyi* and *V. parahaemolyticus*. At 20 mg/l, *in vivo* protection of brine shrimp *Artemia franciscana* was observed against these bacterial pathogens, although the substance did not have any effect on the growth rate of the pathogens in the water. The protective effect was suggested to be due to the disruption of QS, due to the observed inhibition of bioluminescence. In addition to disrupting AHL-mediated quorum sensing, halogenated furanones block the multichannel QS systems of vibrios by decreasing the DNA-binding activity of the quorum sensing master regulator LuxRVh. However, while halogenated furanones protect both fish and crustaceans against vibriosis, they are too toxic to higher organisms to be applied in practice, with toxic concentrations being only slightly higher than QS-disrupting concentrations (Defoirdt, 2013).

Most seaweed extracts, however, are reported to be nontoxic and can be incorporated directly in the feed or added directly into the water, thus these could be used as an alternative to traditional antibiotics, which cause resistance (Vatsos and Rebours, 2014). The water-soluble substances of seaweeds can be released directly from the seaweeds or extracts can be added directly (for long periods) into the aquatic environment of the farmed fish and shrimp, where they would affect the QS mechanism in bacteria in the water column, prior to infection, with limited effects on bacterial growth. On addition of extracts into fish/shrimp feeds (live or dry), the seaweed compounds can act directly against the pathogens or by stimulating the immune system (Vatsos and Rebours, 2014).

1.4 Diversity of seaweed-associated-bacteria

Seaweeds form an integral part of the aquatic environment (Egan *et al*., 2013), where they serve as primary producers, providing shelter and food. They harbor a rich diversity of microorganisms (bacteria, fungi, cyanobacteria and some microalgae), some of which are pathogenic and others symbiotic (Kiuru *et al*., 2014; Singh and Reddy, 2014), due to their nutrient composition. Epiphytic bacterial communities are essential for normal morphological development of the algal host, enhance algal growth and provide nutrients (Goecke *et al*., 2010), influence release and settlement of algal spores (Potin, 2012), and either enhance or deter secondary colonization by other microscopic and macroscopic epibiota. This seaweed and epiphytic bacteria interaction is considered to be a unified functional entity or holobiont (Harder *et al*., 2012).

The diversity of bacteria colonizing seaweed ranges between 10^2 to 10^7 cells /cm^{-2} alongside virus, fungi and protozoa and are found predominantly in association with the thallus tip and base. Surface colonization and diversity of microorganisms differ based on species of seaweed and season (Case *et al*., 2011; Egan *et al*., 2013). Seaweed communities also experience spatial and temporal shifts, which may be a reflection of the changing local conditions, host physiology, or chemical and physical parameters (Egan *et al*., 2013). The varied differences in bacterial communities is indicative of competitive pressure amongst colonizers leading to the production of bioactive compounds, QS signaling molecules, and/or QS inhibitors which are responsible host protection and normal morphology development (Singh and Reddy, 2014).

Seaweed-associated bacteria from seaweed surfaces or within algal thalli belong to the (super)phyla Proteobacteria, Actinobacteria, Bacteroidetes (CFB group), Cyanobacteria, Firmicutes, Planctomycetes, Verrucomicrobia, Chloroflexi, Deinococcus-Thermus, Fusobacteria, Tenericutes, and the candidate division OP11 (Hollants *et al*., 2013). The γ-proteobacteria appear to be the most common bacterial clade associated with seaweeds (37% relative abundance), followed by the CFB group (20%), α-proteobacteria (13%), Firmicutes (10%), and Actinobacteria (9%). On a lower taxonomic level, the orders Flavobacteriales (14% relative abundance), Alteromonadales (12%), Vibrionales (10%), Pseudomonadales (9%), Bacillales (9%), Actinomycetales (8%), and Rhodobacterales (7%) were most abundant in seaweed-associated bacterial communities. It is surprising that only 33 bacterial genera including *Alteromonas, Bacillus, Flavobacterium, Pseudoalteromonas, Pseudomonas*, and *Vibrio* have been described from green, red, and brown seaweeds. Some genera like *Cytophaga, Planococcus* and *Tenacibaculum*, appear to be associated with green and red

seaweeds, but are virtually absent from brown seaweed surfaces (Hollants *et al*., 2013).

Nutrient-rich seaweed surfaces attract many opportunistic micro- and macro-organisms, creating a highly competitive environment in which bacteria attempt to outcompete other surface colonizers by the production of defensive compounds (Penesyan *et al*., 2013). Thus microorganisms synthesize bioactive metabolites in order to ensure their dominant positions on the host (Fig. 1.1). This leads to the production of structurally novel compounds (Singh *et al*., 2013), which could serve as a source of bioactive compounds for use in human therapies. The production of these antimicrobial compounds is not restricted to a certain bacterial group but is widespread across α-proteobacterial, β-proteobacterial, γ-proteobacterial, flavobacterial, actinobacterial, and bacilli clades. *Micrococcus, Phaeobacter, Pseudoalteromonas, Shewanella, Vibrio*, and various *Bacillus* species have been identified as efficient producers of compounds with antimicrobial, anti-fouling, and QS inhibiting potential, which allows them to be highly successful colonizers of seaweed surfaces (JanakiDev *et al*., 2013; Vinoj *et al*., 2014). Many of these compounds are chemical weapons, which have evolved into highly potent inhibitors of physiological processes in the prey, predators or competitors of the marine organisms that utilize them for survival (Suresh *et al*., 2014).

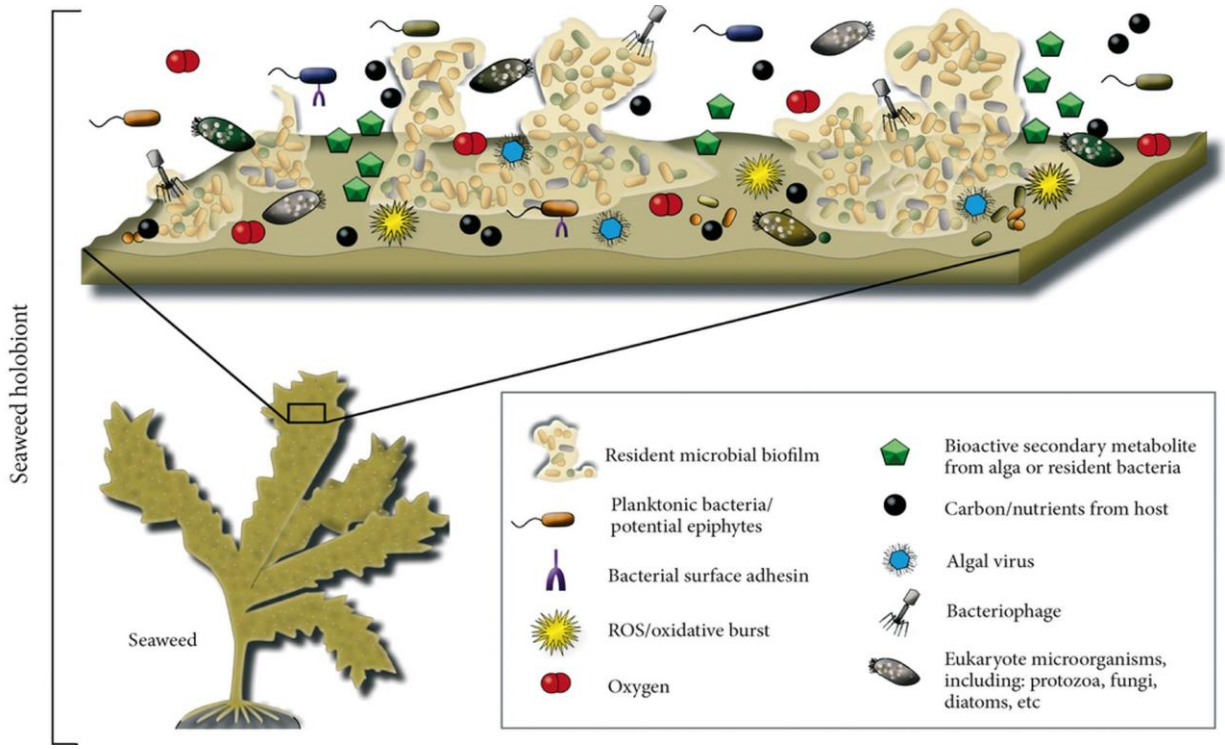

Figure 1.1: The seaweed holobiont and the factors predicted to influence bacterial colonization on macroalgal hosts (Egan *et al*., 2013).

1.5 Bioactive compounds of seaweed-associated-bacteria

Marine surface-associated microorganisms are a rich source of novel bioactive compounds because of the necessity of evolving structurally diverse allelochemicals capable of protecting the producer microorganism from the fierce competition that exists between epiphytic and/or opportunistic microorganisms on the surfaces of marine eukaryotes (Fig. 1.2). The chemically-driven interactions allow for the establishment of cross-relationships between microbes and their eukaryotic hosts, whereby microorganisms producing bioactive compounds may protect the host surface against over-colonization, fouling, etc., in return for nutrient access (Penesyan *et al*., 2010).

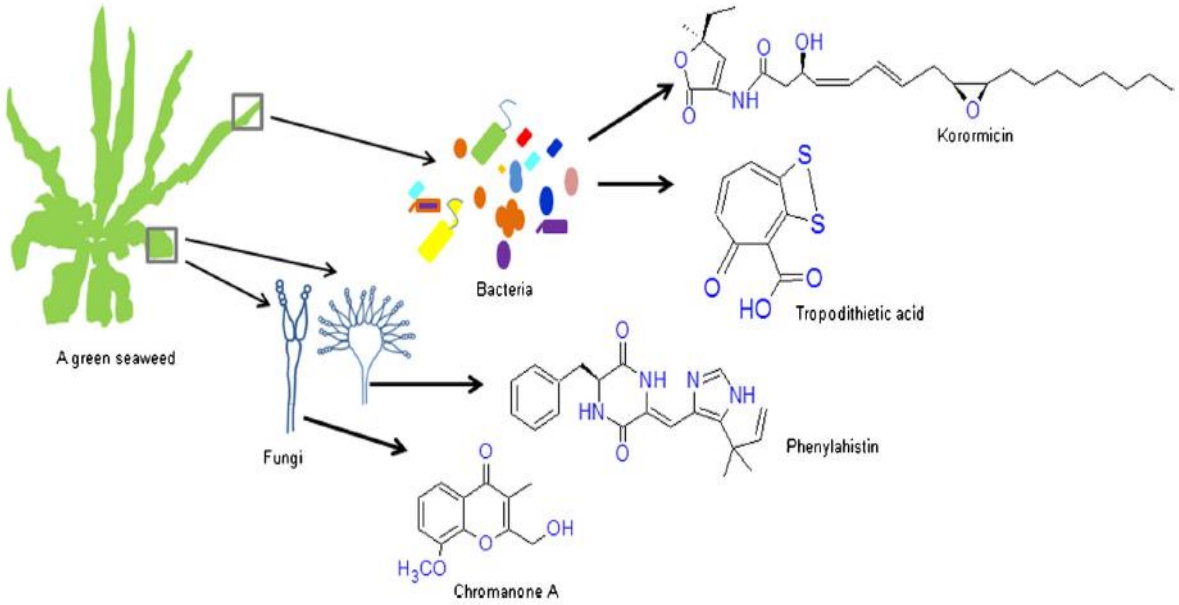

Figure 1.2: Graphical representation of green seaweed, existing of bacterial and fungal communities on their surface and structure of bioactive compounds (Singh *et al*., 2014).

Marine seaweeds are a playground for a wide diversity of bacterial associations ranging from beneficial (mutualistic), harmful (parasitic), and neutral (commensal), to obligate and facultative, to endo- and ecto-phytic interactions (Susilowati *et al*., 2015). Thus, a wide range of chemically-mediated beneficial and detrimental interactions exists between seaweeds and epi- and endo-symbiotic bacteria that reside either on the surface or within the algal cells based on the exchange of nutrients, minerals, and secondary metabolites (Susilowati *et al*., 2015). The observed positive and negative seaweed–bacterial interactions are based on seaweed surfaces providing a protected and nutrient-rich 'hot spot' for opportunistic bacteria that proliferate in the presence of organic material and oxygen (Singh and Reddy, 2014). In return, bacteria produce morphogenic factors, fixed nitrogen, enzymes, and vitamins, which promote

algal growth. In addition, epiphytic bacteria as well as the seaweed hosts themselves produce antibiotic substances that prevent colonization of the algal surface by bacterial competitors and pathogens (Hollants *et al*., 2013). Seaweeds without their own chemical defenses rely on the secondary metabolites produced by their associated bacteria (Goecke *et al*., 2010). The seaweed-associated bacteria produce various bioactive compounds including haliangicin, violacein, pelagiomicin A, korormicin, macrolactines, and chlorophyll d, which demonstrate a wide range of bioactivities including: antifungal, antiprotozoal, anti-settlement, antimicrobial activity against Gram-negative and Gram-positive, and photosynthetic activity, respectively (Singh *et al*., 2015).

1.5.1 Antimicrobial activities of seaweed-associated bacteria

Chemical interactions between different species of seaweed epibiotic bacteria affect the production and secretion of secondary metabolites in these microorganisms (Goecke *et al*., 2010; Egan *et al*., 2014). Since symbiotic bacteria, pathogens, and foulers first select, then settle, and finally attach to the host, the first line of defense against microbial challenge is by the seaweed themselves which produce secondary metabolites that inhibit one or all of these steps. Should bacterial adhesion not be successfully inhibited, other secondary metabolites are synthesized by the seaweeds or by epiphytic and endophytic microbes associated with them, which may inhibit the growth, survival, virulence, or reproduction of invading microbes (Case *et al*., 2011).

Seaweed-based studies have thus led to the isolation of antagonistic bacteria which synthesize compounds with broad spectrum of activities (anti-biofilm, anti-cancer, antimicrobial, and anti-QS) (Case *et al*., 2011; Egan *et al*., 2014; Tebben *et al*., 2014; Singh *et al*., 2015). Penesyan *et al*. (2009) obtained 325 bacterial isolates from the surface of *D. pulchra* and *Ulva australis* in Australia and demonstrated antibiotic activity for 12% of the strains. The majority of these isolates belonged to α- and γ-proteobacteria, but a few antibacterial isolates belonging to the phyla Actinobacteria, Firmicutes and Bacteroidetes were also found (Penesyan *et al*., 2009). Almost 50% of 210 isolates (belonging to 21 genera including *Aeromonas, Bacillus, Pseudoalteromonas, Pseudomonas, Shewanella, Stenotrophomonas, Streptomyces* and *Vibrio*) of the epiphytic bacterial community of the brown alga *Saccharina latissima* (Baltic Sea, Germany) inhibited the growth of at least one microorganism from a panel of Gram-negative and Gram-positive bacteria (Wiese *et al*., 2009).

Antimicrobial activity is thus widespread among seaweed-associated bacteria, with the most represented bacterial genera being the Gram-positive *Bacillus* and *Streptomyces* and

Gram-negative *Pseudomonas* and *Pseudoalteromonas*, which are known for their ability to produce bioactive compounds (Bhatnagar and Kim, 2010). Many *Bacillus* species are efficient producers of antimicrobial compounds (antibacterial and antifungal) and are, therefore, highly successful, dominant colonizers of seaweed surfaces (Kanagasabhapathy *et al*., 2006; Penesyan *et al*., 2010; Burke *et al*., 2011; Lachnit *et al*., 2011). Seaweed-associated *Bacillus* isolates produce peptide compounds with antimicrobial activity (Janakidevi *et al*., 2013). JanakiDevi *et al*. (2013) isolated 126 bacteria from five different seaweeds (*Gracillaria corticata, Geledium pussilum, Hypnea musiformis, Padina gymnosphora,* and *Valoniopsis pachynema*) which showed antibacterial activity (2.6 - 16 mm inhibitory zones) against *E. coli, K. pneumoniae, P. aeruginosa, Salmonella* sp., *Serratia* sp., *Shigella dysenteriae, V. cholerae, Micrococcus* and *Staphylococcus* spp. An important antibacterial protein (30.7 kDa) was obtained from *Bacillus licheniformis* associated with *Fucus serratus*, with activity against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and *L. monocytogenes* (Jamal *et al*., 2006). A bacteriocin (lichenicidin, a class of lentibiotics) was identified from a seaweed-associated (*Polysiphonia lanosa* and *Ulva* spp.) *B. licheniformias* (Prieto *et al*., 2012), while another bacteriocin (~8 kDa molecular weight) was partially characterized from seaweed-associated *Staphylococcus haemolyticus* MSM and exhibited strong antibacterial activity against human pathogenic bacteria (Suresh *et al*., 2014).

Besides *Bacillus* species, *Pseudoalteromonas* spp. which are commonly found on marine seaweeds, also produce biologically active molecules that inhibit or control fouling of other species on the host surface. The γ-proteobacterium *Pseudoalteromonas tunicata* plays a role in defending the algal host against surface colonisation by producing antimicrobial, anti-larval and anti-protozoan compounds. Tebben *et al*. (2014) identified 13 natural products from *Pseudoalteromonas* strain J010, isolated from the surface of the crustose coralline alga *Neogoniolithon fosliei*. Among them, a new bromopyrrole, 4-(3,4,5-tribromo- 1H-pyrrol-2-yl) methyl)phenol and five new korormicins G–K were obtained which exhibited antibacterial activity, in addition to a coral larval metamorphosis inducer compound, tetrabromopyrrole which demonstrates broad-spectrum activity against tested bacteria, fungi, and protozoan (Tebben *et al*., 2014). Two important diketopiperazines, cyclo-(L-prolyl-L-glycine) and cyclo-(L-phenylalanyl-4R-hydroxy-L-proline), and 2,4-dibromo-6-chlorophenol were extracted from *Pseudoalteromonas luteoviolacea* from the seaweed *Padina australis* (Jiang *et al*., 2001). Both diketopiperazines stimulated antibiotic production in this strain whereas 2,4-dibromo-6-chlorophenol showed antibacterial activity against cystic fibrosis-associated *Burkholderia cepacia* and MRSA.

Seaweed-associated *Pseudomonas* sp. strain AMSA, isolated from a red alga *Ceratodyction spongiosum*, produced novel compound 2,4-diacetylphloroglucinol, which exhibited activity against MRSA at a minimal concentration of 1 mg/ml and *V. parahaemolyticus* at 24 mg/L (Wietz *et al*., 2013). Four massetolides A, B, C, and D (novel cyclic depsipeptides) were extracted from ethyl acetate fraction of a *Pseudomonas* sp. isolated from an unidentified red alga and inhibited the growth of *Mycobacterium tuberculosis* and *M. avium-intracellulare* (Gerard *et al*., 1997). Two peptides cyclo-[phenylalanyl-prolyl-leucyl-prolyl] and cyclo-[isoleucyl-prolyl-leucyl-alanyl] obtained from a *Pseudomonas* sp. associated with Japanese seaweed *Diginea* sp., inhibited growth of *S. aureus, Micrococcus luteus, B. subtilis, E. coli,* and *V. anguillarum* (Rungprom *et al*., 2008). Ravisankar *et al*. (2013) identified an alkaloid from *Pseudomonas* sp. associated with *Padina tetrastromatica*, which inhibited growth of *K. pneumoniae* and *Pseudomonas aeruginosa* at a concentration of 300 μg.

Vibrio alginolyticus G16 associated with *Gracilaria gracilis* had broad-spectrum antimicrobial activity against *S. marcescens* and anti-QS activity (Padmavathi *et al*., 2014). An epiphytic bacterium, *Pseudovibrio* sp. D323 isolated from *D. pulchra* produced the antibacterial compound tropodithietic acid, which has a broad-spectrum effect against bacteria belonging to Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (Penesyan *et al*., 2011). Suvega and Kumar (2014) observed that the majority of bioactive compound producing-bacterial isolates (epibiotics, 39.54 % and endobiotics, 40.74 %) were obtained from the surface of seaweeds as compared to the seawater (8.61 %) and marine sediments (11.11 %). These isolates produced antimicrobial compounds, which were active against plant pathogens (*Xanthomonas axonopodis* pv. citri, *X. oryzae* pv. oryzae and *Ustilaginoidea virens*). Proteins present in the extracellular components of these bacterial isolates were highly active at pH 7.0 and showed antibacterial activity up to 40 °C and antifungal property up to 60 °C, whereas nonpolar lipophilic compounds extracted from these active bacteria only displayed antifungal activity (Suvega and Kumar, 2014).

Antimicrobial-producing marine bacteria belong to many taxonomic groups, with some of the most prevalent producers originating from the Actinomycetes. This is not a surprise as most structurally diverse bioactive compounds have been isolated from terrestrial actinomycetes, particularly *Streptomyces* spp (Singh *et al*., 2015). The antibiotics that are in use today are derivatives of novel natural products of actinobacteria such as indigenous marine actinomycetes which includes members of the genera *Actinomadura, Actinosynnema, Amycolatopsis, Arthrobacter, Frigoribacterium, Geodermatophilus, Gordonia, Kitasatospora, Micromonospora, and Micrococcus* (Ravikumar *et al*., 2012; Manivasagana *et al*., 2014).

Bacillus and *Actinomycetes* contain non-ribosomal polyketide synthetase (NRPS) and polyketide synthetase (PKS) pathways, which allow for the production of a wide diversity of secondary metabolites (Li *et al*., 2014). A polyketide, 2-hydroxy-5-((6-hydroxy-4-oxo-4*H*-pyran-2-yl)methyl)-2-propylchroman-4-one, was obtained and structurally characterized from *Streptomyces sundarbansensis* strain, an endophytic actinomycete isolated from the Algerian marine brown algae *Fucus* sp (Djinni *et al*., 2013). This compound exhibited selective activity against MRSA (MIC = 6 μM), with a bacteriostatic effect. Braña *et al*. (2014) identified several bioactive compounds from seaweed-associated *Streptomyces* strains, *Streptomyces cyaneofuscatus* M-27 and *Staphylococcus carnosus* M-40. These compounds: daunomycin (anticancer), cosmomycin B (antitumor), galtamycin B (antitumor), maltophilins (antifungal), and lobophorins (anti-inflammatory, anti-BCG and antituberculosis) display several biological activities. The benzaldehydes (2-hydroxy-5-(3-methylbut-2-enyl)benzaldehyde and 2-hepta-1,5-dienyl-3,6-dihydroxy-5-(3-methylbut-2-enyl)benzaldehyde) produced by seaweed-associated *Streptomyces atrovirens* Pk288-21, demonstrated pathogens *E. tarda* and *S. iniae* (Cho and Kim, 2012). A novel polyketide family member 7-O-methyl-5′-hydroxy-3′-heptenoate-macrolactin was obtained from a *B. subtilis* MTCC 10403 strain associated with the seaweed *Anthophycus longifolius* (Chakraborty *et al*., 2014). It possesses broad-spectrum antibacterial activity against aquaculture pathogens *A. hydrophila, V. vulnificus*, and *V. parahemolyticus* (Chakraborty *et al*., 2014). Sridevi and Dhevendaran (2014) antimicrobial activity against fish isolated 45 *Streptomyces* spp. isolates from 16 seaweeds, of which 25 demonstrated antagonistic activity against *V. alginolyticus, V. harveyi, V. parahaemolyticus* and *V. vulnificus*. Based on *in vitro* and *in vivo* probiotic studies, they have suggested that seaweed-associated *Streptomyces* are a promising source of probiotic and biocontrol agents against vibriosis in aquaculture settings. According to Tan *et al*. (2016), *Streptomyces* as probiotic in aquaculture would be beneficial as anti-pathogenicity agents through the production of various antagonistic compounds (e.g., anti-biofilm, anti-QS and anti-virulence) against *Vibrio* pathogens. In addition to being a good protein source, *Streptomyces* probiotics secrete hydrolytic exoenzymes which improve the amylolytic and proteolytic activity in the digestive tract of the fish, which would allow for more efficient use of the feed allowing better growth performance of the livestock (Tan *et al*., 2016).

Generalist bacterial species such as *Bacillus, Pseudoalteromonas*, or *Streptomyces* which occupy a broad spectrum of environments would be more likely to benefit from producing broad-spectrum antimicrobials or a cocktail of molecules targeting different potential competitors, while obligate epiphytes which are highly specialized for a given habitat

may produce antimicrobials with narrower range, targeting specific competitors (Hibbing *et al*., 2010). While bioactive compound production may promote the colonization of and competition on host surfaces (Rao *et al*., 2005; Wahl *et al*., 2012), they often have a fitness cost in terms of resource allocation, diverting energy away from growth and reproduction (Kumar *et al*., 2011; Wahl *et al*., 2012).

1.6 Role of biofilms and quorum sensing in virulence

Many clinically-relevant bacteria use QS regulons to regulate the collective expression of virulence factors. Of increasing concern is the growing inability to combat these pathogens, due to the toxicity of anti-fouling agents like tributylin (Cho *et al*., 2012) and the rapid spread of antimicrobial resistance, found to be fuelled by the very drugs and treatments which are being employed to fight it (Chankhamhaengdecha *et al*., 2013). Quorum sensing regulates the expression of biofilm formation and is a cooperative group behaviour that involves bacterial populations living within extracellular matrix. Quorum sensing coordinates the switch from planktonic to a biofilm lifestyle when the population density reaches a threshold level (Lee *et al*., 2013). Although pathogenic bacteria can be found as planktonic cells, most prefer a biofilm lifestyle, which provides them with a 1000-fold more resistance than they would possess alone (Padmavathi *et al*., 2014). However, for different bacterial species activation of QS is cell density dependent and is linked to the maturation and disassembly of the biofilm in a coordinated manner (Pandey *et al*., 2014). *Enterococcus faecalis* and *V. harveyi* utilize QS to coordinate their virulence expression and have emerged as important nosocomial and food pathogens (Hamza *et al*., 2015).

1.6.1 Quorum sensing

Quorum sensing (QS) is a bacterial cell–cell communication process that follows three basic principles the production, detection, and response to signaling molecules referred to as autoinducer (AIs). Three main QS systems have been reported, i.e., the acyl-homoserine lactone (AHL) QS system utilized by Gram-negative bacteria, the autoinducing peptide (AIP) QS system in Gram-positive bacteria and the autoinducer-2 system for both Gram-negative and positive organisms (Singh *et al*., 2015). Auto-inducers (AIs) accumulate in the environment as the bacterial population density increases, when the AI reach certain threshold collectively alter gene expression (Kalia, 2014). Phenotypes associated with QS systems include bioluminescence, competence, conjugation, biofilm formation, antibiotic production, swarming, nodulation, sporulation, and expression of virulence factors (toxins, siderophores,

lytic enzymes, and adhesion molecules) which are not related to the growth or survival of the organism (Brackman *et al*., 2011; Tan *et al*., 2012).

Many Gram-negative pathogenic bacteria (*Aeromonas, Edwardsiella, Pseudomonas, Tenacibaculum* and *Vibrio*) utilize QS to express virulence factors (Zhao *et al*., 2015). The Gram-negative QS mechanism involves AHL molecules, which diffuse freely through the plasma membrane and as the population density increases the AHL concentration also increase until a threshold is reached which triggers transcription of targeted genes (Defoirdt, 2013).

The QS system of Gram-positive bacteria typically consists of signalling peptides (Fig. 1.3) such as Agr and RNA-III activating/inhibiting peptides (RAP/RIP) in *Staphylococcus aureus*, and a two-component regulatory system made up of a membrane-bound sensor and an intracellular response regulator (Quave and Horswill, 2014). The autoinducer molecule is an AIP that is detected by a membrane-bound signal transduction system. This system works in a two component signal manner in which the two component regulatory system is cell-density-dependent. The peptide signal is secreted into the surrounding media for other organisms to detect. Once a particular threshold is reached the peptide signal operates by binding to a sensor protein, histidine kinase found on the cell membrane. Activation of histidine kinase leads to phosphorylation reactions, which activate the response regulating protein leading to transcriptional activation (Quave and Horswill, 2014; Singh and Nakayama, 2015).

Four genes which are involved in the QS system (Fig. 1.3) are encoded by the accessory gene regulator (*agr*) locus (Singh *et al*., 2016). The AIP propeptide is translated from *agrD* and is subsequently processed by a cell membrane enzyme encoded by *agrB*. Mature AIP, containing a thiolactone bridge, is secreted out of the cell. As bacterial density increases, the AIP concentration increases, and at a specific threshold, AIP triggers the activation of the two-component regulatory system encoded by *agrC* and *agrA*. The expression of RNAIII, a regulatory RNA molecule as well as mRNA of δ-hemolysin, is promoted by activated AgrA (Fig. 1.3). RNAIII thus transcriptionally or translationally controls the expression of a series of genes involved in virulence (Singh *et al*., 2016).

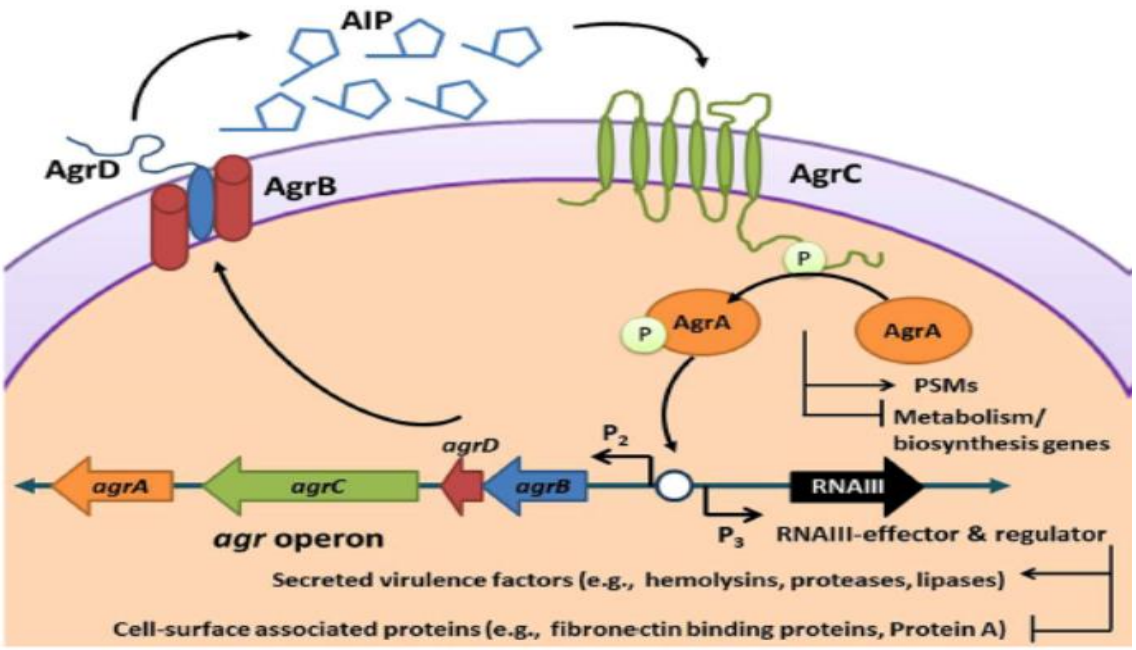

Figure 1.3: Schematic overview of *Staphylococcus aureus* accessory gene regulatory **(***agr***)** system. The RNAII transcript is an operon of four genes, *agrBDCA* that encode factors required to synthesize AIP and activate the regulatory cascade (Quave and Horswill, 2014).

Quorum sensing in Gram-positive bacteria is linked to the expression of extracellular toxins such as α-hemolysin encoded by *hla*, and cell surface adhesion factors such as Protein A encoded by *spa* (Gray *et al*., 2013). Their expression of virulence factors is under the control of the *agr* (accessory gene regulator) QS and *fsr* (*Enterococcus faecalis* regulator) systems. Virulence in *Enterococcus faecalis* is also controlled by a two component QS system utilizing a peptide based autoinducer (AI) encoded by the *fsr* gene locus. The *fsr* locus, comprised of four genes *fsrABDC*, has been identified as homologue of the *S. aureus* Agr QS system (Hirakawa and Tomita, 2013). The *fsr* QS system utilizes gelatinase biosynthesis activating pheromone (GBAP) as antoinducer (AI). FsrC the histidine receptor kinase (Fig. 1.4) is modulated by the binding of mature GBAP to the ligand and promotes kinase activity. FsrC is then able to phosphorylate the DNA binding response regulator FsrA. This actions leads to the upregulation of virulence factor expression (Cook and Federle, 2014). *Enterococcus* virulence is linked to the expression of gelatinase (GelE) and serine proteases (SprE) encoded by *fsr* gene cluster (Fig. 1.4). GelE and SprE are reported as regulators of biofilm formation (Hirakawa and Tomita, 2013; Singh and Nakayama, 2015). Biofilm formation plays an essential role in the development of enterococcal diseases such as endocarditis and persistent infections due to indwelling catheters (Cook and Federle, 2014).

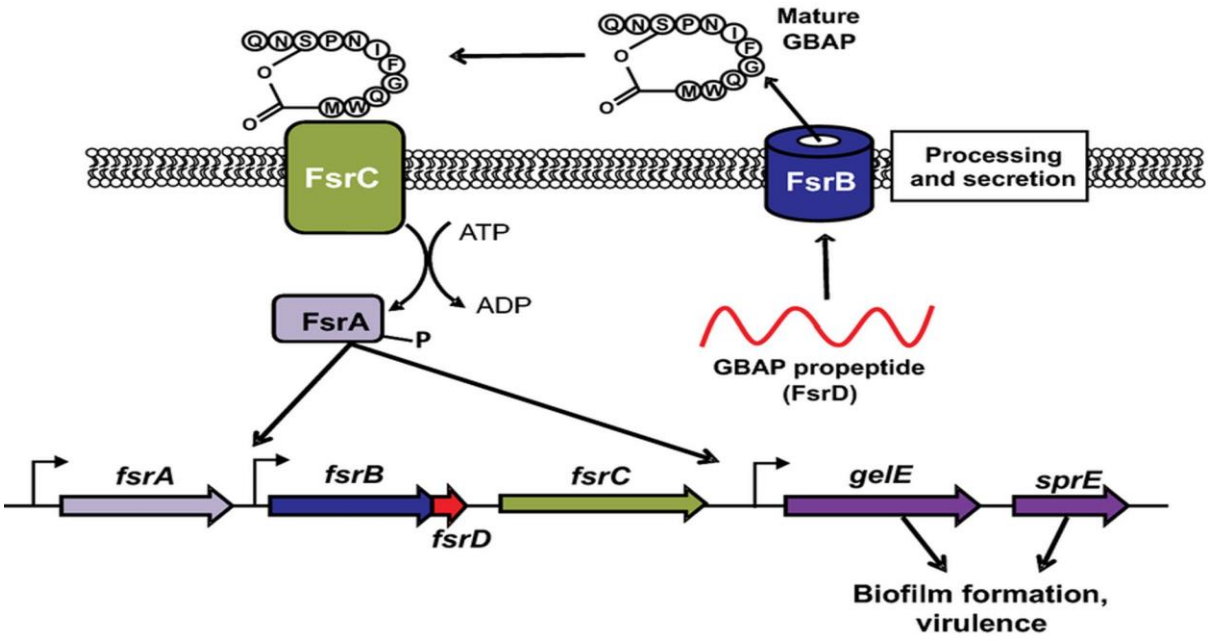

Figure 1.4: The Fsr QS system of *Enterococcus faecalis*. FsrD, the gelatinase biosynthesis activating pheromone (GBAP) precursor, is processed to a cyclical peptide during secretion by FsrB. Mature GBAP pheromone interacts with the FsrC sensor kinase on the surface of surrounding cells causing phosphorylation of the DNA-binding response regulator, FsrA. Phosphorylated FsrA binds to promoters, including those of *fsrB* and *gelE*/ *sprE*, and upregulates gene expression (Cook and Federle, 2014).

1.6.1.1 Mechanisms targeting quorum sensing

To date only three mechanisms targeting QS have been documented and reported, i.e., inhibition of signal generation (LuxI-type synthases, *agrC*), QS signal degradation and inhibition of QS signal biosynthesis (Zhao *et al*., 2015). Quorum sensing signal molecules can be enzymatically degraded, by quorum quenching (QQ) enzymes to prevent their accumulation and subsequent activation of the QS system. Many natural QQ substances have already been identified for Gram-negative QS systems, and may be classified based on their modes of action as AHL-lactonases, AHL-acylases and AHL-oxidoreductases. Lactonases hydrolyze the ester bond of the AHL molecules yielding N-acyl-homoserine and acylases hydrolyze the amide bond to yield homoserine lactones and a fatty acid chain (Tang and Zhang, 2014). Oxidoreductase catalyzes the reduction of 3-oxo-C(8-14)-homoserine lactones (HSLs) to their corresponding 3-hydroxy-HSLs, thereby rendering them unrecognizable to their receptor molecules (Fetzner, 2015).

To date no specific enzymatic quenchers of Gram-positive AIP signals have been described (Singh *et al*., 2016). There are three potential targets in the agr QS system (Fig. 1.5). AIP biosynthesis is the primary choice because it is the initial event in the QS signal circuit (Singh *et al*., 2016). Two enzymes, AgrB and SpsB are involved in AIP biosynthesis, AgrB is

specifically involved in the biosynthesis of cyclic AIP, while SpsB is type-I signal peptidase involved in the secretion of Sec and Tat-dependent proteins. AgrB and SpsB are processing enzymes with protease activity and inhibition of their catalytic functions is anticipated to block QS signaling entirely. Enzyme inhibitors, however, are more or less bactericidal or bacteriostatic even when targeting AgrB that is specific to AIP biosynthesis rather than SpsB that is common for wide range of secretary protein (Singh *et al*., 2016). Blockage of the two component regulatory system AgrC-AgrA is the second target. The combined action of AgrC and AgrA, where AgrC phosphorylates and activates regulator AgrA and leads to activation of promoters P2 and P3 which are responsible for expression of virulence factors (Fig. 1.5) is a viable target for QS inhibitory compounds (Painter *et al*., 2014). Peptide antagonists are expected to have high specificity to AgrC and neither bactericidal nor bacteriostatic activity. A third target, i.e., kinase inhibitors targeting AgrC is a possibility, however, it appears to have bactericidal and/or bacteriostatic activity due to the number of diverse histidine kinases functioning in bacteria. Binding of heterologous AIP causes the linker ligand to bend in the opposite direction thus preventing the action of the kinase receptor (Painter *et al*., 2014). These three targets are available for *agr*-like systems of other Gram-positive bacteria, while there are some targets specific to each QS Gram-positive bacterial group (Singh *et al*., 2016).

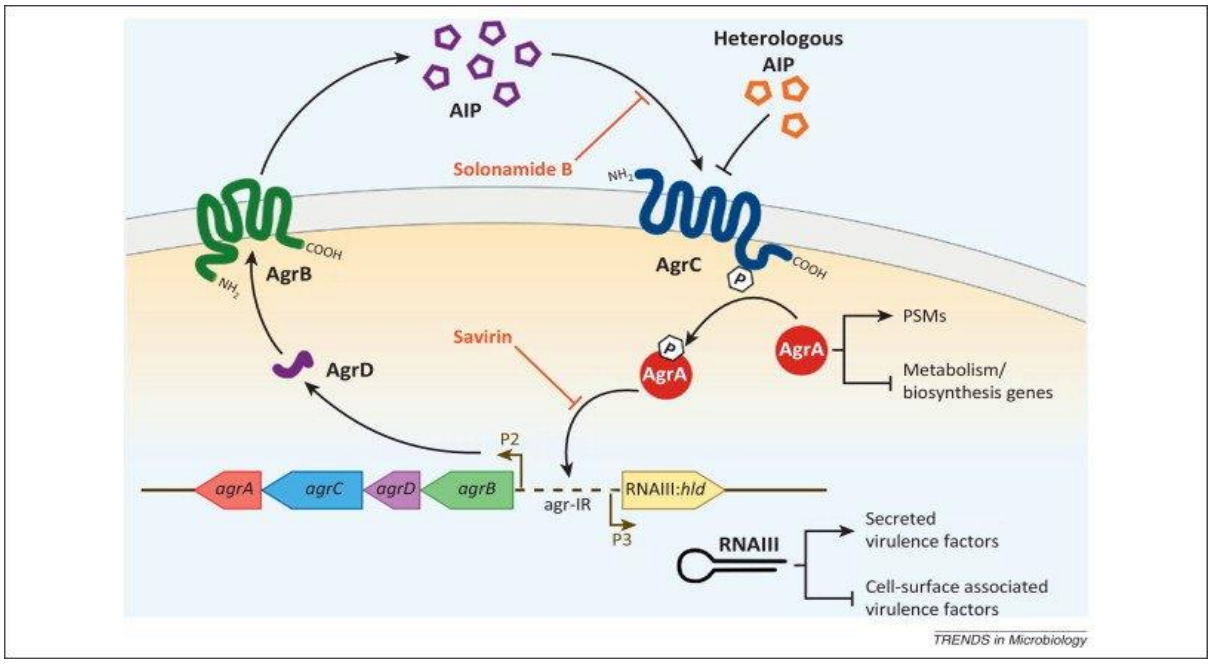

Figure 1.5: The *agr* quorum sensing gene regulatory system. Quorum sensing inhibitory targets are indicated for inhibitory compounds savirin and solonamide B (Painter *et al*., 2014).

A number of natural products studies have led to the discovery of QS compounds (Table 1.1) that target the Gram-positive cyclic peptide-mediated QS (Nakayama *et al*., 2007; 2009; Mansson *et al*., 2011; Desouky *et al*., 2013; Desouky *et al*., 2015). AIPs of *Staphylococcus epidermidis* and *S. lugdunensis* demonstrate cross inhibition (bacterial interference) towards *S. aureus* AIP, as well as interfering with *S. aureus* QS. *Pseudomonas aeruginosa* produces a long chain AHL, which inhibits QS-medited virulence expression in *S. aureus* with an IC_{50} of 6 μM through bacterial intereference (Singh *et al*., 2016). *Pseudomonas aeruginosa* secretes 3-acyltetramic and 3-acyltetronic acids compounds, which act as negative allosteric inhibitors of AgrC (Rampioni *et al*., 2014). Another compound, colostrum hexasaccharide, was reported to inhibit QS-mediated factors associated with *S. aureus* established infections (Srivastava *et al*. 2015).

Daly *et al*. (2015) reported the production of ω-hydroxyemodin by *Penicillium restrictum* that inhibits the QS signaling of all four groups of *S. aureus*. Marine *Photobacterium* produces six AI antagonists, i.e., solonamide A and B, as well as ngercheumicins F, G, H and I, that were able to inhibit QS in a methicillin-resistant *S. aureus* strain by binding to the receptor site but failing to switch on the QS cascade (Reuter *et al*., 2016; Singh *et al*., 2016). Avellanin C, synthesized by the fungus *Hamigera ingelheimensis*, displayed inhibitory effects on QS signaling in *S. aureus* (Igarashi *et al*., 2015). Hamamelitannin (2, 5-di-O-galloyl-d-hamamelose) is a nonpeptide analog of RIP, originally obtained from the bark of Hamamelis virginiana (witch gazel), which does not affect growth of *S. aureus* (Kiran *et al*., 2008). It inhibited production of RNAIII and prevented biofilm formation and cell attachment under *in vitro* conditions (Kiran *et al*. 2008). Hamamelitannin attenuated virulence gene expression in methicillin-resistant *S. aureus* and *S. epidermidis* strains from a device-associated *in vivo* condition in a rat graft model (Table 1.1).

In 2007, Nakayama *et al*. identified siamycin, a tricyclic peptide, produced by *Streptomyces* Y33-1, which inhibited GBAP biosynthesis by blocking the autophosphorylation action of FsrC, and subsequently inhibiting the expression of gelatinase in *E. faecalis* at 10 nM without affecting bacterial growth (Table 1.1). It also affected signal transduction of the two-component regulatory system FsrC-FsrA in a noncompetitive manner. Nakayama *et al*. (2009) also identified ambuic acid as an anti *fsr* molecule, from fungal metabolites. Ambuic acid blocked the biosynthesis of GBAP through the inhibition of FsrB function in *E. faecalis* and inhibited the biosynthesis of AIP in *S. aureus*. Desouky *et al*. (2015) discovered three cyclodepsipeptides (tachykinin - WS9326A, WS9326B, and endothelin - cochinmicin II/III) in

culture extracts of actinomycetes, which were antagonists of FsrC of *E. faecalis*, and WS9326A and WS9326 repressed the production of hemolysis in *S. aureus*.

Table 1.1: Potential quorum quenching auto-inducer peptide analogues or compounds from natural resourcesactive against against Gram-positive bacteria (adapted from Singh *et al*., 2016).

Known inhibitor Natural inhibitors	Strain tested and IC_{50} for QQ	Activity	Reference
Siamycin and Ambuic acid	*E. faecalis* OU510 and OG1RF	Inhibition of gelatinase Production	Nakayama *et al*. (2007); (2009)
Hamamelitannin	Methicillin-resistant *S. aureus* (MRSA)	Preventing device-associated infections *in vivo*; RIP analogous	Kiran *et al*. (2008)
cyclo(L-Tyr-LPro) and cyclo(L-Phe-L-Pro)	*S. aureus* strain RN4220	Inhibition of production of TSST-1	Li *et al*. (2011)
Solonamide A and B Ngercheumicins (F, G, H, and I)	*S. aureus*	Competitive inhibitors of *agrC*	Mansson *et al*. (2011); Kjaerulff *et al*. (2013)
Phytochemicals (chrysin, α-cyperone)	*S. aureus*	Suppress the alpha-haemolysin production in *S. aureus* via *agr* QS inhibition in mouse model	Qiu *et al.* (2011); Wang *et al*. (2011); Luo *et al*. (2012)
3- tetradecanoyltetronic	*S. aureus* Group- 1- RN6390B	Reducing nasal cell colonization and arthritis in a murine infection model	Murray *et al*. (2014)
Avellanin C obtained from the fungus *Hamigera ingelheimensis*	*S. aureus agr* reporter strain (8325–4) with an IC_{50} value of 4.4 μM	Reduction of *agr*-signaling pathway	Igarashi *et al*. (2015a)
Arthoamide from *Arthrobacter* sp.	*S. aureus agr* reporter strain (8325–4) with an IC_{50} value of 0.3 μM	Reduction of *agr*-signaling pathway	Igarashi *et al*. (2015b)
ω-hydroxy-emodin from *Penicillium restrictum*	Group- I- MRSA strain USA300 LAC	Reducing dermonecrosis and inflammatory cytokine transcription in mouse model of skin and soft tissue infectioncytokine	Daly *et al*. (2015)
Cyclodepsipeptide (WS9326A)	*E. faecalis* OU510 and OG1RF (2.7 μM); *S. aureus* strains 8325–4 (type-I AIP), K12 (type-II AIP), well as *C. perfringens* 13 (type A) (0.88)and K9 (type-IV AIP) (19 μM) a=	Inhibition of gelatinase production in *E. faecalis*; Reducing inhibition of hemolysis in *S. aureus*; inhibiting transcription of *pfoA* in *C. perfringens*; inhibiting	Desouky *et al*. (2015)
Cyclodepsipeptide (WS9326B)	*S. aureus* strains-Newman (typeI) and K3 (type-II)	Attenuating the corneal cytotoxicity of *S. aureus*	Desouky *et al*. (2015)

1.6.1.2 Anti-quorum sensing activity of seaweed-associated bacteria

The chemical ecology of the seaweed holobiont is maintained through a diverse chemical communication network of all the cellular components, whereby the host modulates and controls its associated microbiota through bioactive metabolites, while the epibiotic bacteria coordinate and express their various traits through QS (Friedrich, 2012). Seaweed-associated bacteria produce toxins, signaling compounds, and secondary metabolites, which represent an interesting reservoir for the discovery of bioactive compounds (Friedrich, 2012). Quorum sensing inhibitors and antimicrobial compounds produced by numerous epiphytic bacteria work in concert with seaweed-derived metabolites to protect the seaweed surface from pathogens, herbivores, and fouling organisms (Goecke *et al*., 2010). The ecological role of QS regulation in seaweed defense is observed by the lower bacterial abundance on the seaweed surface relative to seaweed with reduced QS inhibition, which harbour a higher abundance of epibiotic bacteria and have different bacterial communities (Goecke *et al*., 2010; Egan *et al*., 2014).

Some seaweed-associated bacteria are capable of producing AHL-like molecules to disrupt QS in other bacteria as a means of competition. Kanagasabhapathy *et al*. (2009) suggested that certain epibiotic bacteria from the brown macroalgae *Colpomenia sinuosa* may play a role in defense mechanisms which influence seaweed colonization patterns and suppress the settlement of other competitive bacteria by producing QS inhibitors or QS inhibitor-like compounds. Of the 96 bacteria isolated from *C. sinuosa*, 12% possessed anti-QS ability against indicator organism *Serratia rubidaea* JCM 14263, with inhibition of red pigment (prodigiosin) production without affecting its growth (Kanagasabhapathy *et al*., 2009). Bacillaceae (Firmicutes), Pseudomonadaceae (Proteobacteria), Pseudoalteromonadaceae (Proteobacteria) and Vibrionaceae (Proteobacteria) were isolated and identified as potential sources of anti-QS compounds (Kanagasabhapathy *et al*., 2009).

Quorum sensing inhibitory activity was also suggested to be widespread among bacteria of the marine genera *Bacillus* and *Halobacillus* that were isolated from diverse marine sources including algae, aquatic biofilms and sediments (Teasdale *et al*., 2011). Although only a few studies have been carried out to assay the AHL-degrading activity of marine bacteria, more than 30 species of quorum quenching (QQ) bacteria belonging to α-proteobacteria, γ-proteobacteria Actinobacteria, Flavobacteria and Firmicutes have been identified thus far (Tang and Zhang, 2014), with some QQ strains demonstrating degradative activity only against long-chain AHLs. Quorum quenching enzymes are classified into three major types according to their mechanisms (Tang and Zhang, 2014): AHL lactonase (lactone hydrolysis), AHL

acylase (amid hydrolysis) and AHL oxidase and reductase (oxidoreduction). AHL lactonases usually exhibit broad AHL-inactivating activities while many acylases are specific to long-chain AHLs, AHL acylases might be more common than lactonases in the ocean, which is consistent with the distribution of acylase and lactonase coding sequences in metagenome collections. Therefore, many marine QQ bacteria may be still undiscovered, and the prevalence of QQ enzymes in marine bacteria may be higher than expected (Tang and Zhang, 2014).

Bacillus, Pseudoalteromonas, *Pseudomonas* and *Vibrio* spp., isolated from marine sources have been reported as a source of QS antagonists (AHL-degrading lactonases and acylases and other small molecule antagonists), which might block the QS systems of their bacterial competitors to obtain a selective advantage over them and may provide the host seaweed with a tool to control biofouling. Most of the isolates with high anti-fouling activity obtained by Burgess *et al*. (2003) from varying algae were identified as *Bacillus* species, i.e. *B. pumilus, B. licheniformis* and *B. subtilis*, suggesting that QS-mediated inhibition resulted in decreased fouling. Almost 40% of the bacterial strains isolated from the brown macroalga *F. vesiculosus* demonstrated the ability to degrade short-chain AHLs, while 21% were able to degrade long chain AHLs (Romero *et al*., 2011). The QQ bacteria from *F. vesiculosus* included *Alteromonas, Hyphomonas, Oceanobacillus, Phaeobacter, Rhodococcus* and *Stappia* spp., which contain acylases and/or lactonases. Three *Bacillus* spp. were isolated from green seaweed, which were able to inhibit QS-mediated luminescence of *V. harveyi* BB120 (Teasdale *et al*., 2011). Jacobs (2015) screened 96 South African seaweed-associated bacteria for QS inhibitory activity against *Chromobacterium violaceum* and *P. aeruginosa*. Quorum quenching biosensor sandwich assays identified 30 potential bacterial isolates capable of quenching short and/or long chain AHLs. Cell-free supernatant extracts from these seaweed-associated bacteria exhibited potential broad-spectrum activity against *Chromobacterium violaceum* ATCC 12472 (Jacobs, 2015).

Padmavathi *et al*. (2014) screened 33 morphologically different *Gracilaria*-associated bacteria for anti-QS activity using the QS reporter strain *Chromobacterium violaceum* ATCC 12472. Phenol, 2,4-bis(1,1-dimethylethyl) of *Gracilaria*-associated *Vibrio alginolyticus* (Padmavathi *et al*., 2014) inhibited QS-regulated virulence factor production in the uropathogen *S. marcescens* and resulted in a significant ($p < 0.05$) reduction in protease (41.9%), haemolysin (69.9%), lipase (84.3%), prodigiosin (84.5%) and extracellular polysaccharide (84.62%) secretion without hampering growth in *S. marcescens.* Lafleur *et al*. (2015) have isolated a marine seaweed epibiont *Cellulophaga* sp. E6, which produces a QS inhibitory compound active against *P. aeruginosa*. Supernatant from *Cellulophaga* sp. E6

culture reduced expression of the 3-oxo-C12-HSL-dependent virulence-associated gene *lasB*, and reduced biofilm formation in a dose-dependent manner. Based on activity-guided purification of the QS inhibitory activity, the active molecule appears to be smaller than 1000 Da, water-soluble, and stable to temperatures of 50°C (Lafleur *et al*., 2015).

The natural product anti-QS research has focused on Gram-negative organism, and there are fewer reports on inhibition of QS as a mechanism to control Gram-positive organisms. This is because the enzymes responsible for auto-inducer synthesis, such as ribosomes and peptidases, are commonly essential for the growth and survival of the bacterial cells (LaSarre and Federle, 2013). Gram-positive bacteria, such as *Bacillus, Enterococcus* and *Staphylococcus*, communicate by production, detection and response to autoinducer peptides (AIP). Although there are currently no reports on marine seaweed-associated bacteria which quench Gram-positive QS, Mansson *et al*. (2011) investigated crude extracts and fractions from a marine *Photobacterium*, which led to the identification of two novel depsipeptides, solonamides A and B, solonamide B, which interfere with *agr* in *S. aureus* 8325-4 and USA300, a community-acquired MRSA strain, respectively. Cyclo (Pro-Leu) extracted from marine *Staphylococcus saprophyticus* exhibited moderate anti-QS activity without affecting growth. Cyclodepsipeptides ngercheumicin F, G, H, and I extracted from *Photobacterium* also demonstrated anti-QS by interfering with expression of virulence genes of *S. aureus*, ngercheumicins increased transcription of *spa* and reduced expression of *hla* and *rnaIII* indicating the potential of ngercheumicins as *agr* inhibitors (Kjaerulff *et al*., 2013).

The major aquatic pathogens are *Aeromonas* and *Vibrio* spp., as well as *P. aeruginosa* (Yuvaraj and Arul, 2014). Attenuation of QS in pathogenic *Aeromonas* spp. and *V. campbellii* resulted in significantly reduced mortality toward their respective hosts, i.e., turbot (Natrah *et al*., 2012), larvae of brine shrimp and giant freshwater prawn (Pande *et al*., 2013). Due to the close association between the QS system and virulence of aquatic pathogens, ecological strategies are the preferred option to overcome the problems of acquisition of antibiotic resistance and the spread of resistance genes when antibiotics or disinfectants are used to treat bacterial diseases (Homem and Santos, 2011; Singh *et al*., 2015). Quorum sensing inhibitory compounds effective against these aquatic pathogens are of either of biological or chemical origin. Bacteria are useful since they are able to interfere with QS-related phenomena either by degrading AHL molecules or by producing small molecule inhibitors that can interfere with and inhibit QS signals in other bacteria. A number of bacteria can utilize AHL molecules as carbon and nitrogen sources and have been isolated and used for controlling aquaculture infections. Many microorganisms belonging to the phyla *Actinobacteria, Bacteroidetes,*

Firmicutes, and *Proteobacteria* produce enzymes that degrade AHLs, which are the main QS autoinducers in Gram-negative bacteria (Defoirdt *et al*., 2011; Romero *et al*., 2011; Torres *et al*., 2013). Several studies have demonstrated the potential application of AHL-degrading enzymes for disrupting QS of fish pathogens such as *Aeromonas* and *Vibrio* spp. as a strategy to control bacterial disease in aquaculture (Chu *et al*., 2014; Romero *et al*., 2014; Torres *et al*., 2016). The addition of AHL-degrading bacteria to diseased aquaculture systems has improved the survival rate of the aquaculture organisms (Cao *et al*., 2012; Romero *et al*., 2014; Vinoj *et al*., 2014; Torres *et al*., 2016). Some seaweeds commonly encountered in aquaculture setups are also reported to inherently produce QS inhibitor molecules. Quorum quenching enzymes may be used in combination with prebiotics, probiotics, immune-stimulants and vaccines to control and protect fish against a wide spectrum of pathogens. By seeding bacterial consortia (capable of degrading signaling molecules) or algae (producing such inhibitors), self-regulated systems limiting the growth of aquatic pathogens may be developed. The use of QSIs in controlling aquatic pathogens may in the future provide an effective and sustainable alternative to the use of commonly used antibiotics or sanitizers (Hamza *et al*., 2015b). The trend of using probiotic microorganisms to control disease in aquaculture is encouraging, as they can disrupt the QS systems of pathogens. The positive effect of AHL-degrading *Bacillus* spp. and *Streptomyces* as probionts in aquaculture may result from inactivation of QS autoinducers, inhibition of virulence, inhibition of biofilm formation, in addition to the production of growth-inhibiting substances (Tan *et al*., 2016; Zhang and Li, 2016).

1.6.2 Biofilms

Biofilms can be described as communities of cells bound to a surface and to each other, embedded into extracellular polymeric substances (EPS) (Spano *et al*., 2015). The initial attachment of bacteria to surfaces is crucial for biofilm formation which is mediated by cell-surface charges, QS and hydrophobicity. The cells that attach to surfaces begin cell division, form microcolonies, and produce the extracellular polymers that define a biofilm (Fig. 1.6). EPS consists primarily of polysaccharides and can be detected microscopically and by chemical analysis. The final step in biofilm formation is cellular dispersion in which bacteria revert back to their planktonic lifestyle to colonize other surfaces (Spano *et al*., 2015).

Biofilms increase the chances of survival of the organisms and also enhance its growth by providing protection and required nutrients. Bacterial biofilm formation is the preferred growth for microorganisms, as this gives them a competitive survival advantage (Rodrigues *et al*., 2015). The virulence stage of pathogenic bacteria is usually characterized by biofilm

formation, and cells enclosed in the biofilm are more resistant to antibiotics. Biofilms are known to be problematic in most environments, having economic, environmental and health implications. The formation of biofilms on water pipes, food processing systems and food contributes to the spread of pathogenic bacteria that are resistant to treatment (Chari *et al.*, 2014). About 60% of all microbial infections are linked to the development of biofilms, thus making biofilm formation an important virulence mechanism. Pathogens such as *P. aeruginosa* are known to cause cystic fibrosis lung disease in humans, *E. coli* causes urinary and gastrointestinal infection, these are just some of the health problems caused by biofilm-forming bacterial pathogens (Majik and Parvatkar, 2014).

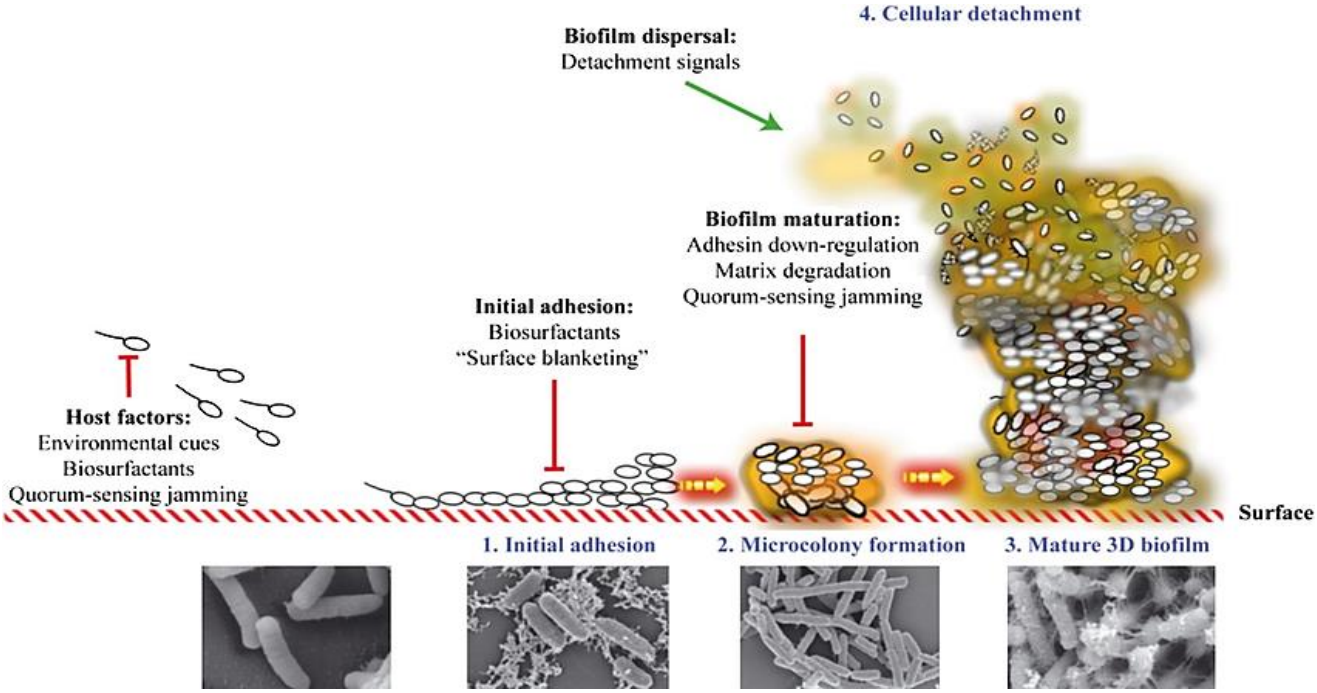

Figure 1.6: Biofilm formation is a process in which bacteria adhere to surfaces, through the production of pili, fimbriae, and exopolysaccharides. After initial attachment, biofilm development starts with the building of microcolonies. Biofilm maturation, is dependent on matrix production, which ensures cohesion and structure of mature biofilms. The final step in biofilm formation is cellular detachment or dispersion, by which bacteria regain the planktonic lifestyle to colonize other surfaces. Microbial interferences can inhibit biofilm formation or enhance biofilm dispersion through different mechanisms and strategies at different stages of their development (Rendueles and Ghigo, 2012).

Opportunistic bacteria *P. aeruginosa* and *S. aureus* are two of the main pathogens responsible for nosocomial and wound infections (Castillo-Juárez *et al.*, 2016). These important bacterial pathogens utilize QS cell communication to coordinate the expression of multiple virulence factors and associated behaviours such as swarming and biofilm formation (Gellatly and Robert, 2013). *Pseudomonas aeruginosa* possesses at least three functional QS

circuits, two of them are mediated by N-acyl homoserine lactones (HSL) signals and the other mediated by quinolones, which function in a hierarchical manner (Dorotkiewcz-Jach *et al*., 2015). The regulation of virulence factors by these three QS systems controls the expression of virulence factors, such as phenazines, exotoxin and improve biofilm formation *via* the Las QS system (Castillo-Juárez *et a*l., 2016). The LasI QS systems is expressed during the initial stage of biofilm formation, while the RhlR/RhlI system is activated during the maturation stage of *P. aeruginosa* biofilm development (Castillo-Juárez *et a*l., 2016). The *rhl* system has been reported to contribute to biofilm formation in *P. aeruginosa* by enhancing Pel polysaccharide biosynthesis. Furthermore, the control of swarming, twitching motilities, as well as rhamnolipids and lectins production in biofilm formation is also coordinated by the *rhl* system (Gellatly and Robert, 2013). LecA and LecB lectins play a role in adhesion and biofilm formation. Swarming motility is implicated in early stages of *P. aeruginosa* biofilm formation, while twitching motility is required for the assembly of a monolayer of *P. aeruginosa* cells into microcolonies (Gellatly and Robert, 2013). *Pseudomonas* quinolone signal (PQS)-mediated QS systems function as regulators for extracellular DNA (eDNA) generation (Al-Wrafy *et al*., 2016).

Implanted medical devices (catheters, prosthetic heart valves, cardiac pacemakers) are susceptible to colonization by staphylococci causing acute to life-threatening infections (McCarthy et al., 2015). Similarly, QS controls the expression of virulence factors such as hemolysins, leukocidins, cell surface adhesins, exoenzymes, and biofilm formation via the Agr system in *S. aureus*, which relies on the autoinducing peptide (AIP). Among the QS-controlled virulence factors in *S. aureus*, RNAIII regulates biofilm formation, antibiotic resistance and the establishment of chronic infections (Castillo-Juárez *et al*., 2015). Activation of the *agr* system increases the production of extracellular proteases *via* RNAIII. The link between QS and biofilm formation in *S. aureus* strongly suggests that QS is important for the development and establishment of its chronic infections (Quave and Horswill, 2014). Thus, antibiofilm compounds could be interesting antibiotic adjuvants to prevent or treat chronic infections (Rasamiravaka *et al*., 2015).

1.6.2.1 Mechanisms of biofilm inhibition

Research is focused on the discovery of anti-biofilm agents that are nontoxic and do not oppose selective pressure on the organisms, as it is believed that such molecules will not contribute to future drug resistance (Galloway *et al*., 2012). A potential drug is one, which will promote the dispersion of pre-formed biofilm or inhibit the initial formation of a biofilm. Anti-biofilm

molecules can have a variety of actions at several stages of the biofilm formation process (Christiaen *et al*., 2014). Strategies, which include utilization of QS inhibition molecules can be applied to disrupt cell-to-cell communication which is required to form a quorum (Fig 1.6). Several reports have indicated that mutations affecting signal synthesis in QS have an effect on biofilm formation (Galloway *et al*., 2012; Brackman and Coenye, 2015; Singh *et al*., 2015). The resulting loss of AIP and RNAIII production affects biofilm formation in *S. aureus* (Brackman and Coenye, 2015). As such, blocking signal production or degrading the signal is a promising strategy. Other strategies include altering surfaces (biosurfactant production, surface blanketing), which reduces the ability of bacteria to colonize by weakening bacteria-surface interactions (Rendueles and Ghigo, 2012). *Bacillus subtilis* synthesizes a surfactin, which is essential for swarming. Interestingly the same surfactin also inhibited biofilm formation of *Proteus mirabilis* and *Salmonella enterica* (Rendueles and Ghigo, 2012).

In hospital settings, patients present with advanced infections (mature biofilms) prior to seeking therapeutic interventions, thus compounds with the ability to disperse mature biofilms are required (Rendueles *et al*., 2011). The EPS play a crucial role in the structural integrity of the biofilm by maintaining bacterial cohesion (Musthafa *et al*., 2011). The EPS is, therefore, an ideal target for compromising the structural matrix of biofilms. Degradation of the matrix could possibly disperse a mature biofilm. Enzymes degrading matrix polysaccharides have been identified (Korea *et al*., 2011; Rendueles and Ghigo, 2012). *Streptococcus salivaris* produces matrix-degrading enzyme that targets sucrose leading to mature biofilm dispersion of *Streptococcus mutans* (Ogawa *et al*., 2011). Degradation of nucleic acid component of the matrix utilizing DNase and RNase was shown to affect integrity of biofilms by degrading nucleic acid scaffold components of the extracellular matrix. Some bacteria release DNase into the medium and can inhibit biofilm formation of other DNA-dependent biofilm-forming strains (Nijland *et al*., 2010). Due to the possible bactericidal effect of DNase/RNase, more natural nontoxic compounds need to be investigated.

1.6.2.2 Anti-biofilm/anti-fouling activity of seaweed-associated bacteria

The aquatic environment favors the development of biofilms on biotic and abiotic surfaces, with seaweed being especially susceptible to epibiosis. Seaweeds not only live in an environment with strong competition for space amongst benthic organisms but the seaweed surfaces also provide a nutrient-rich habitat. Seaweeds release large amounts of organic carbon into the surrounding environment, providing nutrients for microorganisms and triggering chemotactic behavior of bacteria. Most primary metabolites such as carbohydrates, amino

acids, peptides, and proteins are inducers of microbial colonization. The surface of a seaweed provides a protected micro-niche favorable for bacterial colonization and reproduction, thus marine seaweeds are continuously challenged by microorganisms as well as by grazers (Goecke *et al*., 2010). Once attached, epiphytic bacteria must compete with other microbial epiphytes for nutrients and space within the seaweed surface biofilm, producing anti-fouling compounds that work in concert with the seaweed-derived compounds to protect the seaweed surface (Chari *et al.*, 2014).

Bacteria producing antibiotic substances are more prevalent in epibiotic biofilms than in other habitats, such as seawater (Wahl *et al*., 2012). The biofilm communities on seaweed surfaces constitute a highly competitive environment for space and nutrients and thus select for bacteria with inhibitory activities against other surface colonizers (Egan *et al*., 2008). Increased antimicrobial compound production by bacteria when they are exposed to a different strain of bacteria suggests that competition for space between epibiotic bacteria may provide anti-fouling protection to the algal basibiont. Some bacteria that previously identified as non-antimicrobial producers are induced to produce bioactive metabolites on exposure to other bacterial species or extracellular products from other bacteria. Since surface-associated bacteria would be exposed to similar pressures in the seaweed surface biofilm, the numbers of bacteria isolated from seaweed surfaces that are producing active compounds is increased. Bacteria when on the surface of seaweed may thus produce greater amounts of compounds, therefore, protecting the seaweed surface from further fouling (Spano *et al*., 2015).

An ideal seaweed-bacterium anti-biofilm/anti-fouling compound will act in different ways on the targeted organisms. Ideally, the compound should prevent the formation of biofilms, which is considered as a cue for the further settlement of invertebrate larvae in the marine environment. The main mechanisms of these anti-biofilm activities of seaweed-associated microbial strains should include antibiotic activity, anti-adhesion potential and should affect the extracellular polymer production, which is essential for biofilm formation (Satheesh *et al*., 2016). Phenol, 2,4-bis(1,1-dimethylethyl) from *Vibrio alginolyticus* G16-T was effective in controlling the initial stages of *S. marcescens* attachment and subsequent biofilm formation as well as disrupting pre-formed mature biofilms. Phenol, 2,4-bis(1,1-dimethylethyl) control involves a dual approach: it controls QS-mediated biofilm formation and simultaneously increases the hydration of the cell wall, which results in reduced biofilm formation (Padmavathi *et al*., 2014). Cho (2012) isolated two active anti-fouling steroids (17-(1,2-dihydroxyl-5-methyl-hexane)-2,3-dihydroxyl-cholest-4-en-6-one and 13-acetate-17-(1,5-dimethylhexane)-cholest-7-en-3,5,6,15-tetraol) from the seaweed epiphyte, filamentous

bacterium *Leucothrix mucor*. Fouling bacteria, *Alteromonas* sp. KNS-8 and *P. aeruginosa* KNP-3 were sensitive to those compounds. Viju *et al*. (2014) found that extracellular polymeric substances secreted by *Pseudomonas taiwanensis* strain S8, a symbiont of seaweed (*Gracillaria*, *Sargassum,* and *Ulva* spp.) inhibited the formation of biofilms by *Pseudomonas* and *Alteromonas* spp. Epiphytic bacteria *Pseudomonas* sp. (UR4) and *Bacillus* sp. (UR7) isolated from *U. reticulata* demonstrated activity against marine biofilm bacteria (Mahadevan *et al*., 2012). Both *P. tunicata* and *Phaeobacter* sp. 2.10, epibiotic isolates from *U. lactuca,* displayed antibacterial activity against monospecies biofilms under laboratory conditions (Rao *et al*., 2005), and monospecies biofilms of *P. tunicata* and *Phaeobacter* sp. 2.10 inhibited the settlement and attachment of fouling organisms (Rao *et al*., 2007). The green alga, *U. lactuca* relies on the epibiotic bacterium *P. tunicata* to block biofilm formation by the synthesis of pigmented substances that inhibit AHL-dependent transcriptional control (Egan *et al*., 2002). *Pseudoalteromonas tunicata* was able to prevent biofouling by growth inhibition of other surface-associated microorganisms: producing at least five target-specific compounds including a large antibacterial protein, a small polar heat-stable anti-larval molecule, a putative anti-algal peptide, an antifungal alkaloid and violacein, which inhibits protozoan grazing (Matz *et al*., 2008). The seaweed-associated actinomycete *Streptomyces praecox* 291-11 isolated from *Undaria pinnatifida* was evaluated for its anti-fouling activity by Cho *et al*. (2012) who identified two diketopiperazines (6S,3S)-6-benzyl-3-methyl-2,5-diketopiperazine and (6S,3S)-6-isobutyl-3-methyl-2,5-diketopiperazine) as the active metabolites. The epibiotic bacterium *Streptomyces violaceoruber*, isolated from *Undaria pinnatifida,* produces two furanone derivatives: 3-octa-10, 30-dienyl-4-methylfuran-2(5H)-one and 3-octa-10-enyl-4-methylfuran-2(5H)-one, which demonstrate anti-fouling activities against the zoospores *of Ulva pertusa* and *Mytilus* edulis (Hong and Cho, 2013). It has been noted that the presence of the 2-furanone functional group is responsible for inhibition of *P. aeruginosa* biofilm formation (Qian *et al*., 2015). Glycoglycerolipids (2R-1,2-di-12-methylhexadecanoic acid-3-O-[β-D-galactopyranosyl-(1"-6')-O-β-D-galactopyranosyl]-glycerol, 2R-1-12-methylhexadecanoic acid-2-hydroxyl-3-O-[β-D-galactopyranosyl-(1"-6')-O-β-D-galactopyranosyl]-glycerol, 2R-1,2-di-12-methylhexadecanoic acid-3-O-β-D-galactopyranosyl-glycerol, and 2R-1,2-di-14-methylhexadecanoic acid-3-O-β-D-galactopyranosyl-glycerol) were isolated from seaweed-associated actinomycete *Streptomyces coelescens*, and were active against the fouling bacteria *Alteromonas* sp. KNS-8 and *P. aeruginosa* KNP-3 zoospores of *Ulva pertusa*, the diatom *Navicula annexa*, and the mussel *Mytilus edulis*.

Bacterial biofilms are a major problem in the aquaculture sector as they are resistant to antimicrobial treatments (Hamza *et al.*, 2015b). Bacteria belonging to the genera *Vibrio* and *Pseudomonas* genus have been noted in many reports to be important aquaculture pathogens (Yuvaraj and Arul, 2014). The extracellular polymeric substance of *Bacillus* sp. ICN-SS01, an epibiotic bacterium from the surface of the seaweed *Sargassum wightii* reduced the adhesion of biofilm-forming *V. harveyi* on hard coupon surfaces as well as exhibiting inhibitory activity against the settlement of biofouling organisms (Rajasree *et al*., 2012). As indigenous species of the marine environment, actinomycetes predominantly from the genera of *Streptomyces, Micromonospora,* and *Salinispora* could offer interesting options for probiotics in aquaculture (Natrah *et al*., 2011). The diversity of broad-spectrum chemical compounds synthesized by *Streptomyces* suggests that they can be valuable as probiotics in aquaculture due to the production of potential antagonistic and antimicrobial compounds. The ability to produce antagonistic compounds may help the probionts to compete for nutrients and attachment sites in the host gastrointestinal tract. *Streptomyces* spp. have also been implicated in the production of inhibitory compounds and metabolites involved in the attenuation of biofilm formation, anti-quorum sensing activity and anti-virulence activity in *Vibrio* sp. (Tan *et al*. 2016).

1.7 Rationale for study

The use of antimicrobial agents in aquaculture and clinical medicine has significantly reduced options for treating diseases due to emergence of multi-drug resistant organisms. Clinical treatment that focuses on bactericidal and bacteriostatic effects on pathogens has imposed selective pressure on organisms to mutate and become resistant (Tang and Zhang, 2014). The increase in emergence of multi-drug resistant organisms has had a tremendous negative effect on public health and food industries (De Schryver *et al*., 2014; Defoirdt, 2016). Most bacteria coordinate their virulence through QS and biofilm formation (Singh *et al*., 2016). Biofilms pose a serious problem for public health because of the increased resistance to antimicrobial agents and the potential for these organisms to cause persistent infections in patients with indwelling medical devices (Pop-Vicas and Opal, 2014). Given the link between QS and pathogenicity, QS is a promising target for anti-virulence therapy whereby inhibition of virulence occurs instead of a bactericidal effect. This delays the evolution of development of MDR as less selective pressure is imposed (Tang and Zhang, 2014). Quorum sensing inhibitors (QSI) are non-bacteriostatic organisms/molecules that can limit/down-regulate the virulence of pathogens through interference with QS, facilitating the use of host-defense mechanisms to control pathogens (Natrah *et al*., 2011). Research has now shifted to searching for alternative

sources for novel bioactive compounds as traditionally utilized antimicrobials have proved ineffective, and discovery of novel bioactive compounds from terrestrial environment has significantly declined (Chen *et al*., 2013; Cho *et al*., 2013; Viju *et al*., 2014).

Marine waters comprise a high diversity of microbial life including bacteria, fungi, viruses, spores, and actinomycetes (Singh *et al*., 2014). Bioactive compounds obtained from marine-associated microorganisms are known for their broad range of biological effects such as antimicrobial, anti-fouling and antitumor activity (Chakraborty *et al*., 2014; Sri and Dhevendara, 2014; Tan *et al*., 2016). Seaweed has become a prime resource in the search for organisms, which produce novel bioactive products. These organisms produce diverse metabolites that can be used for the development of new drugs to combat antimicrobial resistance (Rodrigues *et al*., 2015). Limited information is currently available on the anti-virulence potential of seaweed-associated bacteria especially those against Gram-positive bacterial pathogens. This study focused on the identifying of seaweed-associated bacteria isolates with the ability to synthesize bioactive compounds with diverse antimicrobial, anti-biofilm, and anti-QS-associated abilities that may be used as a weapon in targeting MDR strains. These isolates may become potential candidates for identifying compounds involved in anti-virulence activity with the ability to inhibit QS-mediated virulence factor production by pathogenic microorganisms such *P. aeruginosa, S. aureus* and *E. faecalis*.

1.8 Objectives

The following objectives have been established:

1.8.1. To establish whether bacteria isolated from seaweed demonstrate antimicrobial activity;

1.8.2. To characterize bioactive compounds synthesized by seaweed-associated bacteria;

1.8.3. To identify the anti-biofilm potential of seaweed-associated isolated bacteria; and

1.8.4 To investigate the ability of isolated bacteria to produce Gram-positive QS inhibitory compounds.

1.9 Aims

The following aims were pursued:

1.9.1. To perform primary screening of isolates for antimicrobial activity against indicator organisms using the cross-streak assay;

1.9.2. To obtain secondary metabolites through fermentation and ethyl acetate extraction;

1.9.3. To perform secondary screening of extracts for antimicrobial activity using the agar well diffusion assay;

1.9.4. To analyse crude extracts of seaweed-associated bacteria using thin-layer chromatography (TLC), gas chromatography mass spectrometry (GCMS), and nuclear magnetic resonance (NMR);

1.9.5. To identify isolates capable of inhibiting initial attachment and mature biofilm using microtitre plate assay; and

1.9.6. To screen for extracts with Gram-positive QSI properties using phospholipase assay, *S. aureus agr* and *E. faecalis fsr* inhibition assays.

1.10 Key questions to be answered

A number of specific questions were relevant to this topic

1.10.1 Do microbial strains isolated from seaweed have the ability to produce bioactive compounds that will inhibit microbial growth?

1.10.2. Do seaweed-associated bacteria produce bioactive compounds, which have an inhibitory effect on microbial adhesion and/ or mature biofilm formation?

1.10.3. Do microbial communities associated with seaweed possess anti-quorum sensing activity?

1.10.4. Do bioactive compounds produced by seaweed-associated bacteria possess anti-virulence compounds with potential to control multi-drug resistant pathogens?

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CHAPTER TWO

ANTIMICROBIAL AND ANTI-BIOFILM POTENTIAL OF SEAWEED-ASSOCIATED-BACTERIA ISOLATED FROM SOUTH AFRICAN SEAWEED AGAINST RESISTANT *Pseudomonas aeruginosa* AND *Staphylococcus aureus*

Abstract

Marine substrata are colonized by a variety of marine microorganisms, which are capable of producing novel compounds due to their diverse and often extreme environmental conditions as well as interactions with their associated eukaryotic host organisms. The antibacterial and anti-biofilm potential of bacteria associated with ten South African seaweeds was assessed. Cultivable seaweed-associated bacteria (n = 96) were screened for antibacterial activity against five resistant clinical bacterial strains utilizing the primary cross streak screening assay. Following shake flask fermentations and ethyl acetate extractions extracts, from 30 selected isolates were screened using the agar-well diffusion assay and minimum inhibitory concentrations were determined for 14 extracts. Selected bacteria were identified by 16S rRNA gene amplification and sequencing. Active crude extract of MAB24-SW1 was then analysed using nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry. Extracts capable of inhibiting initial adhesion and detachment of mature biofilm were identified using the crystal violet microtitre plate assay. Primary screening indicated that 38% (37/96) of isolates displayed varying antimicrobial activity against the five bacterial indicator strains. Isolate MAB24-SW1 was found to be active with MICs as low as 0.39 mg/ml. The isolate was identified as *Bacillus velezensis*, with a potential analogue of surfactin being identified through NMR following partial purification of the crude extract of *B. velezensis*. The initial attachment of *Pseudomonas aeruginosa* was inhibited by 90% (27/30) of extracts in comparison to 53% (16/30) against *Staphylococcus aureus* at 5 mg/ml, with limited antimicrobial effect. Detachment of mature biofilms showed significant variation between the indicators, with 70% (21/30) of extracts dispersing *P. aeruginosa* biofilms and 97% (29/30) effective against *S. aureus* at the highest concentration of 10 mg/ml. The present study indicates that bacteria associated with seaweed inhabiting South African coastal waters could be used as a potential source of biofilm inhibitory bioactive metabolites.

2.1 Introduction

The largely unexplored marine world is a complex ecosystem with an enormous diversity of different life forms often existing in close associations. Among these, microorganism-eukaryote associations have gained significant attention (Penesyan *et al.*, 2013). Marine organisms have become the focus of a worldwide effort for the discovery of novel natural products (Thirunavukkarasu *et al*., 2014), producing natural products that encompass a wide variety of chemical classes such as terpenes, polyketides, acetogenins, peptides and alkaloids with novel modes of action. A small number of marine plants, animals and microbes have already yielded more than 12,000 novel chemicals with hundreds of new compounds still been discovered every year (Penesyan *et al*., 2013; Singh *et al*., 2015). Due to increasing resistance to traditionally utilized antibiotics, marine-derived extracts are of interest as sources of antimicrobial molecules. In contrast to terrestrial bacteria, which are relatively well studied in the context of natural product discovery, marine microorganisms are only beginning to be recognized for their biotechnological potential (Penesyan *et al.,* 2013; Singh *et al*., 2015).

Macroalgae or seaweeds form an integral part of the marine environment as providers of food and shelter. Seaweeds harbor a diversity of microbial life including bacteria, fungi, viruses, spores, and actinomycetes (Singh *et al*., 2014). Due to their nutrient-rich surface, these relationships can be mutualistic or parasitic to the host (Susilowati *et al*., 2015). Furthermore, nutrient limitation leads to high competition on the host surface, forcing bacteria to evolve and produce antagonistic chemical metabolites, which are advantageous for the survival of bacteria (Egan *et al*., 2013). These bioactive compounds and enzymes produced by microorganisms in their interaction with the seaweed might be useful in pharmaceutical and industrial applications for treatment of multi-drug resistant (MDR) organisms (Singh *et al*., 2015). Screening of marine bacteria isolated from the surface of marine algae and invertebrates has shown that a high percentage produce novel bioactive metabolites with a broad range of antimicrobial activity (Djinni *et al*., 2013; Ramalingam and Amutha, 2013; Suvega and Kumar, 2014).

Although the utilization of antimicrobials to treat infections has been a method of choice for many decades, this has also been a contributing factor to MDR bacteria (Defoirdt, 2016). There is, therefore, a need for an alternative disease control strategy against bacterial infections. Shutting down cell-to-cell communication, known as quorum sensing inhibition (QSI), has been identified as one such strategy (Fetzner, 2015). It combats bacterial virulence, instead of targeting bacterial survival and will potentially reduce the risk of evolution of drug resistant microbes (Padmavathi *et al*., 2014).

In recent years, *S. aureus* and *P. aeruginosa* have emerged as problematic pathogens, because of multiple efflux pumps, mobile genetic elements and the presence of quorum sensing signals to regulate biofilm formation and virulence factor production (Gellatly and Robert, 2013; Dorotkiewcz-Jach *et al*., 2015). Biofilms are known to be problematic in most environments, causing economic, environmental and health problems. The formation of biofilms on water pipes, food processing systems and food itself contributes to the spread of pathogenic bacteria that are resistant to treatment (Chari *et al*., 2014). Viju *et al*. (2014) found that extracellular polymeric substances secreted by a *Pseudomonas taiwanensis* strain S8, a symbiont of seaweed (*Gracillaria*, *Sargassum*, and *Ulva* spp.) inhibited the formation of biofilms by *Pseudomonas* and *Alteromonas* spp. Two furanone derivatives: 3-octa-10, 30-dienyl-4-methylfuran-2(5H)-one and 3-octa-10-enyl-4-methylfuran-2(5H)-one from *Streptomyces violaceoruber*, which were isolated from marine seaweed *Undaria pinnatifida* showed anti-biofilm activities (Hong and Cho, 2013). Since seaweed-associated bacteria are effective in preventing colonization and fouling by competitor, often pathogenic bacteria (Singh *et al*., 2015), they can be explored as a source of biofilm and/or quorum sensing inhibitory compounds. The required number of new antimicrobial agents is higher than ever due to the rapid incidence of new infections and emergence of multi-drug resistance in common pathogens (Singh and Reddy, 2014; Singh *et al*., 2015). The use of marine isolates to inhibit the formation of biofilms in clinical settings is been regarded as a potential solution to the problem faced worldwide. The current study explores the potential of seaweed-associated bacteria as a reservoir for the discovery of bioactive compounds with antimicrobial and anti-biofilm activity.

2.2 Materials and methods

2.2.1 Bacterial isolates

Ninety-six bacterial isolates were cultured from ten South African intertidal seaweeds (*Amphiroa bowerbankii* Harvey, *Cheilosporum cultratum* Areschoug (Harvey), *Codium duthieae* (P. Silva), *Codium* spp., *Gelidium pteridifolium* (R.E, Norris, Hommersand & Fredericq), *Gracilaria* spp., *Jania verrucosa* (Lamourous), *Laurencia brongiartii* (J. Agarah), *Ulva rigida* (C. Agarah) collected along the coastline of Durban, South Africa (Fig. 2.1.). Seaweed samples were collected in ziplock bags, transported to the lab on ice, and processed within 3 h of collection.

Figure 2.1: Brown, green and red seaweed collected along the coast of Durban, South Africa for isolation of associated bacteria (**A**) *Amphiroa bowerbankii* Harvey (**B**) *Cheilosporum cultratum* Areschoug (Harvey), (**C**) *Codium duthieae* (P. Silva) (**D**) *Codium* spp., (**E**) *Gelidium pteridifolium* (R.E, Norris, Hommersand & Fredericq), **(F)** *Jania verrucosa* (Lamourous), (**G**) *Laurencia brongiartii* (J. Agarah) and (**H**) *Ulva rigida* (C. Agarah).

At the lab, seaweeds were rinsed with sterile distilled water and macerated in a liquidizer. Serial dilutions were set up (neat, 10^{-2} and 10^{-4}) and 100 µl aliquots were spread, in duplicate, on a selection of media: Enriched Anacker and Ordal's agar + seawater (EAOA-S), Luria-Bertani agar (LBA), Seawater yeast extract (SWYE; Crawford *et al*. 1993), Actinomycetes Isolation agar (AIA, HiMedia), and Glycerol-asparagine agar (GAA, HiMedia). Luria-Bertani agar and EAOA-S were used as nutrient-rich and nutrient-poor media, respectively. Three antibiotics were incorporated into SWYE, AIA and GAA media: nystatin (0.05 g/l) and cycloheximide (0.02 g/L) to inhibit fungal growth, while nalidixic acid (0.02 g/L) was added to inhibit fast-growing bacteria (Qin *et al*., 2009). Plates were incubated at 30 °C for 5-30 d and observed at regular intervals. Every culture that was at least 1 mm in diameter was inoculated on fresh agar plate until pure cultures were obtained, while pin point cultures were incubated further. For short-term storage, isolates were maintained at 4 °C on ISP2 media, while for long-term storage spore suspensions were stored in 50% glycerol at -80 °C. For preliminary characterization, isolates were examined and differentiated according to colony characteristics, Gram reaction and cellular morphology.

2.2.2 Primary screening of isolates for antimicrobial activity

Primary antibacterial screening was conducted using the cross-streak method (Kvennefors *et al*., 2012). A panel of resistant clinical indicators (*Enterococcus faecalis* ATCC 51299, *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27583 and *Staphylococcus aureus* ATCC 43300) were used. *Streptomyces griseus* ATCC 15468 was used as a positive antibiotic-producing control. Mueller-Hinton agar (MHA) plates were prepared and inoculated with seaweed isolates by a single perpendicular streak of inoculum in the centre of the petri-dish and incubated at 30 °C for 5-7 d. Indicator organisms were then streaked perpendicularly to the initial streak (Shnit-Orland and Kushmaro, 2013) and plates were incubated at 37 °C for 24 h. Each indicator organism was preliminarily grown individually on MHA to ensure that any lack of growth was not dependent on the medium used for screening. The experiment was performed in triplicate and the inhibition zones recorded (Kvennefors *et al*., 2012). The antagonistic effect was indicated by the failure of the indicator strain to grow in the confluence area. Inhibition was measured from the edge of the vertical streak with the 'test isolate' to the first colony of 'indicator isolate' cross-streak and divided into distance-dependent categories. Inhibition was graded as follows (- = no activity, + = weak activity [inhibition zone of 1-4 mm], ++ = moderate activity [inhibition zone of 5-8 mm], +++ = strong activity (inhibition zone of 9-15 mm), ++++ = highly active [inhibition zone of 16-25 mm) and total growth inhibition = TGI).

2.2.3 Fermentation and ethyl acetate extraction

Thirty bacterial strains exhibiting antagonistic activity in the cross-streak assay together with a further 30 quorum quenching (QQ) seaweed-associated bacteria (Jacobs, 2015) were pre-cultured in 5 ml of International *Streptomyces* Project media 2 (ISP2; Shirling and Gottlieb 1966) broth for 2 d, then inoculated in 250 ml of ISP2 broth and incubated with shaking for 7 d at 30 °C. The QQ activity had been assessed by Jacobs (2015) using the QQ biosensor sandwich assay and identified isolates capable of quenching short and/or long chain AHLs produced by *C. violaceum*. Bacterial cells were pelleted at 9500 rpm for 10 min to collect supernatants. An equal volume (1:1) of ethyl acetate was added to each cell-free supernatant followed by agitation for 1 h at 30 °C. The ethyl acetate layer was collected and then subjected to a second extraction (1:1 volume ethyl acetate) with agitation of flasks for 4 h after which the ethyl acetate layer was collected. Ethyl acetate was evaporated in a rotary evaporator (Ilmvac, ROdist digital 230V 50/60Hz) and each crude extract obtained was weighed (Nithya *et al*.,

2011). Thereafter, crude extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml.

2.2.4 Secondary screening of isolates for antimicrobial activity

2.2.4.1 Screening of potential antagonistic bacteria against clinical pathogens by disc diffusion method

Antibacterial activity of the 30 crude antagonistic bacterial extracts was assessed by loading 0.5, 1 and 2 mg/ml of respective extracts onto 6 mm blank discs (Oxoid, UK). Mueller-Hinton agar plates were prepared and uniformly swabbed with resistant clinical pathogens: *E. faecalis* ATCC 51299 (vancomycin-resistant), *E. coli* ATCC 35218 (TEM-β-lactamase), *K. pneumoniae* ATCC 700603 (extended spectrum β-lactamase), *P. aeruginosa* ATCC 27583 (multidrug resistant) and *S. aureus* ATCC 43300 (methicillin-resistant). DMSO alone was used as a negative control. Plates were incubated overnight at 37 °C (optimal temperature of indicator bacteria) and observed for the zones of inhibition. The diameter of the inhibition halos after 24 h of incubation was measured and was considered to be indicative of bioactivity (Nithya *et al*., 2011).

2.2.4.2 Minimum inhibitory concentration determination of extracts

The minimum inhibitory concentrations (MIC) of 14 extracts demonstrating antimicrobial activity following secondary screening was performed using a modified broth microdilution assay (Motyl *et al*., 2006). Indicator bacteria suspensions (multidrug-resistant *P. aeruginosa* ATCC 27853 and methicillin-resistant *S. aureus* (MRSA) ATCC 43300), equivalent to a 0.5 McFarland standard, were added to 96-well plates containing Mueller-Hinton broth (Sigma) supplemented with extracts (50 mg/ml stock solution) serially diluted two-fold to give final concentrations ranging from 12.5 – 0.01 mg/ml and incubated at 37 °C for 24 h. After incubation, 30 µl of 0.02% resazurin (oxidation-reduction indicator) dye was added to each well, and plates were again incubated at 37 °C for 4 h in dark and observed for a colour change. A pink colour indicated growth and blue was indicative of inhibition of growth. The MIC was recorded as the lowest concentration at which a colour change occurred (Sarker *et al*., 2007).

2.2.5 Characterization of MAB24-SW1 extract

2.2.5.1 Thin layer chromatography and bioautography

Chemical constituents of the crude MAB24-SW1 extractwere separated on aluminium-backed thin layer chromatography (TLC) plates (Merck, silica gel 60). The TLC plate was spotted with

sample with the help of a capillary tube. The plate was placed in the solvent system (methanol/acetone/ethyl acetate (3:1:1)), placed in a developing beaker and left to run until the solvent front was approximately half a centimeter from the top of the plate. Separated chemical compounds were detected using methanolic 10% H_2SO_4 solution (Dewanjee *et al*., 2015).

Contact bioautography was performed with *Chromobacterium violaceum* ATCC 12472, *E. coli* ATCC 35218, *P. aeruginosa* ATTC 27853 and *S. aureus* ATCC 43300 (Suleiman *et al*., 2010). The developed chromatogram was placed on a swabbed MHA plate for 30 min to facilitate diffusion of compounds. A heavy object (mortar) was placed on top of the chromatogram to ensure good contact between the agar and TLC plate. After diffusion, the TLC plate was removed and the agar plates were incubated at appropriate temperatures for 24 h. After incubation, all plates were sprayed with a 2 % (w/v) 2, 3,5 – triphenyltetrazolium salt stain which stains live cells red. Clear zones represented inhibition of growth due to antimicrobial activity of compound. The areas of inhibition, coloured yellow, were compared with the RF of the related spots on the reference TLC plate (Suleiman *et al*, 2010).

2.2.5.2 Gas chromatography-mass spectrometry

The crude extract of MAB24-SW1 was subjected to GC–MS analysis in order to identify secondary metabolites. A sample ionization energy of 70 eV was used for GC–MS detection. Helium was used as the carrier gas at a pressure of 60 kPa, with the oven temperature programmed at 100 °C (for 2 min) to 280 °C (for 30 min) at a ramping rate of 4 C per min. A 2 ll sample was manually injected. The injection temperature was at 280 °C with a split ratio of 1:50. The system software was driven by Agilent Chemstation software. The relative amount of each component as a percentage was calculated by comparing the area of the peak to the total area. The identification of the various compounds was carried out by comparing their fragmentation peaks with those of known compounds in the NIST/NBS 2005 mass spectral database of the GC–MS (Aliyu *et al*., 2015).

2.2.5.3 Preparative thin layer chromatography and nuclear magnetic resonance analysis

MAB 24-SW1 crude extract was carefully applied to a prep-TLC plate and developed using a mixture of solvent as eluant, MeOH: EtOH: Acetone (3.5:0.8:0.7). NMR data were recorded on a Bruker Avance III 400 MHz spectrometer. The sample was acquired with deuterated chloroform (CDCl3) or deuterated dimethyl sulfoxide (DMSO-d6). The spectra were referenced according to the deuteriochloroform signal at dH 7.24 (for 1 H NMR spectra) and

dC 77.0 (for 13C NMR spectra) for CDCl3 and dH 2.50 and dC 39.51 for DMSO-d6 (Aliyu *et al*., 2015).

2.2.6 Detection of anti-biofilm activity of seaweed-associated bacteria extracts

Prior to the anti-biofilm assay, the 30 selected QQ extracts were tested for antibacterial activity with sub-inhibitory and inhibitory concentrations (1, 5 and 10 mg/ml), utilizing the disc diffusion assay. Extracts were tested against *P. aeruginosa* ATTC 27853 and *S. aureus* ATCC 43300, in order to assess their effect on initial adhesion and detachment of mature biofilms.

Overnight cultures were used to prepare cell suspensions, which were standardized equivalent to a 0.5 McFarland standard (Basson *et al*., 2008). For initial adhesion studies, extracts were added to 90 µl TSB and 10 µl of standardized cell suspension (to a final volume of 200 µl) and incubated for 24 h at 37 ˚C with agitation. For pre-formed biofilm detachment assays, 24 h biofilms were established following addition of 90 µl TSB and 10 µl of 0.5 McFarland standardized cell suspension to microtitre plate wells, and incubation at 37 ºC for 24 h. Microtitre plates were washed three times with sterile deionised water and allowed to air-dry. Following air-drying, 90 µl TSB as well as extracts at the relevant, respective concentrations were added to wells (to a final volume of 200 µl) and microtitre plates were incubated for 24 h with agitation at 37 ˚C. The negative control contained only broth, while positive controls contained respective cell suspensions with no extracts added. After incubation, growth $OD_{600\ nm}$ values were determined using the Glomax multi + detection system (Promega) and wells with ≥50% reduction in growth were considered unsuitable for analysis. Thereafter, planktonic cells were removed by discarding the liquid media. Plates were processed for biofilm inhibition as described by Basson *et al*. (2008). Microtiter plates were washed three times with sterile dH_2O. Cells were fixed with 200 µl of methanol for 15 min, then air-dried. Wells were stained with 150 µl of 2% Hucker's crystal violet for 5 min. Wells were rinsed gently under running tap water then plates were allowed to dry. Glacial acetic acid (150 µl; 33% (v/v)) was used to re-solubilise cells (Basson *et al*., 2008). The OD was read at 600 nm using the Glomax multi + detection system (Promega).

Tests were conducted in triplicate on two separate occasions. A measure of efficacy called percentage reduction was calculated from the blank, control, and treated absorbance values (Pitts *et al*., 2003): Percentage reduction $=\left[\frac{(C-B)-(T-B)}{C-B}\right] \times 100$, where B denoted the average absorbance per well for blank wells (no biofilm, no treatment), C denoted the average absorbance per well for control wells (biofilm, no treatment), and T denoted the average absorbance per well for treated wells (biofilm and treatment). The difference in biofilm OD

values with and without the addition of varying concentrations of extracts was determined using One-way repeated measures ANOVA with $p \leq 0.05$ being considered significant (SigmaPlot 13.0, Systat Software Inc., San Jose, CA, USA). To identify the concentrations that differed from the others, the Holm-Sidak multiple pairwise comparison procedure was carried out, with $p \leq 0.05$ being considered significant.

2.2.7 Molecular identification of bacterial isolates

2.2.7.1 Genomic DNA isolation purification

Genomic DNA isolation was carried out using GeneJet Genomic DNA purification kit (Thermo Scientific) according to to manufacterer's instruction. Following overnight incubation on ISP2 plates, agar plates were flooded with 1 ml sterile distilled water and bacterial cells were scraped off using a glass Pasteur pipette into 1.5 ml microfuge tube. Thereafter the contents in the microfuge tube were centrifuged for 10 min at 5000 × *g*. The DNA was eluted in 100 µl of DNA elution buffer and stored at -20 °C until required.

2.2.7.2 16S rRNA gene amplification and sequencing

The 16S rRNA gene amplification for selected isolates was performed using F1 5'-AGAGTTTGATCCTGGCTCAG-3'; R5 5'-GGYTACCTTGTTAACGACTT-3' (Weisburg *et al*., 1991; Bintrim *et al*., 1997) and F1 5'-AGTTGATCCTGGCTCAG-3'; R5 5' TACCTTGTTACGACTTCACCCA-3' (Vaneechoutte *et al*., 2000) universal primers. PCR reaction mixtures included 2 µl of template DNA, 18 µl ddH_20, 2.5 µl buffer, 1.2 µl dNTPs, 0.2 µl of each primer set, 1.2 µl $MgCl_2$ and 0.1 µl DNA polymerase (Super Therm). Amplification was performed in a PCR machine (MJ MINI^{TM} personal Thermal cycler; Bio-Rad) using the following conditions: DNA denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 52 °C for 1 min and extension at 72 °C for 1 min. A total of 5 µl of PCR mixture and 2 µl gel loading buffer were loaded into agarose gels together with the molecular weight marker, GeneRuler 100 bp plus DNA ladder (Thermo Scienfic). Amplified PCR products were subjected to gel electrophoresis in 1% (w/v) agarose gel at 70 V for 90 min in 1% TAE buffer. PCR products were visualized by UV transillumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min. DNA was sequenced, sequences were processed using BioEdit (version 7.0) and subjected to identification using NCBI-Blast nucleotide database.

2.3 Results

2.3.1 Antimicrobial activity of crude seaweed-associated bacteria extracts

Ninety-six bacterial isolates were cultured from seaweed. Primary screening utilizing cross-streak assay identified 36% (35/99) of active isolates that inhibited one or more of the resistant indicator organism (Fig. 2.2). Of these, 8% (8/96) demonstrated broad-spectrum antimicrobial activity (to varying degrees) against at least one Gram-negative and one Gram-positive indicator (Table 2.1). Majority of active isolates (54.29%; 19/35) demonstrated antibacterial activity against the methicillin-resistant *S. aureus* ATCC 43300, while the least activity was observed against *P. aeruginosa* ATCC 27853.

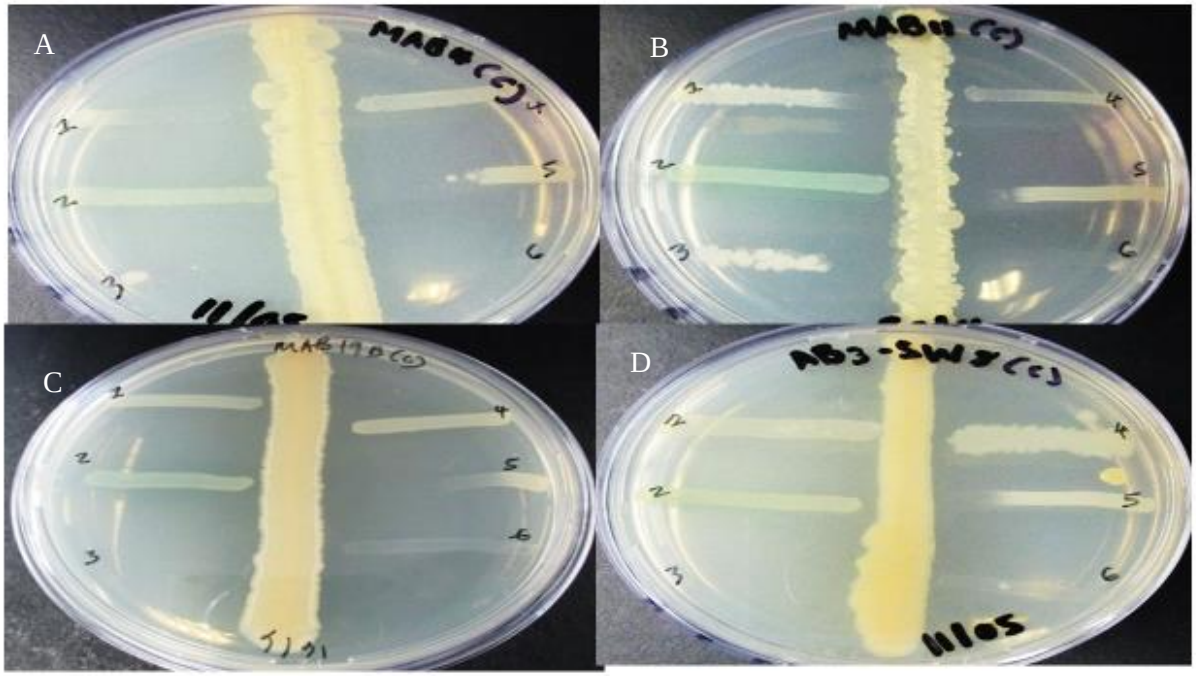

Figure 2.2: Primary screening of (**A**) MAB4-SW1, (**B**) MAB11-SW1, (**C**) MAB19B-SW1 and (**D**) AB3-SW8 against (**1**) *E. coli* ATCC 35218, (**2**) *P. aeruginosa* ATCC 27853, (**3**) *C. violaceum* ATCC 12472, (**4**) *K. pneumoniae* ATCC 700603, (**5**) *E. faecalis* ATCC 51299 and (**6**) *S. aureus* ATCC 43300 using cross streak method.

Table 2.1: Primary cross-streak screening of seaweed-associated bacteria against clinical indicator pathogens.

Isolated bacteria*	*E. coli* ATCC 35218	*K. pneumoniae* ATCC 700603	*P. aeruginosa* ATCC 27583	*E. faecalis* ATCC 51299	*S. aureus* ATCC 43300
MAB4-SW1	+++#	-	-	-	-
MAB7-SW1	+	-	-	++++	-
MAB10A-SW1	-	TGI	-	-	-
MAB10B-SW1	+++	-	-	-	+++
MAB11-SW1	+++	-	-	+	+
MAB12-SW1	-	-	-	-	++
MAB16-SW1	-	-	-	-	+
MAB17-SW1	-	-	-	-	+
MAB19A-SW1	TGI	-	-	-	-
MAB20-SW1	-	+	-	-	-
MAB22-SW1	-	-	-	-	+++
MAB24-SW1	-	TGI	-	-	TGI
MAB25A-SW1	TGI	-	-	TGI	++++
MAB27-SW1	TGI	-	-	TGI	++++
MAB36-SW1	++	-	-	-	-
MAB37-SW1	-	-	-	-	+++
AB1-SW2	-	-	-	++	-
AB5-SW2	-	-	++	-	+++
AB6-SW2	-	-	-	-	++
AB10-SW2	-	+	-	-	-
AB11-SW2	-	-	-	-	++
AB12-SW2	-	++	-	-	-
AB3-SW5	-	-	-	-	+++
AB6-SW5	-	-	-	-	+++
AB7-SW5	+	-	-	-	-
AB8-SW5	-	++	-	-	-
AB5-SW6	-	-	++	-	-
AB6-SW6	-	-	-	+	-
AB1-SW8	-	-	-	-	+++
AB2-SW8	++	-	-	-	-
AB7-SW8	-	-	-	+++	-
AB9-SW8	-	+	-	-	-
AB12-SW8	-	-	-	-	++
AB1-SW9	-	-	-	-	+++
AB1-SW10	-	+++	-	-	++
S. griseus ATCC 15468	++	++	+++	-	-

* SW1 - *Gracilaria* spp, SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

Grading: - = no activity; + = weak activity (zone of inhibition of 1-4 mm); ++ = moderate activity (zone of inhibition of 5-8 mm); +++ = strong activity (zone of inhibition 9-15 mm); ++++ = highly active (zone of inhibition 16-25 mm); TGI = total growth inhibition.

2.3.2 Secondary screening of crude seaweed-associated bacterial extracts

Based on the primary screening results, 60 inhibitory isolates were selected for further investigation: 30 with antimicrobial potential from cross-streak assay and 30 with potential quorum sensing inhibition activity (Jacobs, 2015). Crude extracts of the 30 potential antimicrobial-producing isolates were subjected to secondary screening using the disc diffusion assay. No antibacterial activity was observed for extracts following treatment with 0.5 and 1 mg/ml with the exception of MAB24-SW1, which demonstrated growth inhibitory activity at all concentrations tested. However, at 2 mg/ml the crude extracts exhibited antibacterial activity with the highest activity observed against *E. coli* and *K. pneumoniae* (Table 2.2).

Table 2.2: Antibacterial activity of 30 crude seaweed-associated bacterial extracts using the Kirby–Bauer disk diffusion method.

Extracts*	**Zone of growth inhibition at 2 mg/ml concentration (mm)**				
	***E. coli* ATCC 35218**	***P. aeruginosa* ATCC 27583**	***K. pneumoniae* ATCC 700603**	***E. faecalis* ATCC 51299**	***S. aureus* ATCC 43300**
MAB7-SW1	10	7	14	6	9
MAB10B-SW1	9	0	0	8	0
MAB11-SW1	10	0	15	12	7
MAB12-SW1	11	7	11	10	9
MAB16-SW1	8	6	13	11	8
MAB17-SW1	10	0	11	9	0
MAB20-SW1	11	7	14	11	8
MAB22-SW1	9	0	11	10	6
MAB24-SW1	17	15	15	30	25
MAB25A-SW1	10	0	11	12	6
MAB27-SW1	10	0	9	8	7
MAB37-SW1	13	0	11	9	7
AB1-SW2	9	7	13	7	10
AB5-SW2	11	8	12	8	9
AB6-SW2	10	0	11	0	9
AB10-SW2	9	0	12	0	8
AB11-SW2	10	8	12	8	6
AB12-SW2	11	7	11	10	10
AB3-SW5	9	0	10	13	9
AB6-SW5	12	0	10	9	11
AB7-SW5	8	0	12	11	10
AB8-SW5	7	6	10	10	8
AB6-SW6	6	0	11	9	0
AB1-SW8	8	0	9	9	0
AB2-SW8	11	0	11	8	9
AB7-SW8	10	7	10	7	8
AB9-SW8	11	8	11	9	7
AB12-SW8	8	7	12	6	9
AB1-SW9	11	6	8	9	6
AB1-SW10	10	0	9	8	0

* SW1 - *Gracilaria* spp., SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva)

2.3.3 Minimum inhibitory concentration of crude seaweed-associated bacterial extracts

The MICs of 14 active crude extracts were assessed against MRSA ATCC 43300 and MDR *P. aeruginosa* ATCC 27583. An MIC value of 0.78 mg/ml was exhibited by MAB24-SW1 against *P. aeruginosa* and 0.39 mg/ml against *S. aureus.* In contrast, the remaining thirteen extracts had MIC values of 6.25 mg/ml against MRSA. An MIC value of 12.5 mg/ml was observed for the AB2-SW8, AB1-SW9 and AB1-SW10 extracts against MDR *P. aeruginosa*, whereas the remaining extracts exhibited MIC values of 6.25 mg/ml (Table 2.3). These results were used to inform for the selection of sub-inhibitory and inhibitory concentrations to be used in the biofilm inhibition assays.

Table 2.3: Minimum inhibition concentration (mg/ml) determination of 14 crude seaweed-associated bacterial extracts against clinical indicator bacteria.

Extracts*	***P. aeruginosa* ATCC 27853 (mg/ml)**	***S. aureus* ATCC 43300 (mg/ml)**
MAB7-SW1	6.25	6.25
MAB11-SW1	6.25	6.25
MAB24-SW1	0.78	0.39
AB1-SW2	6.25	6.25
AB5-SW2	6.25	6.25
AB3-SW2	6.25	6.25
AB7-SW5	6.25	6.25
AB8-SW5	6.25	6.25
AB1-SW8	6.25	6.25
AB2-SW8	12.5	6.25
AB12-SW8	6.25	6.25
AB1-SW9	12.5	6.25
AB1-SW10	12.5	6.25
AB4-SW10	12.5	6.25

* SW1= *Gracilaria* spp. ; SW2 – *Codium* spp.; SW5 - *Amphiroa bowerbankii*; SW8 - *Gelidium pteridifolium*; SW9 - *Ulva rigida*; SW10 - *Codium duthieae.*

2.3.4 Characterization of MAB24-SW1

2.3.4.1 Thin layer chromatography and Bioautography

One absorbing band was observed under UV light (254 nm and 336 nm). A dark blue spot with RF value of 0.38 was observed on the TLC plate, which indicated the presence of phenol. The presence of this phytochemical constituents was re-confirmed on the TLC-developed plates by spraying methanolic 10% H_2SO_4 solution (Fig. 2.3).

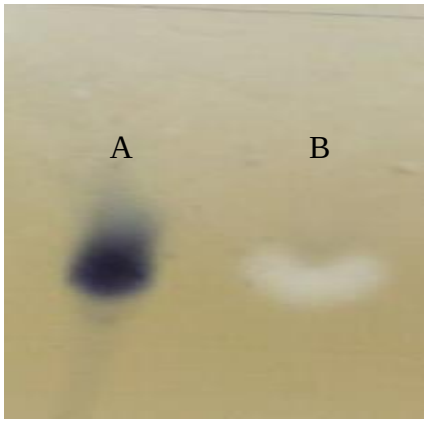

Figure 2.3: Chromatograms of crude extract of (**A**) MAB24-SW1 and (**B**) DMSO control developed in methanol/acetone/ethyl acetate (3:1:1) and sprayed with methanolic 10% H_2SO_4 solution.

TLC bioautography of the ethyl acetate and methanol extract of MAB24-SW1 indicated significant inhibition of *S. aureus*, *E. coli* and *C. violaceum*, respectively. No inhibition was recorded against *P. aeruginosa* for both extracts. (Fig. 2.4).

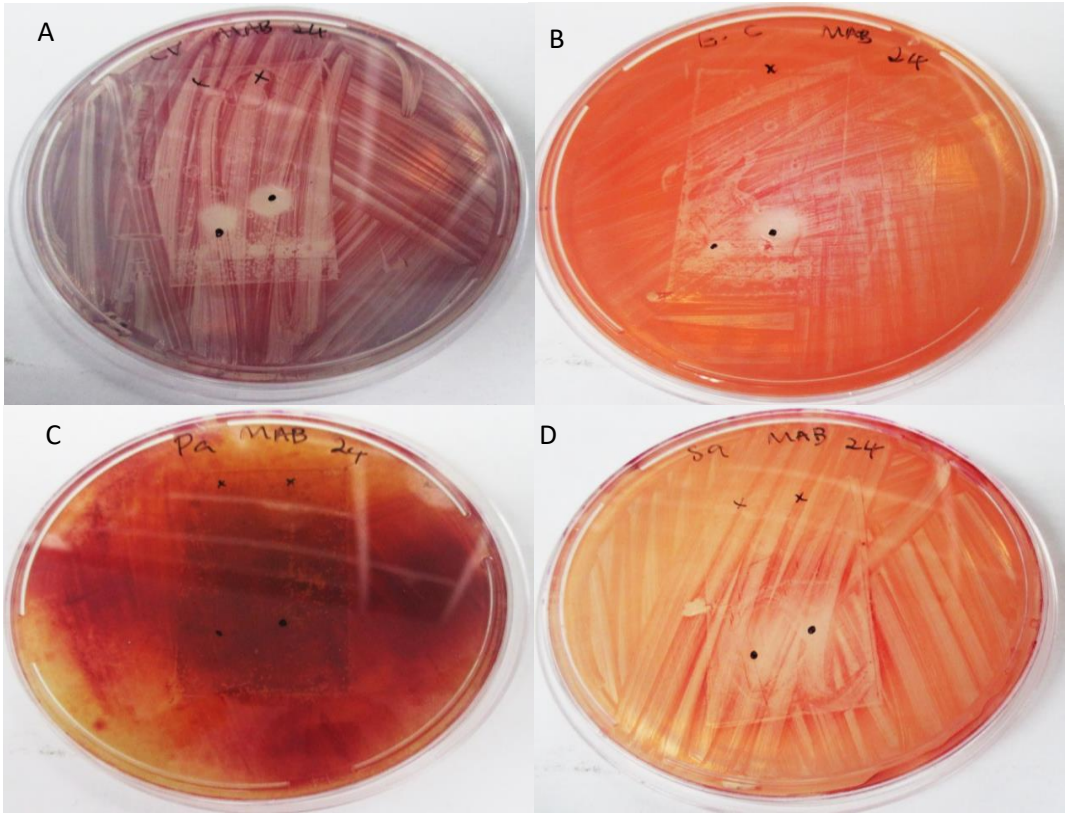

Figure 2.4: Inhibition of growth on bioautographic TLC plates by ethyl and methanol extract of MAB24-SW1 extract against (**A**) *C. violaceum* ATCC 12472, (**B**) *E. coli* ATCC 35218, **(C**) *P. aeruginosa* ATTC 27853, and (**D**) *S. aureus* ATCC 43300.

2.3.4.2 Gas-chromatography-mass spectrometry

The major components in the extract were butanoic acid, 2-amino-3-hydroxy-Threonine, methylsulfonylmethane and acetic acid, however, two compounds provided no hits from the database (Table 2.4). Figure 2.5 shows the major compounds identified from MAB24-SW1 crude extracts using GC-MS and the NIST/NBS 2005 mass spectral database. The compounds are representatives of the various amino acid and fatty acid residues and probable conversion products of cyclohexanone. The five-carbon chain carbonyl fragments may be a result of dihydroxylation of the open chain pentose sugar present in the compound.

Table 2.4: Chemical composition of MAB24-SW1 crude extracts.

Chemical constituents	RT (min)
No hit*	7.338
No hit*	7.364
DL-2-Amino-3-hydroxybutanoic acid	7.450
Threonine L-Threonine, L-butanoic acid,2-amino-3-hydroxy-Threonin	
Butanoic acid, 2-amino-3-hydroxy-Threonine	7.519
Disulfide sulfone Methane, sulfonylbis-MSM Methyl sulfone	
Disulfide, Dimethyl 2.3-Dithiabutane Methyl disulfide (Methyldithio)methane	
Dimethyl methylphosphonate, Phosphonic acid, methyl,-dimethyl ester	
No hit	7.588
Methylsulfonylmethane,Diethylacetic acid	7.763
Pentanonic acid, 2-methyl, methyl ester , Valeric acid	
Acetic acid	7.813
2-amino-2-ethyl-1,3-propanediol 2-amino-1.3-dihydroxy-2-ethylpropane	
3-Methylpyridazine	7.845
Phenol Carbolic acid, Hydroxybenzene	
3-Methylpyridazine, Pyridazine, 3-methyl	
Benzene,[(3-methyl-2-butenyl) oxy]-Ether,3-methyl-2-butenyl phenyl	
No hit	8.623
Phenylethyl alcohol	9.226
Hydrazine, (phenylmethyl)-Hydrazine, benzyl-Benzylhydrazine	
Toluene Benzene, methyl Methacide Methylbenzene	
No hit	10.831
Butylated hydroxytoluene, Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-p-Cresol	16.714
No hit	26.005

Rt= retention time in min

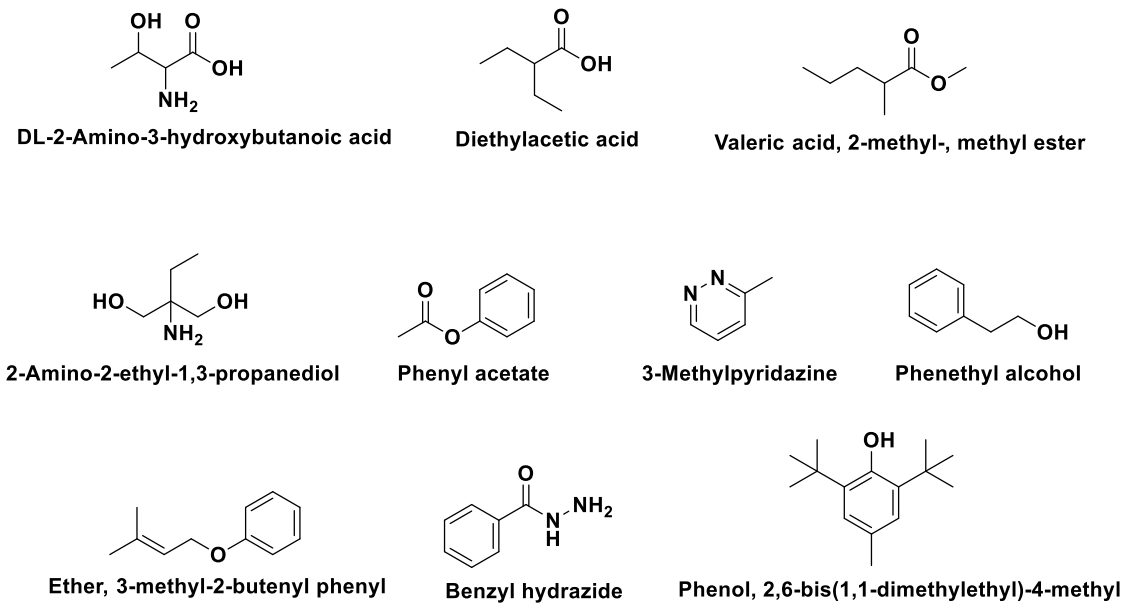

Figure 2.5: Chemical structures of major constituents of MAB24-SW1 crude extract identified by GC–MS.

2.3.4.3 Preparative thin layer chromatography and nuclear magnetic resonance

One zone was observed in the chromatogram. The zone was scrapped off and re-constituted in methanol, vacuum-filtered and concentrated. The extract was subjected to ^{1}H-NMR analysis following a TLC comparison of the zone. The characteristic chemical shifts observed in the ^{1}H-NMR spectrum of the partially purified MAB24-SW1 extract revealed the presence of the known antibiotic surfactin (Fig. 2.6) or perhaps its analogue (Tang *et al*., 2007).

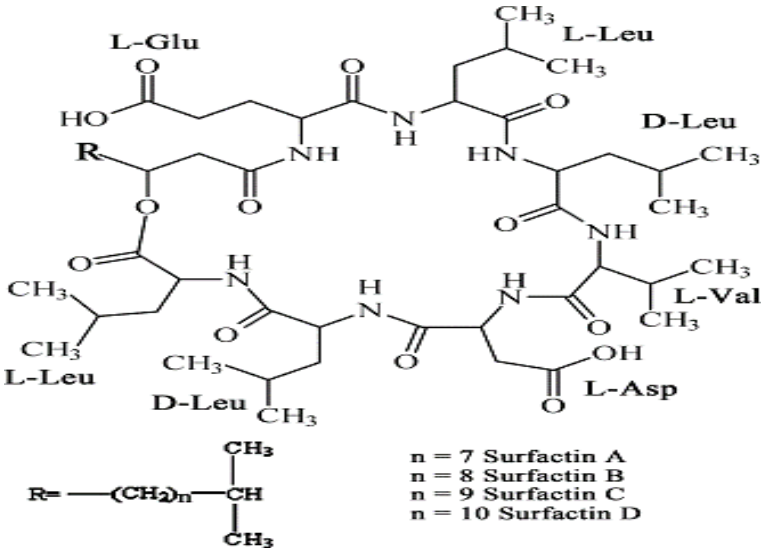

Figure 2.6: General chemical structure of surfactins (Chen *et al*., 2015).

Chemical shifts between 6.7 and 8.1 ppm were assigned to the N-H protons of the peptide backbone. The α-hydrogen resonated between 3.9 and 4.4 ppm. A singlet was observed at 3.58 ppm, which may be assigned to the methoxy group on either of the glutamic or aspartic amino residues. The repeating CH_2 units on the fatty chain were observed between 1.27 and 1.32 ppm while the terminal methyl groups on the surfactin macrocycle and the fatty acid tail have overlapping signals between 0.88 and 0.99 ppm (Fig. 2.7).

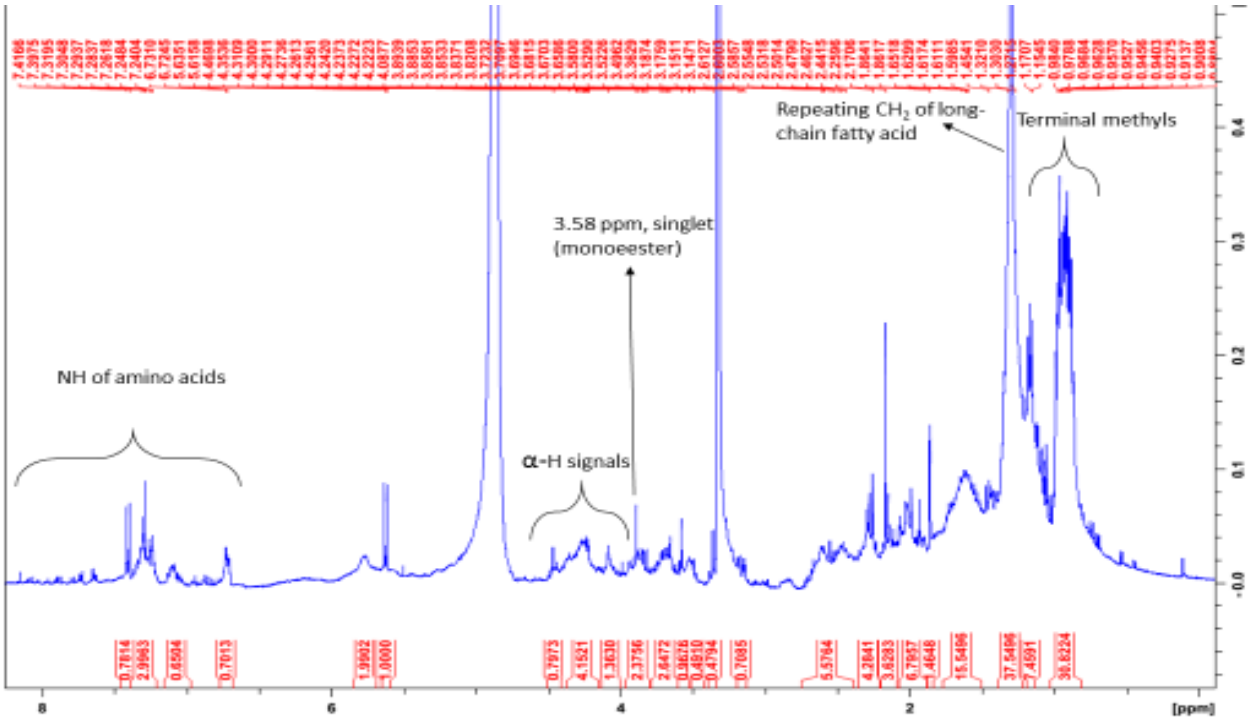

Figure 2.7: ^{1}H-NMR spectrogram of partially purified MAB24-SW1 extract.

2.3.5 Effect of seaweed-associated bacterial extracts on the adhesion and detachment of biofilm-forming bacteria

Potential quorum quenching seaweed-associated bacterial extracts (n=30) were screened for their ability to inhibit initial adherence and for detachment of mature biofilms of *P. aeruginosa* and *S. aureus* on polystyrene microtitre plates. To determine whether the inhibitory effect on biofilm development was related to general growth inhibition or a change in the bacterial growth rate, growth was measured prior to assessing biofilm inhibition. Those extracts noted to have antibacterial activity were excluded from the analysis.

Following treatment (1 and 5 mg/ml) with seaweed-associated bacterial extracts, statistically significant ($p < 0.001$) decreases in initial adhesion of *P. aeruginosa* were noted (Fig 2.8B). After treatment with 1 mg/ml, 90% (27/30) of extracts had an anti-adhesion effect against *P. aeruginosa* ($p < 0.001$), however, 10% (3/30) of the extracts resulted in increased

adhesion (Fig. 2.8B). Due to significant growth inhibition (≥ 50%) at 10 mg/ml in the initial adhesion assay, results for this concentration were not considered as biofilm inhibition and were excluded from analysis (Fig. 2.8A). A ≥ 50% reduction in crystal violet binding was obtained at 1 mg/ml for only two extracts: MAB10B-SW1 and AB4-SW9 against MDR *P. aeruginosa* at the time of inoculation (Table 2.5). While 80% (24/30) of extracts inhibited adhesion of *P. aeruginosa* at 5 mg/ml ($p < 0.001$), exposure to six extracts (MAB5-SW1, AB1-SW6, AB8-SW8, AB4-SW9, AB5-SW9 and AB3-SW10) resulted in ≥ 50% growth inhibition.

Table 2.5: Percentage biofilm reduction of *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 43300 following treatment with 1 – 5 mg/ml crude seaweed-associated bacterial extracts targeting initial attachment.

Extracts[*]	**Percent biofilm reduction at time of inoculation of *P. aeruginosa*[#]**		**Percent biofilm reduction at time of inoculation of *S. aureus*[#]**	
	1 mg/mL[€]	**5 mg/mL**[€]	**1 mg/mL**[€]	**5 mg/mL**[€]
MAB2-SW1	17.68	67.95	39.68	55.92
MAB4-SW1	35.12	71.06	42.28	52.59
MAB5-SW1	24.37	81.62[§]	50.25	62.59
MAB6-SW1	2.60	88.43	74.94	92.28
MAB10A-SW1	72.73	94.05	-30.56[¥]	-36.42
MAB10C-SW1	45.74	83.98	-36.37	-25.79
MAB21-SW1	43.67	88.05	11.63	66.73
MAB24-SW1	36.38	96.90	19.52	70.39[§]
MAB34A-SW1	-3.67	71.45	-55.95	83.79
MAB35-SW1	-0.53	69.23	-37.29	24.48
AB4-SW2	24.09	97.08	24.50	53.47
AB9B-SW2	36.97	77.14	24.59	61.68
AB27-SW2	2.86	77.18	50.41	75.11[§]
AB2-SW5	0.64	47.12	-53.61	-47.60
AB4-SW5	9.95	84.04	-28.64	-12.97
AB1-SW6	28.75	65.84[§]	40.22	33.78
AB3-SW6	22.51	91.97	-13.30	9.62
AB5-SW6	2.58	60.83	25.83	73.78
AB1-SW7	7.90	69.35	5.17	65.55
AB2-SW7	20.65	68.71	33.71	37.12
AB6-SW8	-0.65	68.65	38.59	53.78
AB8-SW8	37.49	71.65[§]	46.36	70.05
AB11-SW8	7.17	60.17	-42.70	8.80
AB14-SW8	25.89	80.35	-38.59	30.81
AB3-SW9	45.43	79.20	23.17	34.23
AB4-SW9	53.38	80.14[§]	27.87	53.38
AB5-SW9	3.16	74.09[§]	14.72	42.75
AB2-SW10	12.27	67.53	29.42	64.57
AB3-SW10	10.86	60.17[§]	8.31	53.41
AB4-SW10	5.96	82.49	-14.90	32.33

[*] SW1 - *Gracilaria* spp., SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

[#] Biofilm reduction calculated according to Pitts *et al*. (2003).

[¥] Negative values are indicative of an increase in attachment/biofilm formation.

[€] Differences in the BFR values among the treatment groups were statistically significant ($p < 0.001$).

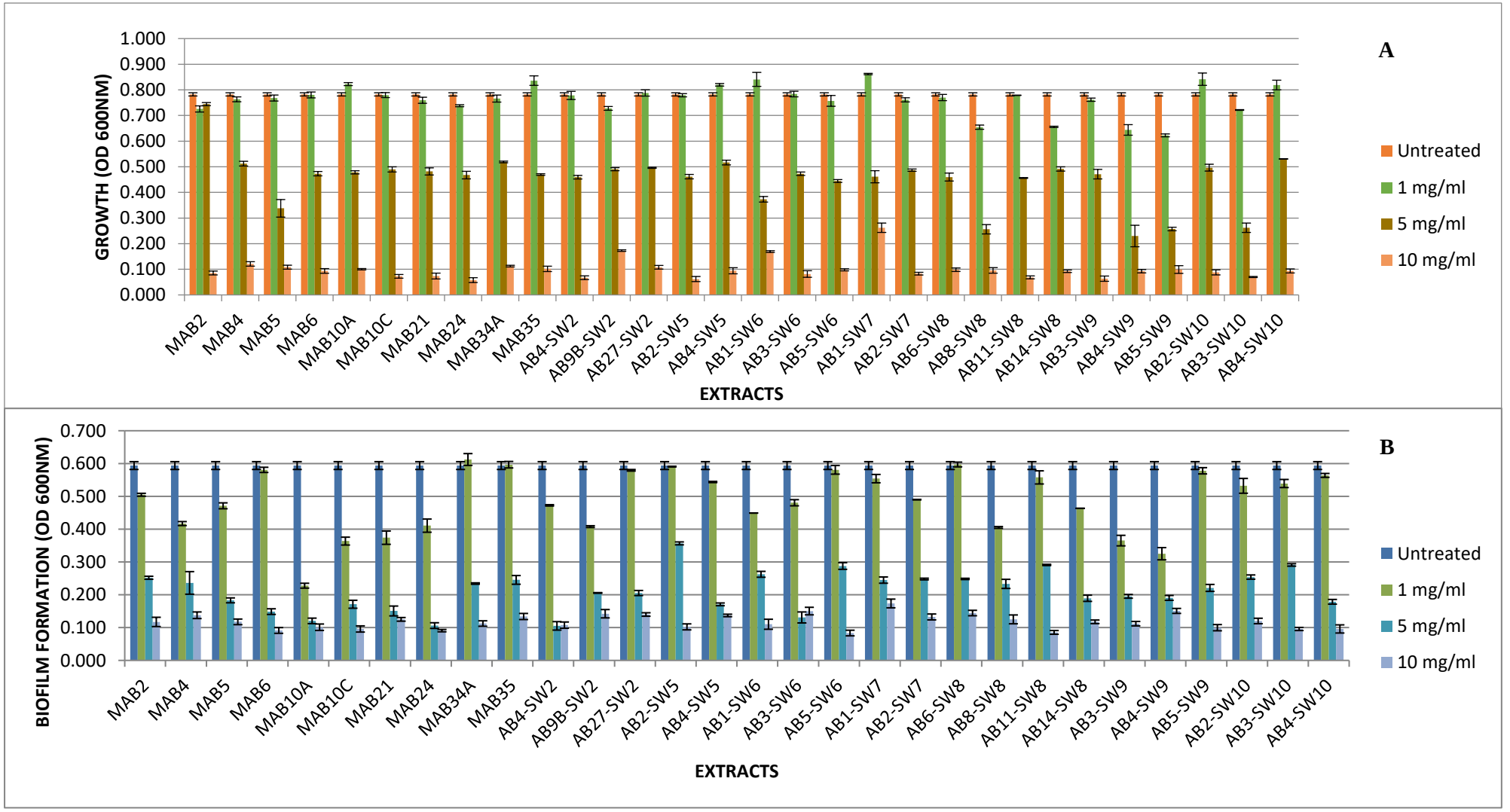

Figure 2.8: The potential effect of crude seaweed-associated bacterial extracts on initial bacterial growth of (**A**) and initial adhesion (**B**) of *Pseudomonas aeruginosa* ATCC 27583 as quantified by crystal violet staining in a microtiter plate assay. The mean values of two independent replicate experiments ± SD are shown. Differences in the mean values of 1 and 5 mg/ml treatment groups were statistically significant ($p < 0.001$).

Of note was that 97% (23/24) of extracts which had a ≥ 50% adhesion reduction potential at 5 mg/ml concentration (Fig. 2.8B; Table 2.5). The increase in activity could be potentially attributed to the higher concentration. Biofilm inhibition activity of ≥ 90% by extracts from MAB10B-SW1, MAB24-SW1, AB4-SW2 and AB3-SW6 was also observed against *P. aeruginosa*. Responses to the treatment targeting initial adhesion appeared to be extract-specific and dose-dependent.

The effects of three concentrations (1, 5 and 10 mg/ml) were investigated during detachment studies, with a higher concentration of extract required to effectively detach mature biofilms of *P. aeruginosa* (Fig. 2.9B). Treatment with extracts resulted in a greater detachment of biofilms as compared to initial adhesion inhibition.

Following treatment with 1 mg/ml, 30% (9/30) of extracts detached biofilms (Fig 2.9B) of *P. aeruginosa,* with the % reduction ranging from 1.20 - 47.27%, while 70% (21/30) of extracts demonstrated increased adhesion (Fig. 2.9B; Table 2.6). From the detachment assay (Fig. 2.9; Table 2.6), majority of extracts enhanced the biofilm formation of MDR *P. aeruginosa* at 1 mg/ml. Upon exposure to 5 mg/ml, 56% (17/30) of extracts were observed to detach *P. aeruginosa,* with a biofilm reduction index ranging from 0.61 - 52.68% ($p = 0.816$). However, only three extracts, i.e., MAB24-SW1, AB2-SW5 and AB4-SW5 were observed to have ≥ 50% detachment activity. None of the extracts had ≥ 90% reduction potential at this concentration (Table 2.6). The best results were noted for 10 mg/ml, with all extracts demonstrating detachment activity with a biofilm reduction index of 33.01 - 104.34% with no growth inhibitory effect ($p < 0.001$). Majority of extracts (70%; 21/30) were observed to have a ≥ 50% biofilm reduction potential. Four extracts (MAB24-SW1, MAB34A-SW1, AB4-SW2 and AB4-SW5) demonstrated ≥ 90% reduction potential at this concentration (Fig. 2.9B). The increase in the number of extracts, together with the increasing percent reduction index of extracts detaching MDR *P. aeruginosa* suggests a dose-dependent effect.

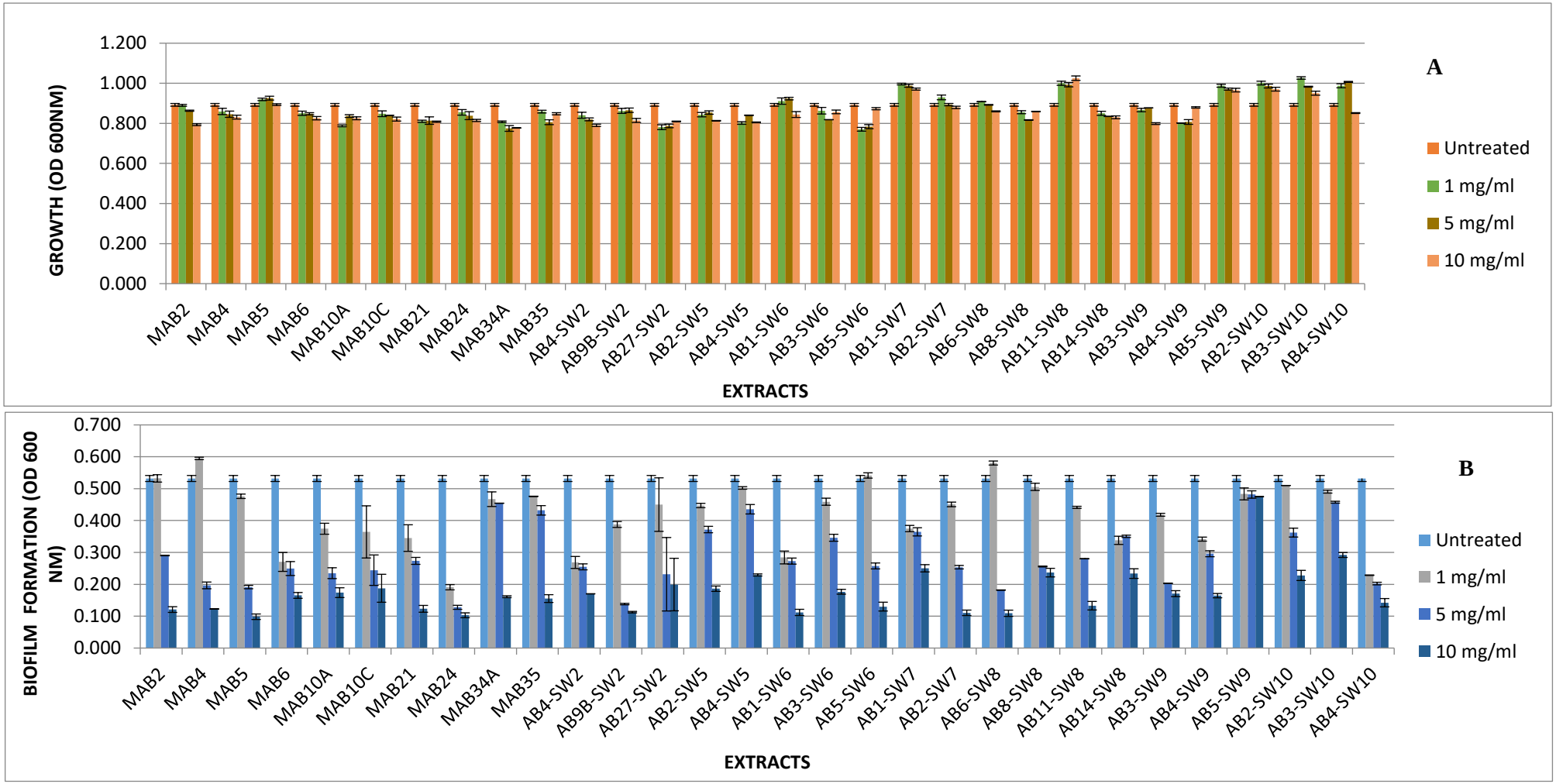

Figure 2.9: The potential effect of crude seaweed-associated bacterial extracts on bacterial growth of (**A**) and mature biofilm detachment (**B**) of *Pseudomonas aeruginosa* ATCC 27583 as quantified by crystal violet staining in a microtiter plate assay. The mean values of two independent replicate experiments ± SD are shown. Differences in the mean values of 1 and 10 mg/ml treatment groups were statistically significant ($p < 0.001$).

Table 2.6: Percentage biofilm reduction following treatment with crude seaweed-associated bacterial extracts against mature biofilm of clinical indicator bacteria.

Extracts*	Percent biofilm reduction against 24 h pre-formed biofilm of *P. aeruginosa*#			Percent biofilm reduction against 24 h pre-formed biofilm of *S. aureus*#		
	1 mg/ml€	5 mg/ml€	10 mg/ml€	1 mg/ml€	5 mg/ml€	10 mg/ml€
MAB2-SW1	-1.06¥	17.26	64.25	-0.07	55.89	94.97
MAB4-SW1	5.63	48.06	64.13	-14.55	77.47	94.56
MAB5-SW1	7.94	37.96	78.46	12.90	78.69	100.19
MAB6-SW1	-60.77	-63.02	61.87	60.47	65.26	84.73
MAB10A-SW1	-79.22	-81.98	27.59	36.41	68.63	82.73
MAB10C-SW1	47.27	46.75	52.76	38.73	66.45	79.54
MAB21-SW1	-14.50	-77.97	33.89	43.18	59.71	94.45
MAB24-SW1	-73.31	50.88	91.46	78.81	93.36	99.12
MAB34A-SW1	-14.79	25.83	104.34	15.02	17.92	85.68
MAB35-SW1	-17.83	0.61	47.82	12.97	23.11	87.02
AB4-SW2	31.72	30.93	101.54	60.84	64.05	83.57
AB9B-SW2	-68.70	-64.20	28.93	33.33	91.03	96.83
AB27-SW2	-37.00	-28.09	76.64	18.94	69.41	76.86
AB2-SW5	-39.72	52.68	97.34	19.60	36.99	79.88
AB4-SW5	39.54	51.13	102.27	6.86	22.29	69.93
AB1-SW6	-69.81	0.66	69.09	57.24	59.86	97.04
AB3-SW6	19.18	25.14	66.57	16.83	42.95	82.10
AB5-SW6	-88.57	-75.03	57.72	-2.21	63.37	92.84
AB1-SW7	-26.36	-11.37	40.42	36.28	38.67	65.08
AB2-SW7	-28.75	0.25	38.78	18.77	64.28	97.34
AB6-SW8	1.20	31.42	74.97	-11.22	80.88	97.67
AB8-SW8	-89.73	-44.93	52.78	6.05	63.78	68.21
AB11-SW8	-27.74	-7.06	45.83	20.90	58.18	92.19
AB14-SW8	2.00	44.60	68.89	44.82	41.93	68.90
AB3-SW9	-33.90	-10.63	32.92	26.30	76.02	83.42
AB4-SW9	-10.73	-30.56	65.43	43.95	54.55	84.89
AB5-SW9	-61.01	3.20	77.52	11.18	11.61	13.21
AB2-SW10	-20.46	-24.38	53.04	5.07	39.22	70.25
AB3-SW10	-61.67	-7.39	33.01	9.59	17.20	55.43
AB4-SW10	5.00	14.45	55.27	70.09	76.14	90.02

* SW1= unidentified red seaweed, SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

Biofilm reduction calculated according to Pitts *et al*. (2003).

¥ Negative values are indicative of an increase in attachment/biofilm formation.

€ Differences in the BFR values among the treatment groups were statistically significant ($p < 0.001$).

Upon exposure to low concentrations of seaweed-associated bacterial extracts, inhibition of initial adhesion was noted for 67% (20/30) of extracts against MRSA (Fig. 2.10) in the range of 5.17-74.94% (Table 2.5), however, 30% (10/30) stimulated biofilm formation ($p = 0.132$). Four extracts (MAB5-SW1, MAB6-SW1 and AB27-SW2) demonstrated ≥ 50% reduction potential. None of the extracts could effectively reduce adherence by ≥ 90% without causing any growth inhibitory effect (Table. 2.5). At 5 mg/ml, 80% (24/26) of extracts demonstrated biofilm reduction potential, without a growth inhibitory effect (Fig 2.10B). While

53% (16/30) of extracts reduced biofilm adherence by ≥ 50% at 5 mg/ml ($p < 0.001$), the best result was noted for extract MAB6-SW1, which was the only extract capable of reducing biofilm adherence by ≥ 90% (Table 2.5).

Similarly, when detachment assays were conducted, 10% (4/30) of extracts increased adhesion of MRSA at 1 mg/ml (Fig. 2.11B), while 90% (26/30) of extracts effectively dispersed the biofilm ($p < 0.001$) with biofilm reduction index of 6.05 - 78.81% (Table 2.6). Extract MAB24-SW1 was the most potent with ≥ 70% biofilm reduction potential and no significant effect on growth. At 5 mg/ml, all extracts dispersed mature biofilms of MRSA ($p < 0.001$). Detachment of ≥ 50% was observed for 60% (18/30) of the extracts, with ≥ 90% inhibition by extracts AB8-SW2 and AB6-SW8. Furthermore, extract AB6-SW8 also demonstrated inhibition of initial adhesion suggesting broad-spectrum anti-biofilm activity (Table 2.5). The most prominent effect of these extracts was their ability to detach mature biofilms at 10 mg/ml, without inhibiting growth ($p < 0.001$). At 10 mg/ml, 97% (29/30) of extracts demonstrated ≥ 50% reduction potential, with 40% (12/30) having a ≥ 90% reduction potential.

Overall the best activity was observed against *P. aeruginosa* in initial adherence assay with a biofilm reduction index of 0.64 - 97.08% (Table 2.5). However, extracts' activity against mature biofilm of *P. aeruginosa* was much weaker (Table 2.6). Pronounced activity of extracts against mature biofilm of MRSA was observed at 10 mg/ml, with ≥ 90% reduction potential noted for 40% (12/29) of extracts (Table 2.7).

Table 2.7: Comparison of biofilm reduction potential of seaweed-associated bacterial extracts against inhibition of initial adherence and pre-formed biofilms.

Indicator bacteria	Concentration	Percent biofilm reduction against initial adherence		Percent biofilm reduction against 24 h preformed biofilm	
		Biofilm reduction ≥ 50%	Biofilm reduction ≥ 90%	Biofilm reduction ≥ 50%	Biofilm reduction ≥ 90%
P. aeruginosa	1 mg/ml	2	0	0	0
	5 mg/ml	23	4	3	0
	10 mg/ml	-	-	21	5
S. aureus	1 mg/ml	3	0	5	0
	5 mg/ml	15	1	20	2
	10 mg/ml	-	-	29	12

*-Results omitted due to significant growth inhibition (≥ 50%) compared to untreated control.

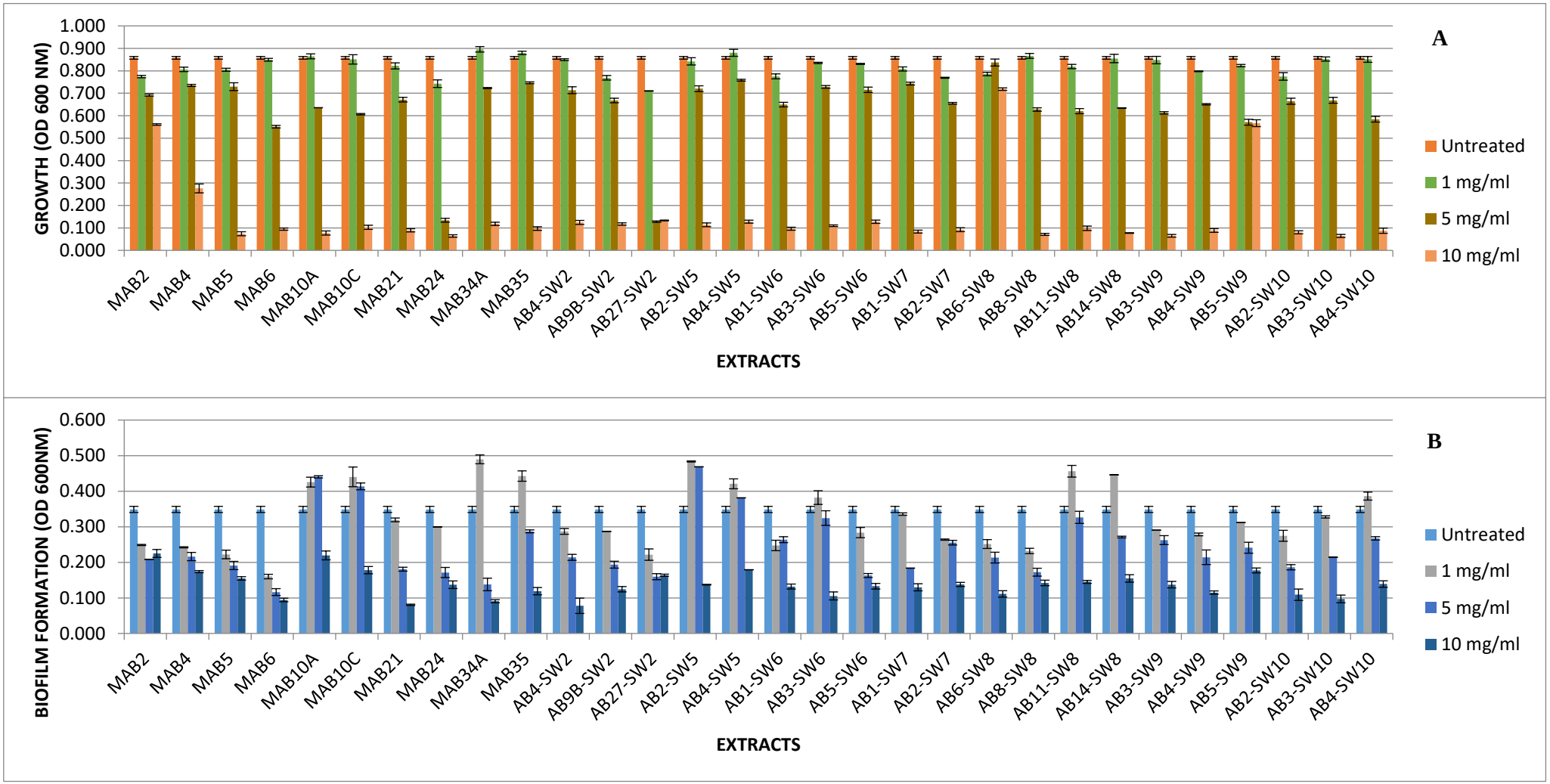

Figure 2.10: The potential effect of crude seaweed-associated bacterial extracts on growth (**A**) and initial adhesion (**B**) of *Staphylococcus. aureus* ATCC 43300 as quantified by crystal violet staining in a microtiter plate assay. The mean values of two independent replicate experiments ± SD are shown. Differences in the mean values of 1 and 5 mg/ml treatment groups were statistically significant ($p < 0.001$).

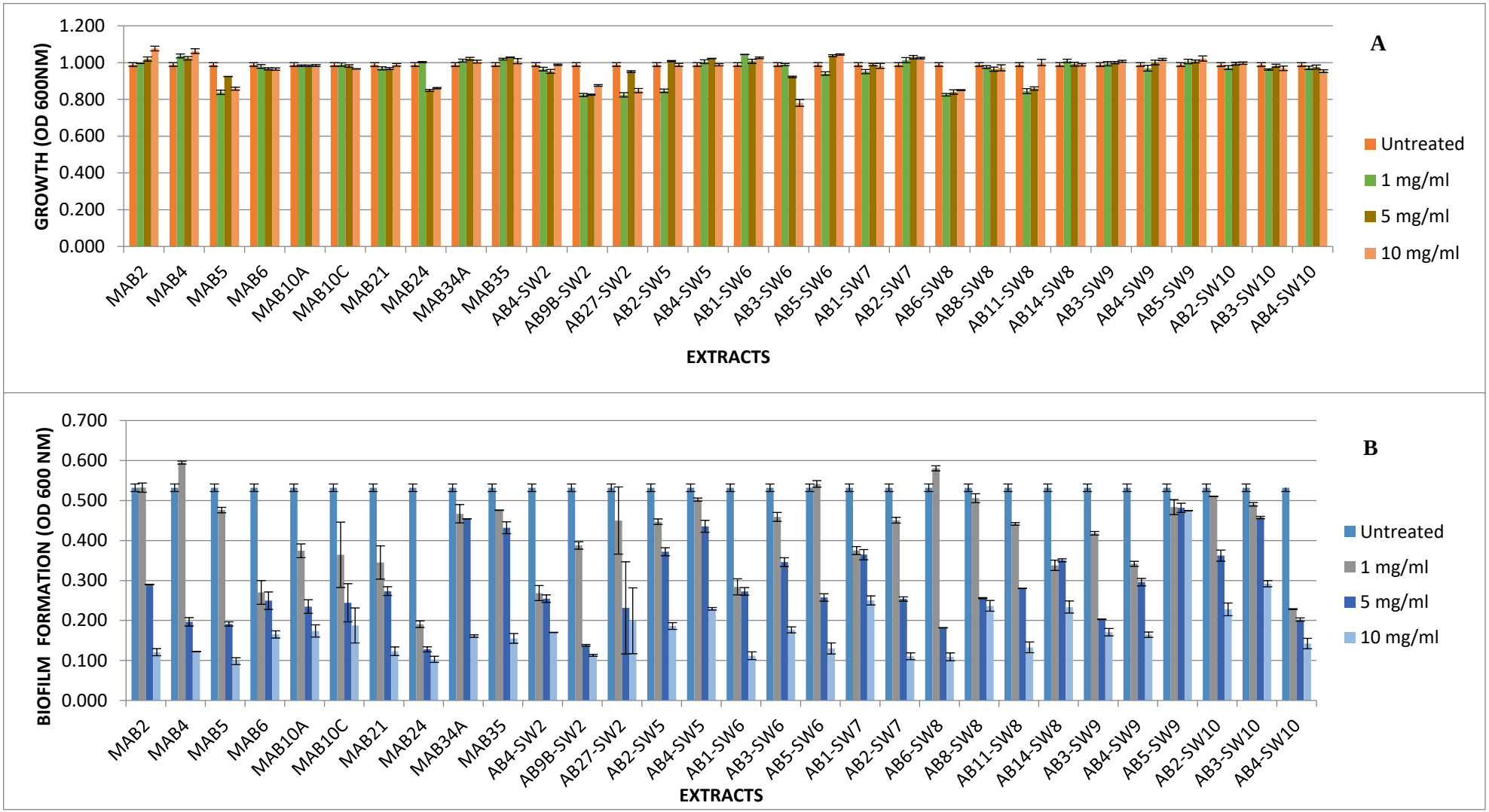

Figure 2.11: The potential effect of crude seaweed-associated bacterial extracts on mature biofilm growth (**A**) and mature biofilm detachment (**B**) of *Staphylococcus aureus* ATCC 43300 as quantified by crystal violet staining in microtiter plate assay. The mean values of two independent replicate experiments ±SD are shown. Differences in the mean values of 1 and 10 mg/ml treatment groups were statistically significant ($p < 0.001$).

2.3.6 Molecular characterization of seaweed-associated bacteria

Genomic DNA was isolated from selected seaweed-associated bacteria and amplified to obtain 16S rRNA gene fragments of ~1500 bp, which were compared with 16S rRNA gene sequences in the GenBank database following sequencing of amplimers. The predominant isolates were *Bacillus* and *Streptomyces* spp. (Table 2.8).

Table 2.8: Colony characteristics of selected antimicrobial and anti-qourum sensing seaweed-associated bacteria and their identities.

Isolation code	Colony characteristics	16S rRNA confirmation
MAB6-SW1	smooth glistening orange	*Rhodococcus fascians*
MAB7-SW1	smooth bright yellow	*Bacillus pumilus*
MAB10B-SW1	whitish cream glossy surface	*Bacillus* sp.
MAB11-SW1	glistening cream opaque	*Bacillus pumilus*
MAB12-SW1	glossy white with white borders	*Bacillus cereus*
MAB24-SW1	cream wrinkled butyrous surface	*Bacillus velezensis*
MAB25A-SW1	glistening smooth cream transparent	*Bacillus pumilus*
MAB37-SW1	glossy cream with white boarders	*Bacillus cereus*
AB1-SW2	cream pink-purple; powdery	*Streptomyces* sp.
AB5-SW2	light yellow smooth	*Stenotrophomonas maltophila*
AB8-SW2	Pinkish with rough boarders	*Streptomyces* sp.
AB7-SW5	milky white light yellow transparent; mucoid	*Stenotrophomonas maltophila*
AB8-SW5	grey circles	*Streptomyces diastaticus*
AB6-SW6	milky white butyrous very shiny	*Streptomyces* sp.
AB1-SW8	white matt	*Streptomyces* sp.
AB2-SW8	olive green with white spores	*Streptomyces* sp.
AB6-SW8	White powdery	*Streptomyces* sp.
AB7-SW8	Greyish yellow with wrinkles	*Streptomyces* sp.
AB12-SW8	Cream transparent and smooth	*Streptomyces* sp.
AB1-SW9	opaque white milky and smooth	*Microbacterium oxydans*
AB4-SW10	grey spores with white edges; powdery	*Streptomyces labedae*

2.4 Discussion

The lack of treatment options for MDR pathogens highlights the urgent need for the discovery and development of alternative therapeutic strategies. The marine environment remains relatively untapped, thus is in the limelight for novel natural product discovery (Suvega and Kumar, 2014; Suresh *et al*., 2014). Marine seaweed and their associated bacteria have become an important target for the biotechnology industry because of the large number of bioactive compounds discovered from them (Yung *et al*., 2011; Tebben *et al*., 2014). Seaweeds and seaweed-associated bacteria contain many different secondary metabolites, which have a wide spectrum of biological activities (anti-viral, anti-bacterial, anti-biofilm, etc.) (Prieto *et al*., 2012; Ravisankar *et al*., 2013b). It has been suggested that, in addition to their own defense

mechanisms, seaweed-associated bacteria have the ability to mimic secondary metabolites synthesized by their host (Egan *et al*., 2014). The highly competitive, nutrient-limited environments in which seaweed-associated bacteria live (Cho and Kim, 2012) induces them to produce secondary metabolites, such as allelochemicals, (Singh *et al*., 2015), antibiotics (Sugathan *et al*., 2012), siderophores, proteases, bacteriocins, lysozymes, biosurfactants and organic acids which threaten the survival of their competitors (Soria-Mercado *et al*., 2012). Seaweed-associated bacterial communities produce plant-growth promoting substances, specific vitamins bioactive compounds, quorum sensing (QS) signalling molecules and other substances which play an important role in the health (Sugathan *et al*., 2012) and development of their hosts (Singh and Reddy, 2014).

In the present study, *Gracilaria* spp. (SW1) yielded the most microorganisms with antimicrobial activity. Red algae are generally considered as the most important source of many biologically active metabolites in comparison to other algal classes (Ali and Gamal, 2009). Majority of seaweed-associated bacteria displayed good antibacterial activity (Table 2.1). Isolate MAB24-SW1 exhibited the highest level of antimicrobial activity against all pathogens tested. These results reflect those of Susilowati *et al*. (2015) who isolated 23 marine bacterial strains from three species of brown algae *Sargassum,* ultimately acquiring only one strain active against MRSA and *S. epidermidis*. Bacterial isolate MAB24-SW1 was distinguished as *Bacillus velezensis,* a heterotypic synonym of *B. amyloliquefaciens* (Silva *et al*., 2015). The genome of a plant endophytic *B. velezensis* was sequenced and reported to harbor an array of gene clusters that produce novel secondary metabolites with antimicrobial activity (Cai *et al*., 2016). Characteristics of this strain include plant growth promotion, biocontrol of phytopathogens and methanol utilization (Cai *et al*., 2016). Analysis of crude extract of MAB24-SW1 through NMR and GC-MS supported the elucidation of a possible surfactin analogue. The biosynthesis of surfactin a secondary metabolite from *B. subtilis* has been described previously by Chen *et al*. (2008) and is encoded by non-ribosomal peptide synthetase (NRPS) gene cluster. Several cyclic lipopetides biosurfactants are produced mainly by members of *Bacillus* spp. (Chen *et al*., 2008; Cai *et al*., 2016) and possess good antimicrobial activity and surface tension reduction (Cai *et al*., 2016; Das *et al*., 2008). Biosurfactant have been reported to exhibit antimicrobial activity against clinical pathogens, anti-biofilm activity and detachment of pre-formed biofilms (Gudiña *et al*., 2016). *Bacillus circulans* produces a biosurfactant with antimicrobial activity against *Acinetobacter calcoaceticus, Citrobacter freundii, Enterobacter cloacae, E. coli, Micrococcus luteus, P. mirabilis, Proteus vulgaris, Serratia marcescens* and MDR *E. coli, K. pneumoniae* and *S. aureus* (Gudiña *et al.*, 2016).

Although biosurfactants have been widely studied in past few years, the marine environment still remains mostly unexplored and only a few reports regarding biosurfactant production by marine micro-organisms have been reported (Cai *et al*., 2016). Microorganisms from marine origin have shown to produce novel biosurfactants with excellent bioactivity (Padmavathi and Pandian, 2014). The possible surfactin analogue characterized in the present study might provide a potential novel biosurfactant from the seaweed environment with potential applications in pharmaceutical and food industries. Our preliminary results suggest that a substantial fraction of secondary metabolites from this isolate still remains to be explored and could include a wide array of compounds such as lipopeptides, and other antimicrobial metabolites.

The incidence of biofilm-mediated infections, especially as a result of the medical use of implantable devices and catheters, is increasing tremendously (Gellatly and Robert, 2013). This indicates an urgent need for the discovery and identification of novel compounds with the capability to inhibit bacterial colonization and biofilm formation. Through the production of secondary metabolites, seaweed-associated bacteria are capable of producing AHL-like molecules to disrupt QS in other bacteria as a means of competition. The production of bioactives, in particular antimicrobials synthesized by seaweed-associated bacteria is well documented (Egan *et al*., 2014; Tebben *et al.,* 2014; Singh *et al*., 2015). In contrast, limited studies have covered the anti-biofilm potential of these strains (Braña *et al*., 2014; Egan *et al*., 2008). Quorum sensing controls gene expression that is beneficial when performed by groups of bacteria acting in synchrony. Processes controlled by QS include pigment formation, biolumniscence, sporulation, antibiotic production and biofilm formation (Jha *et al*., 2013). Inhibition of QS-mediated virulence factors including biofilm formation is a recognized anti-pathogenic drug target. The search for safe and effective anti-QS and anti-biofilm agents is expected to be useful to combat diseases caused by multidrug-resistant bacteria (Braña *et al*., 2014).

In this study, 30 potential QQ seaweed-associated bacterial extracts (predominantly *Streptomyces* and *Bacillus* spp.) were screened for the production of anti-biofilm compounds against MDR *P. aeruginosa* and MRSA, which are commonly associated with biofilm-related infections such as cystic fibrosis and nosocomial infections (Hurley *et al*., 2012). Initial adhesion of *P. aeruginosa* biofilms was found to be susceptible to seaweed-associated bacterial extracts, whereas MRSA was found to be less susceptible. Overall, extracts' activity against initial adhesion was extract- and dose-dependent. These results are similar to those of Lafleur *et al*. (2015) who isolated a marine seaweed epibiont *Cellulophaga* sp. E6, which produces a

QS inhibitory compound active against *P. aeruginosa*. Supernatant from *Cellulophaga* sp. E6 culture reduced expression of the 3-oxo-C12-HSL-dependent virulence-associated gene *lasB*, and reduced biofilm formation in a dose-dependent manner (Lafleur *et al*., 2015).

Several bacteria with QQ enzymes capable of inhibiting biofilm formation have been reported (Romero *et al*., 2012; Chankhamhaengdecha *et al*., 2013). The majority of the seaweed-associated bacterial isolates identified in this study were *Bacillus* species. *Bacillus* species inhibit QS through the production of lactonases which hydrolyze the ester bond of the AHL molecules yielding N-acyl-homoserine thus degrading QS signals (Martin *et al*., 2014), *Bacillus* spp., utilize AHL-lactonases in QQ to boost competitive strength in their immediate environment (Vinoj *et al*., 2014). Actinobacteria are one of the most efficient groups of secondary metabolite producers. They have been investigated as potential sources of anti-biofilm compounds due to their production of QQ enzyme acylase (Tan *et al*., 2016). *Streptomyces* sp. produce AhIM acylases, which degrades short and long chain AHL, thus inhibiting QS-mediated virulence factor production (Chankhamhaengdecha *et al*., 2013). Younis *et al.* (2016) reported the anti-biofilm activity of marine *Streptomyces* against biofilm formation of *Proteus mirabilis* on urinary catheter. Analysis of extracts derived from *Streptomyces gandocaensis* resulted in the discovery of three peptidic metabolites (cahuitamycins A–C) which inhibited biofilm formation of *Acinetobacter baumannii* (Park *et al*., 2016). Manickam *et al*. (2014) emphasized the role of *Streptomyces* spp. crude fatty acid extract, inhibiting the biofilm formation of *S. pyogenes*. Seaweeed-associated *Streptomyces* with biofilm reduction activity were identified in this study. The potential production of QQ enzymes or inhibitory small molecules by the seaweed-associated actinomycetes could be responsible for degrading AHL signals, shutting down QS and limiting biofilm adhesion or stimulating biofilm dispersal of *P. aeruginosa.*

Blockage of the QS circuit and thus biofilm formation in *S. aureus* can occur through inhibition of biosynthesis of auto-inducer peptides, inhibition of processing enzymes, blockage of receptor interactions by use of peptide antagonists (Singh *et al*., 2016). Due to the relatively non-bactericidal nature of tested extracts, data obtained suggests that seaweed-associated bacterial extracts could be analogues of AIP thus acting as receptor antagonists of *agrC* or they interfere with phosphorylation of *agrA* as cell viability was maintained. Nakayama *et al*. (2009) identified ambuic acid as AIP biosynthesis inhibitor of *S. aureus* and *Listeria innocua* suggesting that the compound targets a common point in the biosynthesis process of cyclic peptide quormone. To date, no specific enzymes that degrade AIP signals have been described (Fetzner, 2015).

Biofilms are enclosed within an exopolymer matrix that can restrict the diffusion of substances and bind antimicrobials. This provides effective resistance for biofilm cells against large molecules such as antimicrobials (Nithya *et al*., 2011). Hence, mature biofilms are more difficult to eradicate than the planktonic bacteria. When detachment studies were conducted the lowest concentration tested (1 mg/ml), had no effect on detachment of mature biofilm. However, upon exposure to higher concentrations the number of extracts observed to detach the isolates increased. Only the extract of MAB24-SW1 (*B. velezensis*) demonstrated ≥ 90% reduction potential against mature biofilms of both MRSA and MDR *P. aeruginosa* at 10 mg/ml, suggesting broad-spectrum anti-biofilm activity. The possible production of an analogue of surfactin by this isolate, as well as other lipopeptides synthesised by non-ribosomal peptide synthetases could have contributed to its anti-biofilm activity. Gudiña *et al*. (2016) demonstrated that biosurfactant produced by *B. circulans* exhibited the highest anti-biofilm activity at 10 mg/ml, microbial adhesion was inhibited between 84 - 89%, and pre-formed biofilms were removed (ranging from 59 - 94%) for all the pathogenic microorganisms tested including *S. aureus*. Quinn *et al*. (2012) reported lipopeptide biosurfactant from *B. cereus* that inhibited biofilm and dispersed biofilms of *S. aureus, P. aeruginosa, Streptococcus bovis and Micrococcus luteus*. Biosurfactants are an emerging therapy, which possess antibacterial, antifungal and viral properties with the ability to disperse or disrupt biofilms and seaweed-associated bacterial extracts will have to be chemically characterized in order to ascertain their role in the documented anti-biofilm activity.

The mature MRSA biofilms were susceptible to seaweed-associated bacterial extracts, whereas mature *P. aeruginosa* biofilms were less susceptible. *Pseudomonas aeruginosa* makes use of three distinct QS systems (LasI/LasR system, the RhlI/RhlR system, *Pseudomonas* quinolone signalling system) often simultaneously and in a heirachical manner (Dorotkiewcz-Jach *et al*., 2015). This renders *P. aeruginosa* difficult to eradicate, as inhibition of all three systems would be required to inhibit QS whereas MRSA only utilizes one QS system making it easier to eradicate (Nithya *et al*., 2010). These findings are similar to those of Nithya et al. (2010) who reported on mature biofilm disruption of *P. aeruginosa* by 70–74% after treatment with an anti-biofilm compound S6-15 isolated from the marine environment.

Furthermore, it was noted that a few extracts enhanced the formation of biofilms. This indicates that some of the extracts are potentially capable of mimicking the actions of auto-inducers, which subsequently stimulate transcriptional activity of the QS cascade. A greater number of extracts stimulated biofilm formation at the lower concentrations (1 mg/ml) but above this concentration, biofilm formation was inhibited by most extracts. Corral-Lugo *et al*.

(2016) reported similar results from rosmarinic acid, a plant-derived compound that functioned as a homoserine lactone mimic. *In vitro* assays showed that rosmarinic acid bound to the quorum-sensing regulator RhlR of *P. aeruginosa* PAO1 and competed with the bacterial ligand N-butanoyl-homoserine lactone (C4-HSL). Furthermore, rosmarinic acid induced QS–dependent gene expression and increased biofilm formation and the production of the virulence factors pyocyanin and elastase.

The biofilm inhibiting activity of the bacterial extracts observed in the current study, without any/or limited antibacterial activity demonstrates their potential as anti-biofilm agents. Interfering with QS, which is linked to biofilm formation, is expected to reduce resistance due to a decrease in selective pressure on pathogens (Quinn *et al*., 2012). The combination of QS inhibitory agents and antibiotics has also been suggested as a novel strategy to combat MDR strains (Elbur *et al*., 2014). Lipopeptides isolated from *B. licheniformis* were reported to possess synergistic activity against mature biofilms of *E. coli* when combined with ampicillin, cefazolin and ciprofloxacin, these combinations led to total eradication of biofilms (Rivardo *et al*., 2011). The pronounced antimicrobial and anti-biofilm bioactivities observed for *B. velezensis* (MAB24-SW1) suggests the potential of this extract to be utilized on its own or perhaps in conjunction, with commonly used antibiotics, which may prove more effective against MDR pathogens. This may also equate to lower doses or antimicrobials being used, thereby reducing the toxicity to the patients.

Based on the data, seaweed-associated bacterial extracts have the potential to produce bioactive compounds with broad-spectrum activity. While some extracts demonstrated antibacterial activity, other extracts with QQ potential demonstrated biofilm dispersion and disruption activity. Such extracts would have great clinical significance, given the current difficulties encountered with treating multi-drug resistant pathogens. Furthermore, the current study highlights the potential of marine bacterial extracts as a valuable target for developing pharmaceutically relevant products.

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CHAPTER 3

ANTIMICROBIAL AND ANTI-BIOFILM POTENTIAL OF SEAWEED-ASSOCIATED BACTERIA ISOLATED FROM SOUTH AFRICAN SEAWEED AGAINST RESISTANT AQUACULTURE PATHOGENS

Abstract

The use of antimicrobial agents in aquaculture has significantly reduced options for treating fish diseases, due to the emergence of antimicrobial resistant fish and opportunistic human pathogens. Research is now focused on the discovery of novel bioactive compounds, with seaweed being a prime resource in the search for microorganisms, which demonstrate novel bioactivities. The antimicrobial and anti-biofilm potential of (n=96) seaweed-associated-bacteria from ten South African seaweeds, was thus investigated. Isolates were tested for their antimicrobial activity utilizing primary screening (cross streak assay) against six bacterial fish pathogens. Following shake flask fermentation and ethyl acetate extraction, the efficacy of extracts was assessed by secondary screening (agar well diffusion assay) against six bacterial fish pathogens and their minimum inhibitory concentrations were determined. Extracts, at concentrations ranging from 1 - 10 mg/ml, capable of inhibiting initial adhesion and mature biofilm were assessed using utilizing the crystal violet microtiter plate assay. Primary screening indicated that 67% of isolates displayed activity against *Aeromonas salmonicida*, 51% against *Edwardsiella tarda*, 16% against *Vibrio parahaemolyticus*, 12% against *Salmonella enterica*, 11% against *Yersinia ruckeri* and 4% against *Aeromonas hydrophila*. Extract MAB24-SW1 (*B. velezensis*) demonstrated antibacterial effect against all aquaculture indicator strains. The total range of biofilm inhibition by extracts was between 0.08 - 113.84%. Inhibition of initial adhesion was observed with 70% of extracts, with ≥ 50% activity against *Y. ruckeri, V. parahaemolyticus,* and *A. hydrophila* in a dose-dependent manner with limited effect on growth. Extracts were most effective in the dispersal of mature biofilms with the best results been observed against *V. parahaemolyticus, A. hydrophila* and *Edwardsiella tarda*. Biofilm reduction activity of ≥ 90% was noted for 53% of the extracts against *V. parahaemolyticus*. Seaweed-associated bacteria could be used as a potential source for the isolation of bioactive metabolites to combat biofilm production and the associated antimicrobial resistance of aquaculture pathogens.

3.1 Introduction

Aquaculture is becoming a more intensive industry with a growing number of larger farms (Romero *et al*., 2012; Tan *et al*., 2016). Although there is rapid growth within the sector, it is also faced with challenges, such as water scarcity and the lack of control of infectious microbiota in aquaculture systems (Santhakumari *et al.*, 2015). A major group of causative bacterial strains include *Aeromonas* and *Vibrio* spp. (Defoirdt *et al*., 2011a). The water column of aquaculture systems harbour pathogenic bacteria due to the abundance of faecal pellets and uneaten feed stocks these become the breeding ground and can lead to high mortality rates (Attramadal *et al*., 2014). The excessive use of antimicrobial agents in aquaculture systems to either treat established infections or as prophylactic measure has resulted in the development of multi-drug resistant (MDR) bacteria (Santhakumari *et al.*, 2015). It is well-recognized that issues of antimicrobial use in food animals is a global concern (Defoirdt *et al*., 2016). Prevention and control of bacterial disease in aquatic animals is essential to minimize the use of antimicrobial agents and to avoid negative impacts of MDR. Thus, new strategies and novel compounds that will target virulence of pathogens need to be investigated (Natrah *et al*., 2011).

Marine flora and fauna have a variable relationship with the diverse community of associated marine microbes. The relationship between marine fauna and flora with marine microbes can be either beneficial or fatal to the host organism. Marine substrata are colonized by a variety of marine microorganisms, which are capable of producing novel compounds due to their diverse and often extreme environmental conditions and interactions with their associated marine eukaryotic host organisms (Egan *et al*., 2013). Studies have indicated that seaweeds produce secondary metabolites that can be used against harmful marine microbes (Harder *et al.*, 2012; Thanigaviel *et al*., 2015). Secondary metabolites from green, brown and red marine algae have been intensively studied for their bioactive compounds demonstrating anticancer, antimicrobial and antitumor activity (Martin *et al*., 2014). Research is now focused on bioactive discovery from marine-associated microorganisms (Chen *et al*., 2013; Singh *et al*., 2015; Defoirdt, 2016). Bioactive compounds such as benzaldehydes, isolated from seaweed-associated *Streptomyces atrovirens* Pk288-21 were reported to demonstrate antimicrobial activity against fish pathogens *Edwardsiella tarda* and *Streptococcus iniae* (Cho and Kim, 2012). Macrolactin which was isolated from *Bacillus subtilis* MTCC 10403, was reported to possess antibacterial activity against *Vibrio vulnificus* and *V. parahaemolyticus* (Chakraborty *et al.*, 2014), which are known to cause significant bacterial infections in aquaculture systems.

Quorum sensing (QS) is an intercellular communication mechanisms utilized by microorganism, whereby signal molecules known as autoinducers are synthesized and secreted by bacteria to communicate (Vinoj *et al*., 2014). Many aquatic bacterial pathogens such as *Aeromonas*, *Vibrio*, and *Edwardsiella* spp. use QS to regulate virulence factor production (Chu and Mclean, 2016). Gram-negative bacteria use acyl homoserine lactones (AHL), while Gram-positive bacteria use processed peptide-signalling molecules known as autoinducing peptides (AIPs) (Padmavathi *et al*., 2014). Quorum sensing regulates and coordinates the expression of virulence factors, and other functions that are important to the survival of bacteria; such as biofilm formation, pigment production, swarming, and the biosynthesis of antibiotics (Brackman *et al*., 2011). Studies have suggested that by inhibiting cell-to-cell communication (QS) within or among bacterial species could prevent biofilm formation and the spread of pathogenicity (Harder *et al.*, 2012; Santhakumari *et al*., 2015; Chu and McLean, 2016). Marine microorganisms and macroalgae demonstrates the ability to produce bioactive compounds that influence bacterial settlement, cell multiplication and biofilm formation by interfering with bacterial metabolism or bacterial QS (Salaün *et al*., 2013). Sethupathy *et al*. (2016) demonstrated the anti-biofilm and QS inhibitory potential of the brown macroalga *Padina gymnospora* against the nosocomial pathogen *Serratia marcescens*. Padmavathi *et al*. (2014) reported the anti-QS activity of *Gracilaria*-associated bacteria against *Chromobacterium violaceum*. The potential benefits of inhabiting QS, is that it will reduce MDR bacteria and also aid in the destruction of persistent bacterial infections (Padmavathi *et al*., 2014).

In most aquatic ecosystems bacteria can be found mostly in two forms, either as a bacterial community known as a biofilm that attach themselves to abiotic and biotic surfaces or as planktonic cells (Salaün *et al*., 2013). Biofilms are simply cells that are encased in extracellular polymeric substances (EPS) (Mieszkin *et al*., 2013), which protects bacteria from harsh environmental conditions, and contributes to their resistance to antimicrobial agents (Padmavathi *et al*., 2014). Economic problem arises when biofilms cause disease outbreaks in aquaculture production systems (Salta *et al.*, 2013). Bacteria belonging to the genera *Vibrio* and *Pseudomonas* are important aquaculture pathogens that form biofilms (Yuvaraj and Arul, 2014). Biofilms are a major concern in food and medical environments where their presence serves as a reservoir of contamination for humans or animals (Dheilly *et al*., 2010). The discovery of anti-biofilm agents is, therefore, of major importance in which environmentally-friendly, anti-biofilm molecules or organisms are highly valuable (Natrah *et al*., 2011).

Marine bacteria are often found in association with marine eukaryotes, and their ability to produce a variety of biological activities has attracted particular attention (Nithya and

Pandian, 2010; Defoirdt *et al*., 2011; Natrah *et al*., 2011). Viju *et al*. (2014) observed that extracellular polymeric substances secreted by a *Pseudomonas taiwanensis* strain S8, a symbiont of seaweed (*Gracillaria*, *Sargassum*, and *Ulva* spp.) inhibited the formation of biofilms by *Pseudomonas* and *Alteromonas* spp. Substances currently being utilized to control biofilms, have proved to have detrimental side effects and accumulate in the environment (Prabhakaran *et al.,* 2012), thus environmentally-friendly treatment options are required. The current study places special emphasis on the ability of seaweed-associated-bacterial isolates to serve as reservoir for discovery of bioactive compounds with potential therapeutic activity to combat biofilms and MDR bacteria in an aquaculture setting.

3.2 Materials and methods

3.2.1 Bacterial isolates

Ninety-six bacterial strains were isolated previously from ten South African intertidal seaweeds (*Amphiroa bowerbankii* Harvey, *Cheilosporum cultratum* Areschoug (Harvey), *Codium duthieae* (P. Silva), *Codium* spp., *Gelidium pteridifolium* (R.E, Norris, Hommersand & Fredericq), *Gracilaria* sp., *Jania verrucosa* (Lamourous), *Laurencia brongiartii* (J. Agarah), *Ulva rigida* (C. Agarah) collected along the coastline of Durban, South Africa. Isolates were examined and differentiated according to colony characteristics, Gram reaction and cellular morphology.

3.2.2 Primary screening of isolates for antimicrobial activity

Primary antibacterial screening was conducted using cross-streak method (Kvennefors *et al*., 2012). A panel of resistant aquaculture indicators (*Aeromonas hydrophila* ATCC 7966, *Aeromonas salmonicida* ATCC 33658, *Edwardsiella tarda* ATCC 15947, *Salmonella enterica* serovar Arizonae ATCC 13314, *Vibrio parahaemolyticus* ATCC 17802 and *Yersinia ruckeri* ATCC 29473) was used. *Streptomyces griseus* ATCC 15468 was used as a positive antibiotic-producing control. Mueller-Hinton agar (MHA) plates were prepared and inoculated with seaweed-associated bacterial isolates by a single perpendicular streak of inoculum in the centre of the petri-dish and incubated at 30 °C for 5-7 d. Indicator organisms were then streaked perpendicularly to the initial streak (Shnit-Orland and Kushmaro, 2013) and plates were incubated at 30 °C for 24 h. Each indicator organism was first grown individually on MHA to ensure that any lack of growth was not dependent on the medium used for screening. The antagonistic effect was indicated by the failure of the indicator strain to grow in the confluence area. Inhibition was measured from the edge of the vertical streak with the ‘test isolate’ to the

first colony of 'indicator isolate' cross-streak and divided into distance-dependent categories. Inhibition was graded as follows (- = no activity, + = weak activity [inhibition zone of 1-4 mm], ++ = moderate activity [inhibition zone of 5-8 mm], +++ = strong activity (inhibition zone of 9-15 mm), ++++ = highly active [inhibition zone of 16-25 mm) and total growth inhibition = TGI). Experiments were performed in triplicate and the inhibition zones recorded (Kvennefors *et al*., 2012).

3.2.3 Fermentation and ethyl acetate extraction

Thirty bacterial isolates which exhibited antagonistic activity in the cross-streak assay (Table 3.1) and 30 potential quorum quenching (QQ) isolates (Jacobs, 2015) were pre-cultured in 5 ml of ISP2 broth (Shirling and Gottlieb 1966) for 2 d, then inoculated in 250 ml of ISP2 broth and incubated with shaking for 7 d at 30 °C. Bacterial cells were pelleted at 9500 rpm for 10 min to collect supernatants. An equal volume (1:1) of ethyl acetate was added to each cell-free supernatant followed by agitation for 1 h at 30 °C. The ethyl acetate layer was collected and then subjected to a second extraction (1:1 volume ethyl acetate) with agitation of flasks for 4 h after which the ethyl acetate layer was collected. Ethyl acetate was evaporated in a rotary evaporator (Ilmvac, ROdist digital 230V 50/60Hz) and each crude extract obtained was weighed (Nithya *et al*., 2011). Thereafter, crude extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml.

3.2.4 Secondary screening of isolates for antimicrobial activity

3.2.4.1 Screening of potential antagonistic bacteria against clinical pathogens by disc diffusion method

Antibacterial activity of the 30 antagonistic crude bacterial extracts was assessed by loading 0.5, 1 and 2 mg/ml of respective extracts onto 6 mm blank discs (Oxoid, UK). Muller-hinton plates were prepared and uniformly swabbed with resistant aquaculture pathogens: *A. hydrophila* ATCC 7966, *A. salmonicida* ATCC 33658, *E. tarda* ATCC 15947, *S. enterica* serovar Arizonae ATCC 13314, *V. parahaemolyticus* ATCC 17802 and *Y. ruckeri* ATCC 29473. DMSO was used as a negative control. Plates were incubated overnight at 30 °C (optimal temperature of indicator bacteria) and observed for the zones of inhibition. The diameter of the inhibition halos after 24 h of incubation was measured and was considered to be indicative of bioactivity (Nithya *et al*., 2011).

3.2.4.2 Minimum inhibitory concentration determination of extracts

The minimum inhibitory concentrations (MIC) of 14 extracts demonstrating antimicrobial activity following secondary screening were determined using a modified broth microdilution assay (Motyl *et al*., 2006). Indicator bacteria suspensions (*A. hydrophila* ATCC 7966, *E. tarda* ATCC 15947 and *Y. ruckeri* ATCC 29473), equivalent to a 0.5 McFarland standard, were added to 96-well plates containing Mueller-Hinton broth (Sigma). Wells were supplemented with extracts (50 mg/ml stock solution) serially diluted two-fold to give final concentrations ranging from 12.5 – 0.01 mg/ml and incubated at 30 °C for 24 h. After incubation, 30 µl of 0.02% resazurin (oxidation-reduction indicator) dye was added to each well, and plates were again incubated at 30 °C for 4 h in dark and observed for a colour change. A pink colour indicated growth and blue was indicative of inhibition of growth. The MIC was recorded as the lowest concentration at which a colour change occurred (Sarker *et al*., 2007).

3.2.5 Detection of anti-biofilm activity of seaweed-associated bacteria extracts

Prior to the anti-biofilm assay, the 30 selected potential QQ extracts were tested for antibacterial activity to determine sub-inhibitory and inhibitory concentrations (1, 5 and 10 mg/ml), utilizing the disc diffusion assay. Extracts were tested against *A. hydrophila* ATCC 7966, *E. tarda* ATCC 15947, *V. parahaemolyticus* ATCC 17802 and *Y. ruckeri* ATCC 29473, in order to assess their effect on initial adhesion and detachment of mature biofilms.

Overnight cultures were used to prepare cell suspensions, which were standardized equivalent to a 0.5 McFarland standard (Basson *et al*., 2008). For initial adhesion studies, extracts were added to 90 µl TSB and 10 µl of standardized cell suspension (to a final volume of 200 µl) and incubated for 24 h at 24 ˚C with agitation. For pre-formed biofilm detachment assays, 24 h biofilms were established following addition of 90 µl TSB and 10 µl of 0.5 McFarland standardized cell suspension to microtitre plate wells, and incubation at 30 ºC for 24 h. Microtitre plates were washed three times with sterile deionised water and allowed to air-dry. Following air-drying, 90 µl TSB as well as extracts at the relevant, respective concentrations were added to wells (to a final volume of 200 µl) and microtitre plates were incubated for 24 h with agitation at 30 ˚C. The negative control contained only broth, while positive controls contained respective cell suspensions with no extracts added. After incubation, growth $OD_{600\ nm}$ values were determined using the Glomax multi + detection system (Promega) and wells with ≥ 50% reduction in growth were considered unsuitable for analysis. Thereafter, planktonic cells were removed by discarding the liquid media. Plates were processed for biofilm inhibition as described by Basson *et al*. (2008). Microtiter plates were washed three times with sterile

dH_2O. Cells were fixed with 200 µl of methanol for 15 min, then air-dried. Wells were stained with 150 µl of 2% Hucker's crystal violet for 5 min. Wells were rinsed getly under running tap water then plates were allowed to dry. Glacial acetic acid150 µl; 33% (v/v)) was used to re-solubilise cells (Basson *et al*., 2008). The OD was read at 600 nm using the Glomax multi + detection system (Promega).

Tests were conducted in triplicate on two separate occasions. A measure of efficacy called percentage reduction was calculated from the blank, control, and treated absorbance values (Pitts *et al*., 2003): Percentage reduction $=\left[\frac{(C-B)-(T-B)}{C-B}\right] \times 100$, where B denoted the average absorbance per well for blank wells (no biofilm, no treatment), C denoted the average absorbance per well for control wells (biofilm, no treatment), and T denoted the average absorbance per well for treated wells (biofilm and treatment). The difference in biofilm OD values with and without the addition of varying concentrations of extracts was determined using One-way repeated measures ANOVA with $p \leq 0.05$ being considered significant (SigmaPlot 13.0, Systat Software Inc., San Jose, CA, USA). To identify the concentrations that differed from the others, the Holm-Sidak multiple pairwise comparison procedure was carried out, with $p \leq 0.05$ being considered significant.

3.3 Results

3.3.1 Primary antimicrobial activity screening of seaweed-associated bacteria

Preliminary screening of antibacterial activity of 96 seaweed-associated bacteria against resistant pathogenic bacteria identified 73% (71/96) of isolates, which demonstrated antibacterial activity (Fig. 3.1).

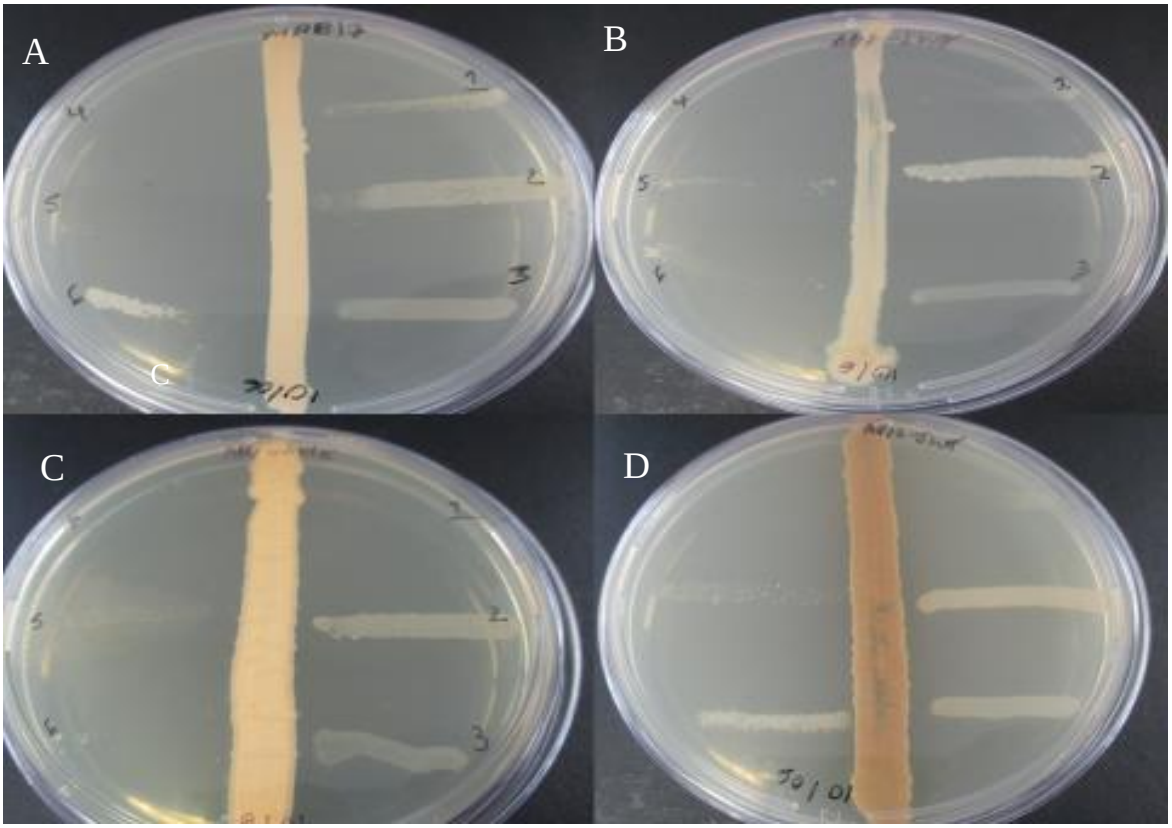

Figure 3.1: Primary screening results of (**A**) MAB17-SW1, (**B**) AB8-SW8, (**C**) AB9-SW8 and (**D**) AB12-SW8 against (**1**) *E. tarda* ATCC 15947, (**2**) *A. hydrophila* ATCC 7966, (**3**) *S. enterica Arizone* ATCC 13314, (**4**) *A. salmonicidia* ATCC 33658, (**5**) *Y. ruckeri* ATCC 29473 and (**6**) *V. parahaemolyticus* ATCC 17802 aquaculture indicator bacteria using cross streak method. The antagonistic activity was indicated by inhibition of growth away from the vertically streaked indicator.

Of those, 14% (14/96) demonstrated activity against three indicator organisms. Majority of isolates (67%; 64/96) demonstrated antibacterial activity against *A. salmonicida*, while isolates had weak activity against *A. hydrophila* (4%; 4/96) and *S. enterica* (6%; 6/96). Based on primary screening, 30 isolates were selected for further screening (Table 3.1).

Table 3.1: Primary antibacterial activity screening of seaweed-associated bacterial isolates against aquaculture pathogens.

Extract*	Zone of growth inhibition (mm)					
	***A. hydrophila* ATCC 7966**	***A. salmonicida* ATCC 33658**	***E. tarda* ATCC 15947**	***S. enterica* serovar Arizonae ATCC 13314**	***V. parahaemolyticus* ATCC 17802**	***Y. ruckeri* ATCC 29473**
MAB7-SW1	-	TGI#	TGI	-	-	-
MAB10B-SW1	-	TGI	-	-	-	-
MAB11-SW1	-	TGI	-	-	-	-
MAB12-SW1	-	TGI	+	-	-	-
MAB16-SW1	-	-	-	-	+++	-
MAB17-SW1	++	TGI	+	-	+++	TGI
MAB20-SW1	-	TGI	5	-		-
MAB22-SW1	-	TGI	++	-	-	-
MAB24-SW1	+	+	-	TGI	-	-
MAB25A-SW1	-	TGI	++	+++		+++
MAB27-SW1	-	+++	-	-		-
MAB37-SW1	-	TGI	+++	-	+++	+++
AB1-SW2	-	TGI	++++	-	-	-
AB5-SW2	-	-	-	-	-	-
AB6-SW2	-	TGI	+++	-	-	-
AB10-SW2	-	TGI	+	-	-	-
AB11-SW2	-	TGI	+++	-	-	-
AB12-SW2	-	0	-	-	-	-
AB3-SW5	-	TGI	TGI	-	+	TGI
AB6-SW5	-	TGI	TGI	+	-	-
AB7-SW5	-	-	-	-	-	-
AB8-SW5	-	TGI	+++	-	-	-
AB6-SW6	-	TGI	++	-	-	-
AB1-SW8	-	TGI	TGI	-	-	-
AB2-SW8	-	TGI	++	-	-	-
AB7-SW8	-	TGI	++	-	+	++
AB9-SW8	-	TGI	TGI	-	TGI	-
AB12-SW8	-	TGI	+++	-	-	+
AB1-SW9	-	TGI	TGI	-	-	-
AB1-SW10	-	TGI	+++	-	-	++
AB4-SW10	-	TGI	+++	-	-	-
S. griseus ATCC 15468		++	+	+++	++	TGI

*SW1 – *Gracilaria* spp., SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

#Grading: - = no activity; + = weak activity (zone of inhibition of 1-4 mm); ++ = moderate activity (zone of inhibition of 5-8 mm); +++ = strong activity (zone of inhibition 9-15 mm); ++++ = highly active (zone of inhibition 16-25 mm); TGI = total growth inhibition.

3.3.2. Secondary screening of crude seaweed-associated bacterial extracts

Thirty isolates, selected from primary screening for shake-flask fermentation and secondary metabolite extraction using ethyl acetate were subjected to secondary screening using the disc diffusion assay. No antibacterial activity was observed for extracts at 0.5 and 1 mg/ml with the exception of MAB24-SW1 (Fig. 3.2; Table 3.2), which demonstrated growth inhibitory activity at all concentrations tested. However, at 2 mg/ml, the crude extracts exhibited antibacterial activity predominantly against *A. salmonicida* ATCC 33658 and *A. hydrophila* ATCC 7966 (Table 3.2).

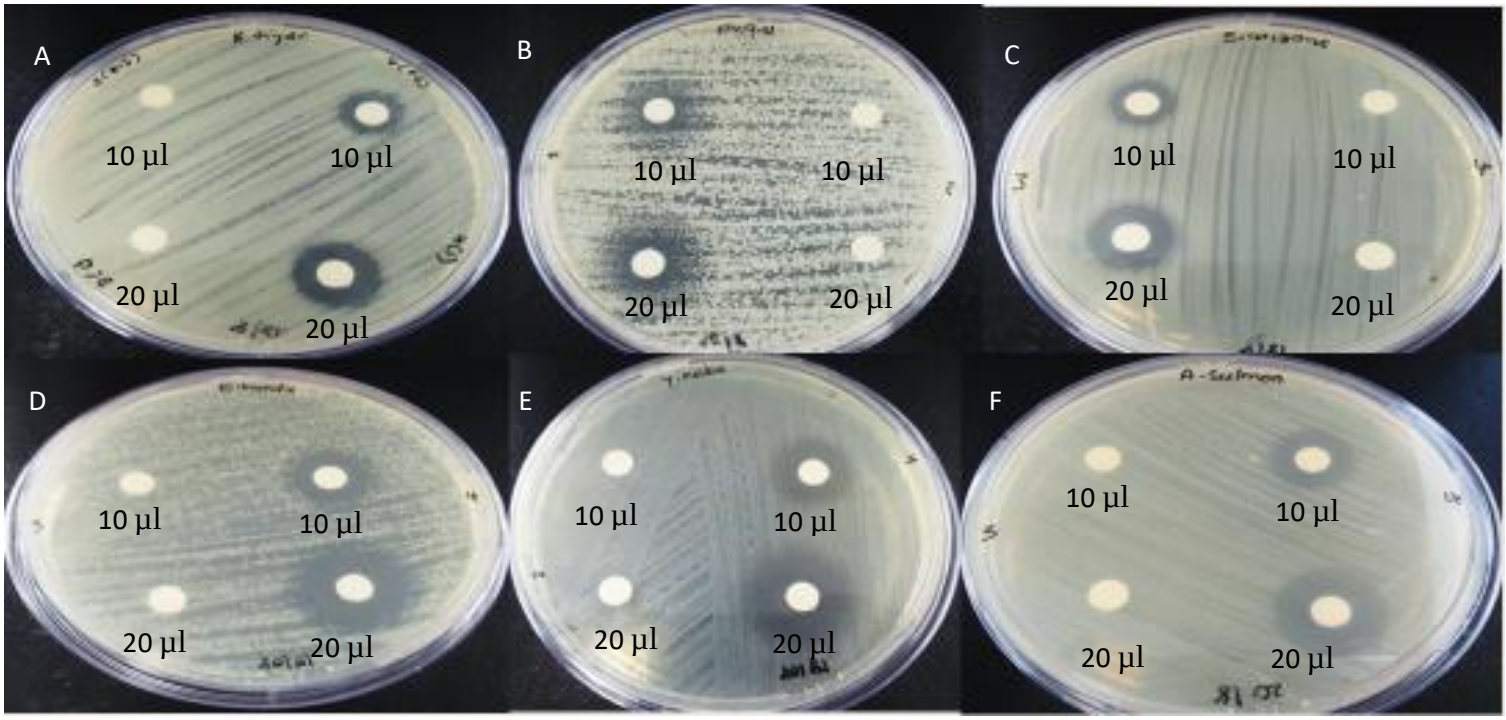

Figure 3.2: Bacteriostatic and bactericidal activity of crude MAB24-SW1 extract (0.5 - 1 mg/ml) against (**A**) *A. hydrophila* ATCC 7966, (**B**) *V. parahaemolyticus* ATCC 17802, (**C**) *S. enterica serovar Arizone* ATCC 13314, (**D**) *E. tarda* ATCC 15947, (**E**) *Y. ruckeri* ATCC 29473 and (**F**) *A. salmonicidia* ATCC 33658 indicator organisms using agar-well diffusion assay**.**

Table 3.2: Antibacterial activity of extracts from seaweed-associated bacterial isolates against aquaculture indicators using the Kirby–Bauer disk diffusion method.

Extract*	Zone of growth inhibition at 2 mg/ml concentration (mm)					
	***A. hydrophila* ATCC 7966**	***A. salmonicida* ATCC 33658**	***E. tarda* ATCC 15947**	***S. enterica* serovar Arizonae ATCC 13314**	***V. parahaemolyticus* ATCC 17802**	***Y. ruckeri* ATCC 29473**
MAB7-SW1	10	10	0	10	0	0
MAB10B-SW1	11	7	0	11	0	0
MAB11-SW1	7	9	8	9	0	8
MAB12-SW1	10	0	8	0	0	9
MAB16-SW1	9	9	0	10	0	0
MAB17-SW1	10	7	9	0	0	0
MAB20-SW1	0	7	0	9	0	0
MAB22-SW1	7	8	0	0	0	0
MAB24-SW1	15	14	13	10	12	16
MAB25A-SW1	0	0	9	0	0	0
MAB27-SW1	0	0	8	0	0	0
MAB37-SW1	10	10	0	11	0	9
AB1-SW2	9	12	0	10	0	10
AB5-SW2	10	0	0	9	0	9
AB6-SW2	12	9	0	0	0	10
AB10-SW2	10	8	0	9	0	10
AB11-SW2	9	10	0	8	0	0
AB12-SW2	10	7	0	9	0	8
AB3-SW5	11	8	0	10	0	0
AB6-SW5	0	8	0	0	0	10
AB7-SW5	0	10	0	9	7	11
AB8-SW5	11	8	12	10	0	0
AB6-SW6	10	8	8	11	0	8
AB1-SW8	8	7	8	10	0	10
AB2-SW8	9	7	9	11	0	12
AB7-SW8	9	0	0	10	8	0
AB9-SW8	10	0	0	12	0	9
AB12-SW8	8	7	10	9	0	8
AB1-SW9	10	8	0	10	0	10
AB1-SW10	10	10	9	10	0	11
AB4-SW10	9	11	10	12	0	10

*SW1 – *Gracilaria* spp., SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

3.3.3 Minimum inhibitory concentration of crude seaweed-associated bacterial extracts

The MICs of fourteen active seaweed-associated bacteria crude extracts were determined for *A. hydrophila* ATCC 7966, *E. tarda* ATCC 15947 and *Y. ruckeri* ATCC 29473. An MIC of 0.78 mg/ml was obtained with MAB24-SW1 against *Y. ruckeri* ATCC 29473, *A. hydrophila* ATCC 7966 and 0.39 mg/ml against *E. tarda* ATCC 15947. In contrast, the remaining thirteen extracts had MIC values of 6.25 mg/ml against all three indicators (Table 3.3). These results were used to inform for the selection of sub-inhibitory and inhibitory concentrations to be used in the biofilm inhibition assays.

Table 3.3: Minimum inhibitory concentration of seaweed-associated bacterial extracts against selected aquaculture pathogens.

Extracts	*A. hydrophila* ATCC 7966	*E. tarda* ATCC 15947	*Y. ruckeri* ATCC 29473
MAB7-SW1	6.25	6.25	6.25
MAB11-SW1	6.25	6.25	6.25
MAB24-SW1	0.78	0.39	0.78
AB1-SW2	6.25	6.25	6.25
AB5-SW2	6.25	6.25	6.25
AB3-SW5	6.25	6.25	6.25
AB7-SW5	6.25	6.25	6.25
AB8-SW5	6.25	6.25	6.25
AB1-SW8	6.25	6.25	6.25
AB2-SW8	6.25	6.25	6.25
AB12-SW8	6.25	6.25	6.25
AB1-SW9	6.25	6.25	6.25
AB1-SW10	6.25	6.25	6.25
AB4-SW10	6.25	6.25	6.25

*SW1= *Gracilaria* spp.; SW2 – *Codium* spp.; SW5 - *Amphiroa bowerbankii*; SW8 - *Gelidium pteridifolium*; SW9 - *Ulva rigida;* SW10 - *Codium duthieae*.

3.3.4. Effect of seaweed-associated bacterial extracts on the adhesion and detachment of biofilm-forming bacteria

Thirty seaweed-associated bacterial extracts with quorom quenching potential (Jacobs, 2015) were screened for their ability to inhibit initial adherence and to disperse mature biofilms of *A. hydrophila* ATCC 7966, *E. tarda* ATCC 15947, *V. parahaemolyticus* ATCC 17802 and *Y. ruckeri* ATCC 29473 on polystyrene microtitre plates. To determine whether the inhibitory effect on biofilm development was related to general growth inhibition or a change in the bacterial growth rate, growth was measured prior to biofilm inhibition calculations. Those extracts noted to have antibacterial activity were excluded from the analysis. The effect of

seaweed-associated bacterial extracts relative to untreated samples are depicted in Figs. 3.3-10.

Aquaculture indicators were treated with 1, 5 and 10 mg/ml concentrations. Due to significant growth inhibition (≥ 50%) been observed at 10 mg/ml during initial adherence assay, this concentration was not considered as inhibition. At 1 mg/ml, 43% (13/30) of extracts increased the initial adherence of *A. hydrophila*, however, 57% (17/30) decreased initial adherence ($p = 0.296$) with biofilm reduction ranging from 0.28-58.66 (Table 3.4). When the extracts' concentration was increased to 5 mg/ml, only 7% (2/30) retained their ability to increase adherence (Fig. 3.3B; $p < 0.001$). Of note was that 36% (11/30) of extracts demonstrated ≥ 50% reduction potential with 13% (4/30) demonstrating ≥ 90% reduction potential (Table 3.4).

In the present study, effects of three concentrations (1, 5 and 10 mg/ml) were investigated during detachment studies. Upon exposure to 1 mg/ml, biofilm detachment activity was noted for 77% (23/30) of extracts against *A. hydrophila* (Fig. 3.4; $p = 0.228$), with biofilm reduction ranging from 0.18-77.96% (Table 3.5). At 5 mg/ml, AB5-SW9 was the only extract observed to increase adhesion of *A. hydrophila* (Fig. 3.4B), while 97% (29/30) of the extracts detached biofilm (ranging from 4.49-83.08%; $p < 0.001$). Of the extracts with inhibitory potential, 50% (15/30) demonstrated ≥ 50% reduction potential. Biofilm inhibition activity of ≥ 90% by 27% (8/30) of the extracts, could also be detected against *A. hydrophila* after treatment with 10 mg/ml ($p < 0.001$). Moreover, 73% (22/30) of extracts demonstrated ≥ 50% biofilm reduction potential. The results obtained showed a concentration-dependent inhibition (Table 3.5).

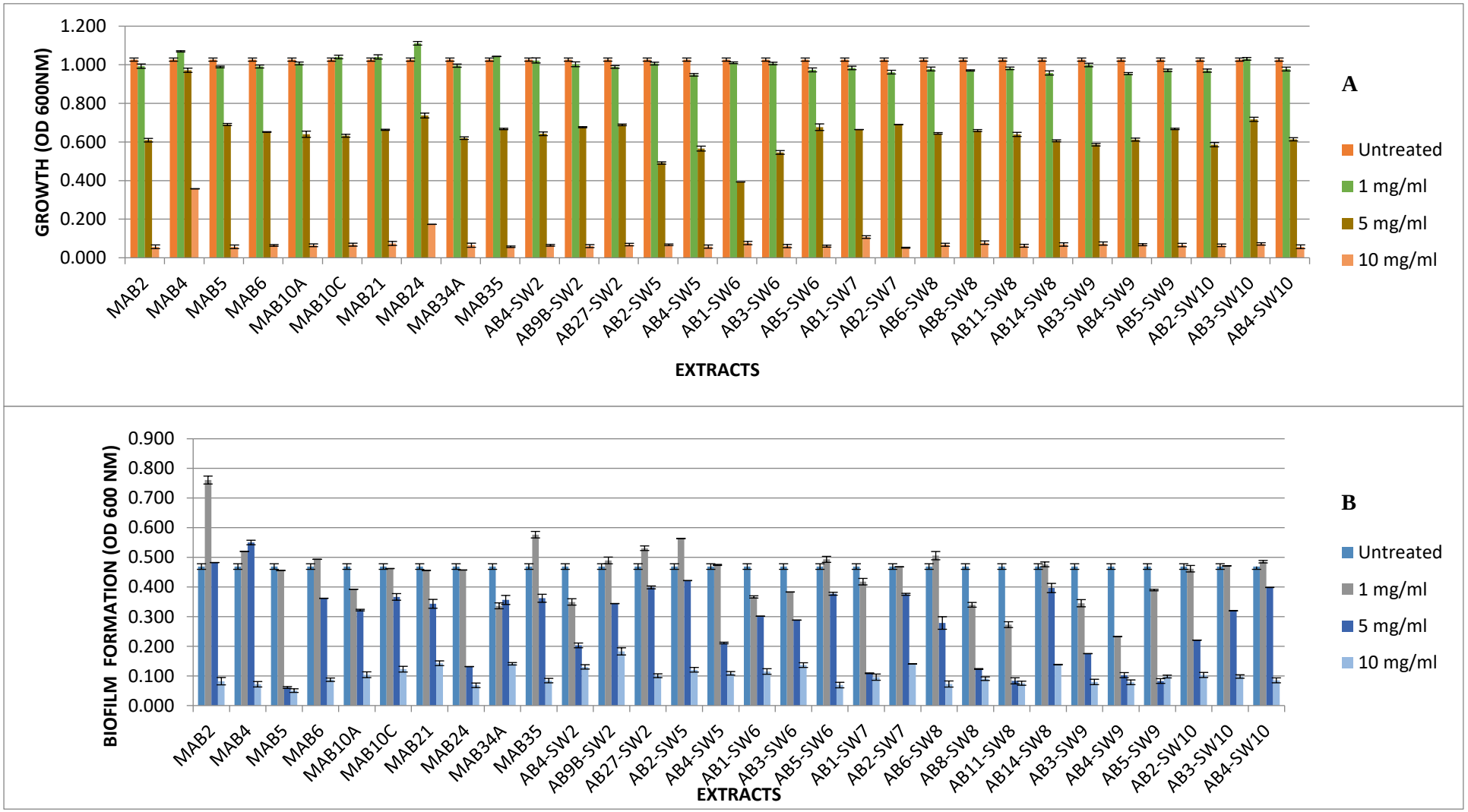

Figure 3.3: The effect of crude seaweed-associated bacterial extracts on growth (**A**) and initial adhesion (**B**) of *Aeromonas hydrophila* ATCC 7966 as quantified by crystal violet staining in microtitre plate assay. Data represents the mean values of two independent, replicate experiments ± SD are shown. Differences in the mean values of 5 mg/ml treatment group was statistically significant ($p < 0.001$).

Table 3.4: Percentage biofilm reduction following treatment with seaweed-associated bacterial extracts against initial adherence of aquaculture indicator bacteria

Extract*	Percent biofilm reduction at time of inoculation of *A. hydrophila* ATCC 7966		Percent biofilm reduction at time of inoculation of *E. tarda* ATCC 15947		Percent biofilm reduction at time of inoculation of *V. parahaemolyticus* ATCC 17802		Percent biofilm reduction at time of inoculation of *Y. ruckeri* ATCC 29473	
	1 mg/ml€	5 mg/ml€	1 mg/ml€	5 mg/ml€	1 mg/ml€	5 mg/ml€	1 mg/ml€	5 mg/ml€
MAB2-SW1	-72.53¥	-3.37	38.23	9.21	39.53	96.57	52.12	95.35
MAB4-SW1	-12.70	-20.18	-5.12	29.28	83.47	97.17	27.20	88.19
MAB5-SW1	3.31	101.41	63.58	20.97	84.67§	100.16§	43.12	62.07
MAB6-SW1	-6.05	26.61	63.67	40.17	76.84	93.42	24.17	62.88§
MAB10A-SW1	18.98	36.49	33.67	29.83	76.96§	98.76§	19.86	38.50
MAB10C-SW1	1.68	25.44	50.94	17.81	65.00	85.79§	51.13	56.67§
MAB21-SW1	3.30	31.23	15.51	24.89	63.30§	38.40§	23.39	87.76
MAB24-SW1	2.92	83.85	81.51	54.32	81.67§	78.75§	95.29§	100.07§
MAB34A-SW1	32.92	28.01	22.95	48.98	68.02§	97.78§	13.60	67.97
MAB35-SW1	-26.73	26.59	79.33	18.07	77.67§	80.77§	5.81	57.54§
AB4-SW2	29.65	66.08	72.67	37.77	76.58	89.64	22.41	28.87
AB9B-SW2	-5.13	31.01	83.25	46.31	68.69§	82.30§	54.19	58.64
AB27-SW2	-15.37	17.45	74.02	10.39	81.37§	95.23§	44.41	70.21§
AB2-SW5	-23.47	11.80§	57.84	38.35	81.06§	75.30	3.68	39.27
AB4-SW5	-1.37	64.09	41.01	-23.25	68.98§	73.63§	11.92	4.61
AB1-SW6	25.46	41.66§	38.81	39.86	71.92§	90.32§	54.78	76.20
AB3-SW6	21.36	44.92	5.18	-5.26	78.82§	70.79§	2.02	38.53
AB5-SW6	-6.04	22.80	64.14	-14.48	81.69§	77.84§	71.07	65.72
AB1-SW7	12.65	89.49	-1.09	30.56	68.91	84.44	20.43	38.69
AB2-SW7	0.28	23.26	46.36	-23.80	89.69§	85.32§	43.20	72.95
AB6-SW8	-9.14	47.33	39.59	28.39	86.60§	100.54§	44.44	66.45§
AB8-SW8	32.08	85.89	2.77	-41.74	63.21	73.96	12.35	68.03
AB11-SW8	48.68	95.79	14.88	-35.14	73.53	79.20	36.44	47.67
AB14-SW8	-1.85	17.97	38.09	-37.88	76.21	102.08§	6.69	59.79
AB3-SW9	30.73	72.98	7.41	-23.55	49.89	74.85	29.46	73.01
AB4-SW9	58.66	91.01	42.55	-2.94	57.06	82.70	26.95	73.08
AB5-SW9	19.73	95.96	20.63	7.59	58.86	79.34	28.32	55.03
AB2-SW10	1.80	61.67	61.96	-3.19	71.61§	89.22	21.63	65.61
AB3-SW10	-0.60	37.11	55.55	-35.90	50.23§	88.75	23.75	54.13
AB4-SW10	-4.12	17.55	46.86	25.09	60.50§	94.39	53.71	65.24

* SW1 – *Gracilaria* spp., SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

Biofilm reduction calculated according to Pitts *et al*. (2003). § ≥50% growth reduction in comparison to untreated control reflected growth inhibitory effect. ¥ Negative values are indicative of an increase in attachment/biofilm formation. € Differences in the BFR values among the treatment groups were statistically significant ($p < 0.001$).

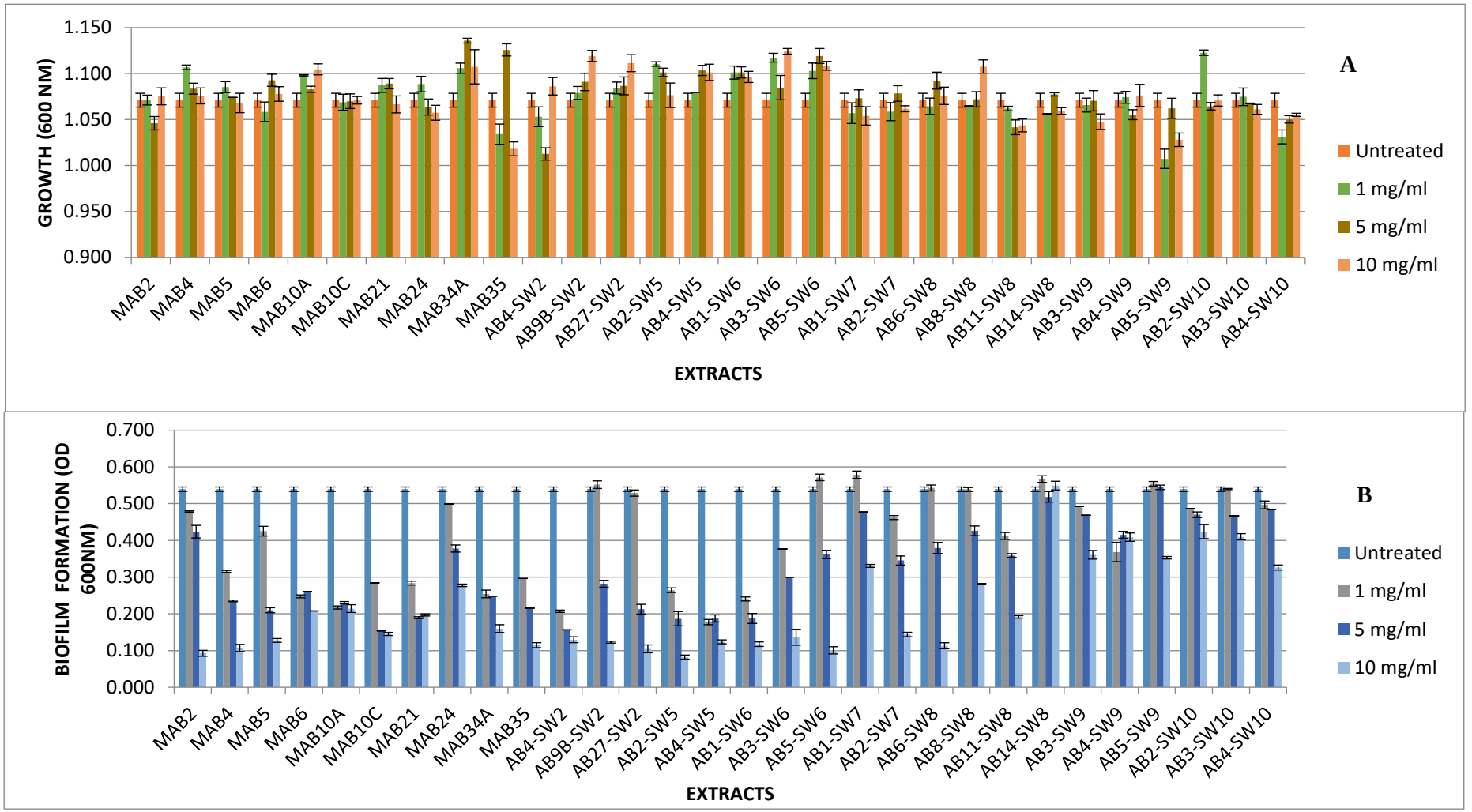

Figure 3.4: The effect of crude seaweed-associated bacterial extracts on **(A)** mature biofilm growth and **(B)** mature biofilm detachment of *Aeromonas hydrophila* ATCC 7966 quantified by crystal violet staining.in microtitre plate assay. Data represents the mean values of two independent, replicate experiments ± SD are shown. Differences in the mean values of 5 and 10 mg/ml treatment groups were statistically significant ($p < 0.001$).

Table 3.5: Percentage biofilm reduction following treatment with seaweed-associated bacterial extracts against mature biofilm of aquaculture indicator bacteria

Extract	Percent biofilm reduction against 24 h pre-formed biofilm of *A. hydrophila* ATCC 7966			Percent biofilm reduction against 24 h pre-formed biofilm of *E. tarda* ATCC 15947			Percent biofilm reduction against 24 h pre-formed biofilm of *V. parahaemolyticus* ATCC 17802			Percent biofilm reduction against 24 h pre-formed biofilm of *Y. ruckeri* ATCC 29473		
	1 mg/ml[€]	5 mg/ml[€]	10mg/ml[€]	1 mg/ml[€]	5 mg/ml[€]	10mg/ml[€]	1 mg/ml[€]	5 mg/ml[€]	10mg/ml[€]	1 mg/ml[€]	5 mg/ml[€]	10mg/ml[€]
MAB2-SW1	13.04	24.93	96.27	46.48	40.41	99.81	42.78	102.65	104.41	-9.81	23.62	90.24
MAB4-SW1	48.23	65.58	93.16	49.03	67.00	100.84	77.76	100.48	110.13	29.10	19.63	92.60
MAB5-SW1	24.61	71.00	88.70	39.64	70.00	95.96	98.48	107.29	113.84	-3.35[¥]	-9.72	41.93
MAB6-SW1	62.87	60.06	71.42	21.35	30.09	50.54	47.87	70.59	102.54	15.86	47.80	33.69
MAB10A-SW1	69.38	66.62	70.04	18.25	21.49	73.38	29.70	52.19	70.12	0.71	1.93	36.71
MAB10C-SW1	55.01	83.08	84.90	29.33	38.22	45.23	14.48	22.30	94.58	-3.81	-10.47	8.50
MAB21-SW1	55.09	75.35	73.75	14.07	10.59	60.11	-11.10	66.86	86.33	2.81	15.29	51.90
MAB24-SW1	8.59	34.81	56.40	43.99	74.31	93.59	41.25	60.03	96.51	20.22	32.57	95.87
MAB34A-SW1	61.43	62.71	81.78	0.68	17.45	48.29	3.09	67.14	80.59	-0.27	-6.84	7.40
MAB35-SW1	52.36	69.76	91.50	1.24	8.18	46.94	53.71	84.85	75.38	65.33	49.10	40.87
AB4-SW2	71.59	82.51	88.29	8.07	3.96	70.32	68.12	95.57	106.97	4.69	43.37	53.78
AB9B-SW2	-2.65	55.49	89.76	30.36	34.32	73.38	70.77	95.61	101.39	31.55	5.16	97.23
AB27-SW2	2.25	70.38	93.65	41.97	58.46	99.55	79.64	91.28	107.23	-7.27	15.59	103.24
AB2-SW5	59.22	75.97	98.50	9.01	15.08	67.68	30.72	95.48	97.35	9.57	55.27	46.20
AB4-SW5	77.96	75.82	89.53	12.68	14.68	18.54	90.36	97.17	88.00	15.45	29.11	53.91
AB1-SW6	64.41	75.80	90.93	24.79	45.96	64.54	43.38	95.13	96.84	-30.78	-23.79	61.50
AB3-SW6	35.07	51.58	86.86	3.17	10.75	37.58	0.93	85.80	96.74	0.08	45.38	48.63
AB5-SW6	-6.93	38.25	94.52	15.54	75.94	63.87	84.24	94.18	97.35	0.67	34.10	88.58
AB1-SW7	-8.50	13.38	44.98	21.63	30.19	27.67	10.93	24.34	67.23	-3.25	-2.01	37.14
AB2-SW7	16.63	41.83	85.22	32.40	41.08	86.94	9.43	86.97	109.14	-24.09	-0.04	97.05
AB6-SW8	-0.74	34.48	91.85	12.20	47.68	89.33	43.78	105.86	107.42	-8.42	46.76	94.77
AB8-SW8	0.18	24.42	55.39	22.93	24.64	35.04	14.72	49.87	71.51	-4.18	-3.03	26.41
AB11-SW8	27.41	38.90	74.93	28.09	49.37	49.04	28.00	78.06	84.96	5.17	48.27	54.61
AB14-SW8	-5.94	4.49	-2.13	12.70	32.48	42.88	10.14	62.55	93.81	19.30	40.21	75.81
AB3-SW9	10.08	15.27	38.49	13.14	7.51	41.61	12.31	65.18	80.71	-1.66	4.00	19.82
AB4-SW9	36.81	26.73	28.12	31.08	38.72	60.63	51.54	60.17	49.44	7.91	17.72	32.71
AB5-SW9	-3.21	-1.10	40.19	30.33	24.58	29.03	21.20	60.12	86.83	16.44	34.91	52.66
AB2-SW10	11.41	14.97	24.90	26.40	30.06	25.83	9.71	49.49	50.97	-5.40	-0.34	51.94
AB3-SW10	-0.09	15.68	27.92	15.92	6.09	8.12	-6.94	69.67	70.27	-19.51	40.21	38.24
AB4-SW10	9.21	12.06	45.95	20.70	25.65	46.93	17.86	42.66	49.28	-7.47	20.17	22.49

[*] SW1 – *Gracilaria* spp., SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

[#] Biofilm reduction calculated according to Pitts *et al*. (2003). [¥] Negative values are indicative of an increase in attachment/biofilm formation.

[€] Differences in the BFR values among the treatment groups were statistically significant ($p < 0.05$).

The extracts' ability to decrease initial adhesion of *E. tarda* was also observed, following treatment with 1 mg/ml ($p < 0.001$), where 93% (28/30) of extracts had anti-adhesion effect (Fig. 3.5) with biofilm reduction ranging from 2.77 - 83.25%. A ≥ 50% biofilm reduction potential for 40% of extracts was also observed, however, 7% (2/30) of the extracts demonstrated increased adhesion. Following treatment with 5 mg/ml, 63% (19/30) of extracts decreased adhesion of *E. tarda* (Fig. 3.5; $p < 0.001$) with biofilm reduction ranging between 7.59 - 54.32% (Table 3.4). MAB24-SW1 demonstrated the highest reduction potential of 54.32% at this concentration.

Detachment of mature biofilm of *E. tarda* was observed with all extracts tested (Fig. 3.6), with percentage biofilm reduction ranging from 0.68 - 49.03%. None of the extracts demonstrated ≥ 50% reduction potential at 1 mg/ml (Table 3.4; $p = 0.026$). At 5 mg/ml, all extracts maintained their detachment activity ($p < 0.001$) with an increase in percentage biofilm reduction (3.96 - 75.94%). The best anti-biofilm activity was observed for extract AB5-SW6. Pronounced activity was observed for 10 mg/ml ($p < 0.001$), with a reduction range of 8.12 - 100.84%. Greater than 50% biofilm reduction was also observed for 53% (16/30) of the extracts. Extracts MAB2-SW1, MAB4-SW1, MAB5-SW1, MAB24-SW1 and AB9B-SW2 demonstrated ≥ 90% reduction potential (Fig 3.6B).

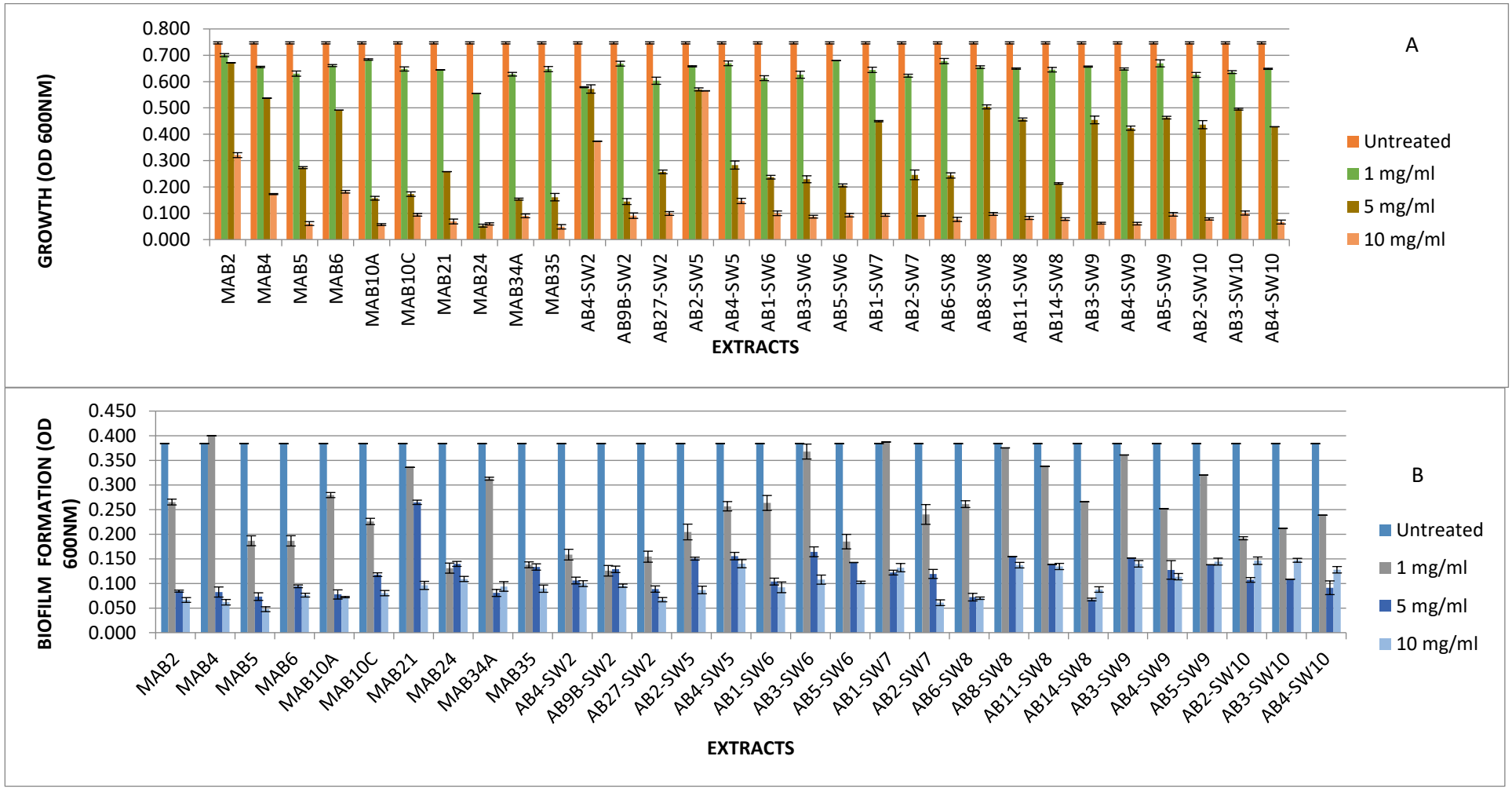

Figure 3.5: The effect of crude seaweed associated bacterial extracts on (**A**) growth and (**B**) initial adhesion of *Edwardsiella tarda* ATCC 15947 quantified by crystal violet staining using microtitre plate assay. Data represents the mean values of two independent, replicate experiments ± SD are shown. Differences in the mean values of 1 and 5 mg/ml treatment groups were statistically significant ($p < 0.001$).

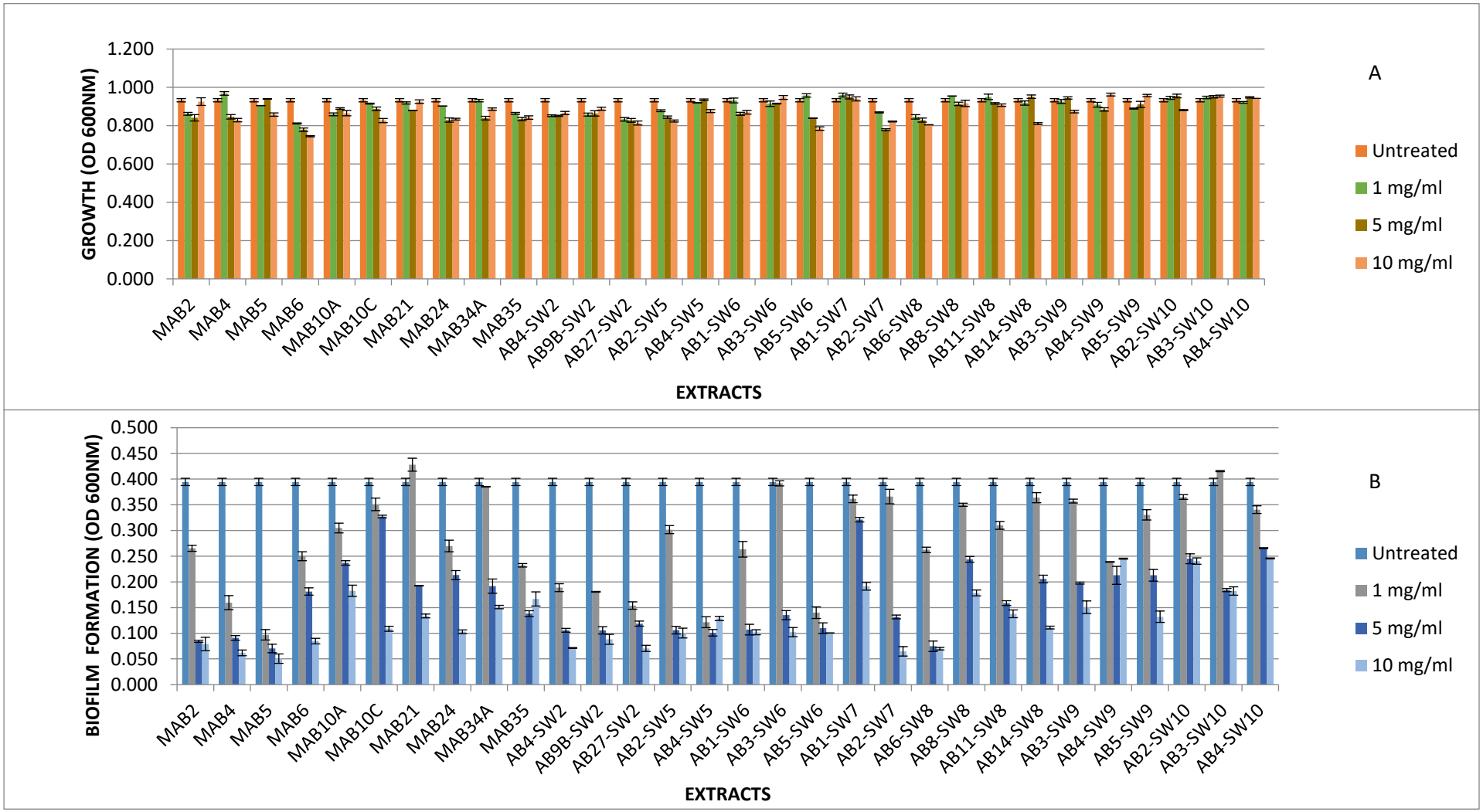

Figure 3.6: The effect of crude seaweed-associated bacterial extracts on (**A**) mature biofilm growth and (**B**) mature biofilm detachment of *Edwardsiella tarda* ATCC 15947 quantified by crystal violet staining using microtitre plate assay. Data represents the mean values of two independent, replicate experiments ± SD are shown. Differences in the mean values of 1, 5 and 10 mg/ml treatment groups were statistically significant ($p < 0.05$).

At 1 mg/ml, 40% (12/30) of extracts were noted to have anti-adhesion (Fig 3.7; $p < 0.001$)), with % biofilm reduction ranging from 39.53 - 83.47% against *V. parahaemolyticu*s. Of those extracts, 33% (10/30) demonstrated ≥ 50% biofilm reduction activity (Table 3.4). Majority of the extracts (60%; 18/30) were observed to have an antibacterial effect rather than anti-adhesion effect. A similar growth inhibitory effect was observed at 5 mg/ml for 53% (16/30) of the extracts (Fig 3.7A). However, 47% (14/30) of the extracts demonstrated ≥ 50% reduction potential and 13% (4/30) demonstrated ≥ 90% biofilm reduction (Fig. 3.7B; $p < 0.001$).

The mature biofilm of *V. parahaemolyticus* was detached by 93% (28/30) of extracts (Fig. 3.8; $p = 1.00$) with percentage biofilm reduction ranging from 0.93 - 98.48% at 1 mg/ml. Of those extracts, MAB5-SW1 demonstrated the highest reduction potential (Table 3.5). Treatment with extracts MAB21-SW1 and AB3-SW10 increased the adhesion of *V. parahaemolyticus*. Upon exposure to 5 mg/ml, all extracts displayed detachment activity ($p = 0.026$), with biofilm reduction ranging from 22.30 - 107.29%. Of these 37% (11/30) demonstrated ≥ 90% reduction potential (Table 3.6). At 10 mg/ml ($p < 0.001$), 53% (16/30) had ≥ 90% reduction potential, with MAB5-SW1 maintaining the best activity against the mature biofilm of *V. parahaemolyticus* (Fig 3.8).

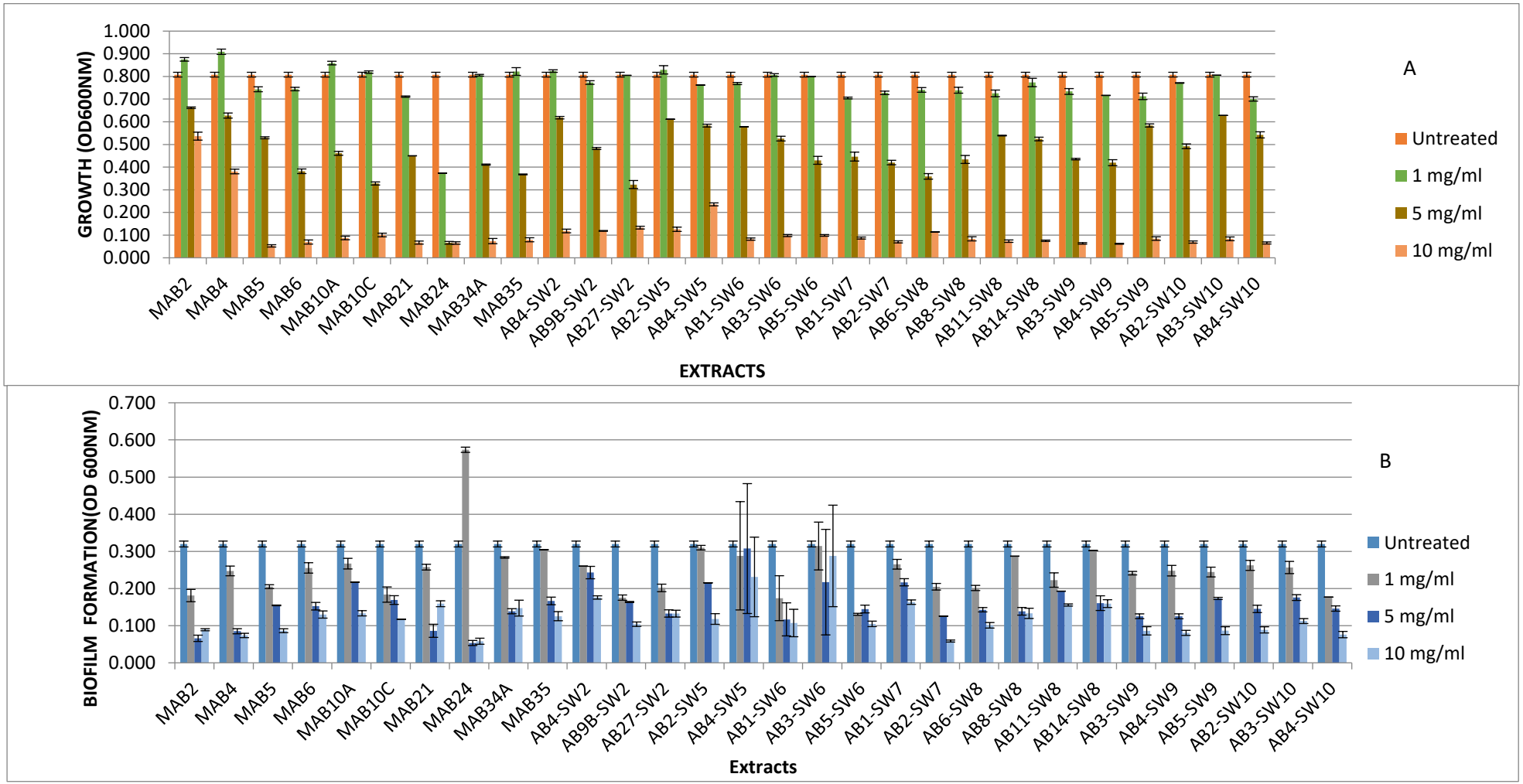

Figure 3.7: The effect of crude seaweed extracts on (**A**) growth of and (**B**) initial adhesion of *Vibrio parahaemolyticus* ATCC 17802 quantified by crystal violet staining in microtitre plate. Data represents the mean values of two independent, replicate experiments ± SD are shown. Differences in the mean values of 1 and 5 mg/ml treatment groups were statistically significant ($p < 0.001$).

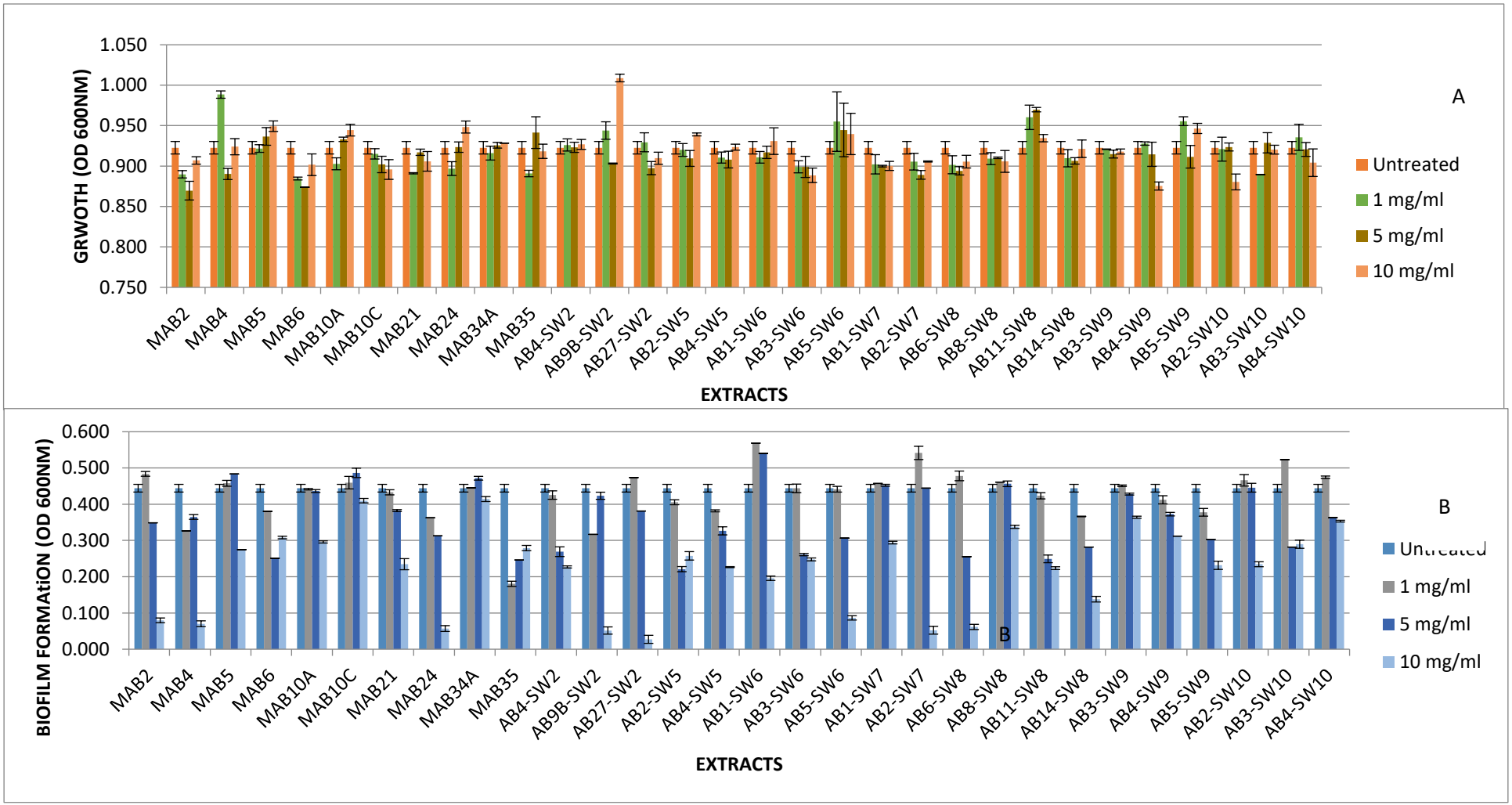

Figure 3.8: The effect of crude seaweed-associated bacterial extracts on (**A**) mature biofilm growth and (**B**) mature biofilm detachment of *Vibrio parahaemolyticus* ATCC 17802 quantified by crystal violet staining in microtitre plate assay. Data represents the mean values of two independent replicate experiments ± SD are shown. Differences in the mean values of 5 and 10 mg/ml treatment groups were statistically significant ($p < 0.05$).

Anti-adherence activity ranging from 2.2 - 71.09% was observed against *Y. ruckeri* at 1 mg/ml with 97% (29/30) of the extracts ($p = 0.556$). Biofilm reduction of ≥ 50% was observed for 16% (5/30) extracts. The activity of MAB24-SW1, however, was due to its antibacterial activity (Fig. 3.9A). At 5 mg/ml, 20% (6/30) of the extracts had a growth inhibitory effect, while 80% (24/30) decreased attachment ($p < 0.001$) with biofilm reduction ranging from 4.61 - 93.35% (Table 3.4). Biofilm reduction of ≥ 50% for 56% (17/30) extracts was observed at this concentration. Extract MAB2-SW1 was the only extract that effectively decreased adhesion with ≥ 90% biofilm reduction.

Furthermore, 53% (16/30) of extracts demonstrated weak detachment of *Y. ruckeri* (Fig. 3.10; $p < 0.001$) with percentage biofilm reduction ranging from 0.08 - 65.33%. None of the extracts had ≥ 90% reduction potential, with only MAB43-SW1 demonstrating ≥ 50% biofilm reduction activity. Inhibition was observed at 5 mg/ml with 73% (22/30) of extracts without growth inhibitory effect ($p < 0.001$). None of the extracts could effectively disperse the biofilm by ≥ 90% (Table 3.5). The most promising results were displayed at 10 mg/ml ($p < 0.001$), with percentage biofilm reduction ranging from 7.40 - 103.24% (Fig. 3.10; Table 3.5). Inhibition of ≥ 50% was noted for 53% (16/30) of extracts. Futhermore, 23% (7/30) of the extracts had ≥ 90% biofilm reduction activity. Extract AB27-SW2 was observed to completely eradicate the mature biofilm of *Y. ruckeri*.

Overall from the inhibiton of initial adherence assay, extracts demonstrated the best effect against *Y. ruckeri*, *V. parahaemolyticus*, and *A. hydrophila* at 5 mg/ml with majority of extracts demonstrating ≥ 50% biofilm reduction activity (Table 3.6). Based on the detachment assay, extracts demonstrated stronger activity against *V. parahaemolyticus* and *A. hydrophila* when compared to the other indicators. Biofilm reduction activity of ≥ 90% was noted for 53% of the extracts against *V. parahaemolyticus.* The activity of extracts was more pronounced against detachment rather than inhibition of initial adherence (Table 3.6).

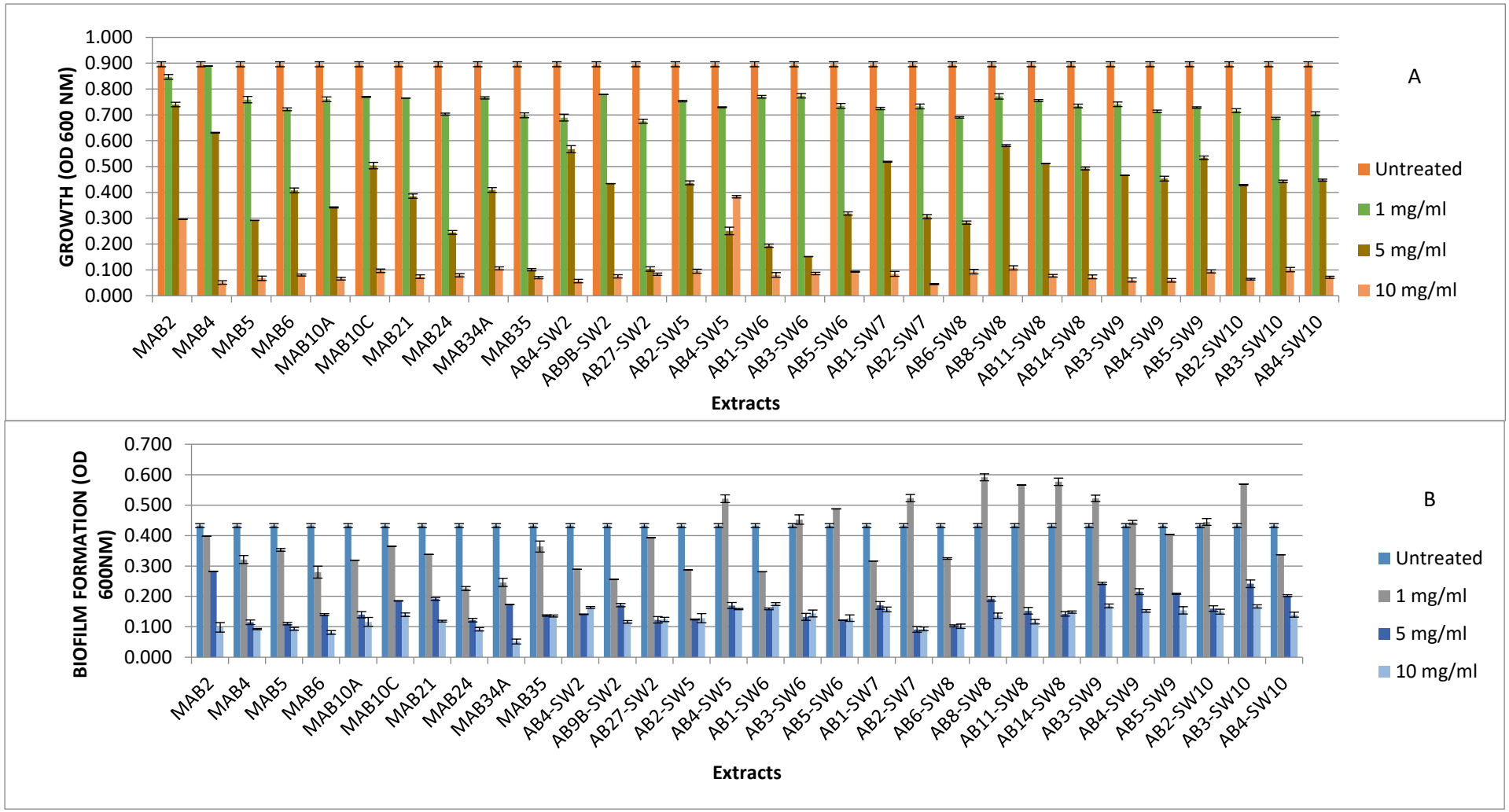

Figure 3.9: The effect of crude seaweed-associated bacterial extracts on (**A**) growth and (**B**) inhibition of adhesion of *Yersinia ruckeri* ATCC 29473 quantified by crystal violet staining in microtitre plate assay. Data represents the mean values of two independent replicate experiments ± SD are shown. Differences in the mean values of the 5 mg/ml treatment group was statistically significant ($p < 0.001$).

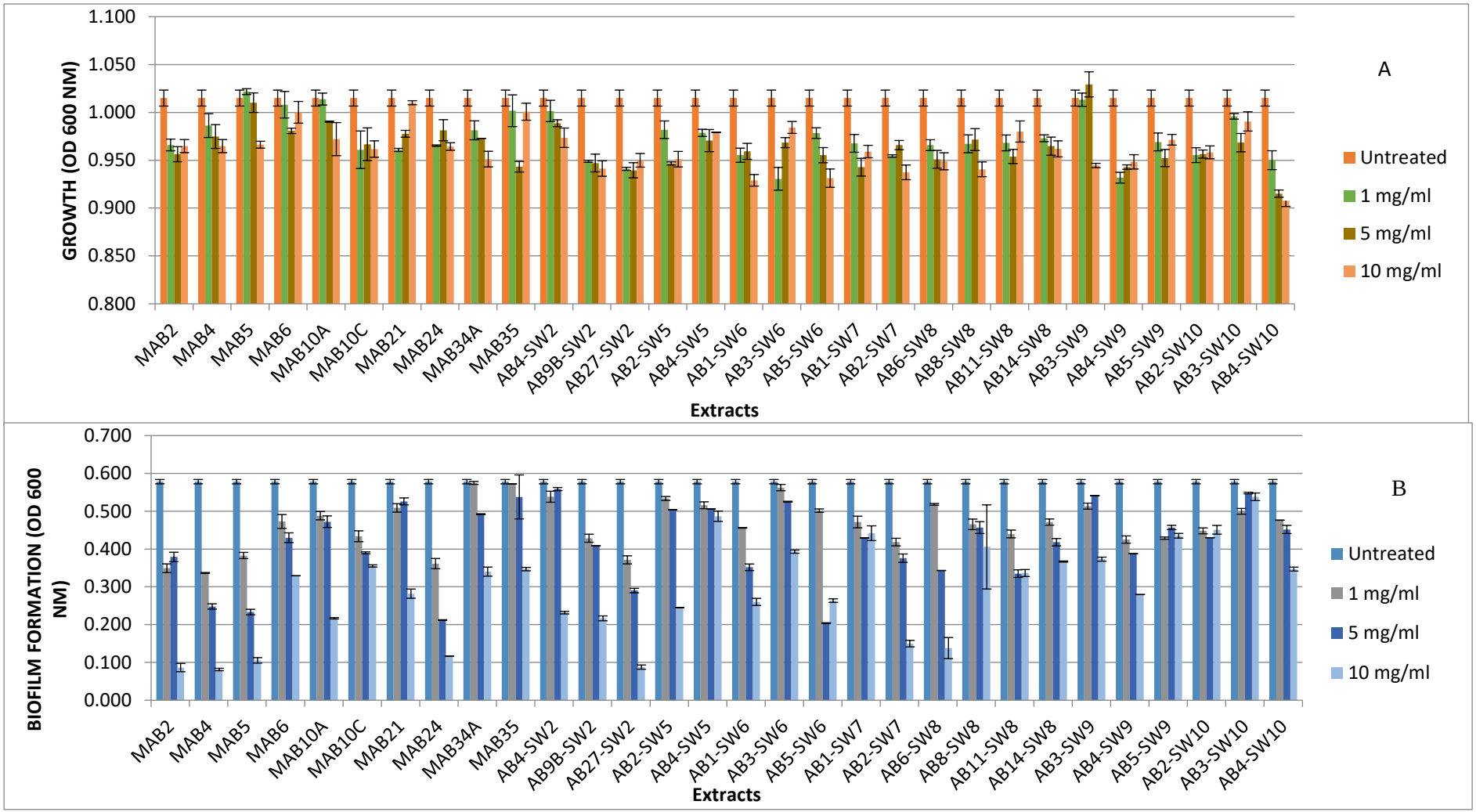

Figure 3.10: The effect of crude seaweed-associated bacterial extracts on (**A**) mature biofilm growth and (**B**) mature biofilm detachment of *Yersinia ruckeri* ATCC 29473 as quantified by crystal violet staining. Data represents the mean values of two independent replicate experiments and ± SD are shown. Differences in the mean values of 1, 5 and 10 mg/ml treatment groups were statistically significant ($p < 0.001$).

Table 3.6: Comparison of biofilm reduction potential of extracts against inhibition of initial adherence and pre-formed biofilms.

Indicator bacteria	Concentration	Percent biofilm reduction against initial adherence		Percent biofilm reduction against 24 h preformed biofilm	
		Biofilm reduction ≥ 50%	Biofilm reduction ≥ 90%	Biofilm reduction ≥ 50%	Biofilm reduction ≥ 90%
A. hydrophila **ATCC7966**	1 mg/ml	1	0	10	0
	5 mg/ml	11	2	25	0
	10 mg/ml	-	-	22	8
E. tarda **ATCC 15947**	1 mg/ml	12	0	0	0
	5 mg/ml	1	0	5	0
	10 mg/ml	-	-	16	5
V. parahaemolyticus **ATCC 17802**	1 mg/ml	10	0	9	2
	5 mg/ml	14	4	25	11
	10 mg/ml	-	-	28	16
Y. ruckeri **ATCC 29473**	1 mg/ml	5	0	1	0
	5 mg/ml	17	1	1	0
	10 mg/ml	-	-	16	7

*- Results omitted due to significant growth inhibition (≥ 50%)

3.4 Discussion

Aquaculture is a fast developing industry, which has the potential to alleviate food shortages due to its remarkable production increases. However, this growth is associated with problems, which threaten its production globally, including disease outbreaks (Vatsos and Rebours, 2015). The use of seaweed-associated bacteria as a source of new therapeutic products is of huge interest because the surface of seaweed is known as a "gold-mine" for microorganisms that produce bioactive compounds (Sugathan *et al*., 2012; Egan *et al*., 2013). The bacteria found on the surface of seaweed have anti-fouling, cytotoxic activities, antibacterial, anticoagulant and anti-virulence potential (Goecke *et al*., 2013).

Screening of marine bacteria isolated from the surface of seaweed and invertebrates has shown that a high percentage produce bioactive metabolites (Singh and Reddy, 2014). To the best of our knowledge, there is no previous report on the antibacterial and anti-biofilm activity of seaweed-associated bacterial strains isolated from the Durban coast against aquaculture pathogens. In the present study, antagonism by seaweed-associated bacteria was indicated by interruption in the growth of the tested aquaculture pathogens. Antimicrobial activity of seaweed-associated bacteria was more pronounced against *A. salmonicidia, E. tarda* and *V. parahaemolyticus. Bacillus pumilus* isolated from brown algae *Padina pavonica*, displayed the largest spectrum of growth inhibition against a panel of 12 pathogenic bacteria (Ismail *et al*.,

2016). Thilakan *et al*. (2016) observed that 22% of bacteria from two major phyla *Firmicutes* and *Proteobacteria* were active against at least one tested pathogen (*A. hydrophila, V. alginolyticus, V. anguillarum, V. harveyi, V. parahaemolyticus,* and *V. vulnificus*). Among the isolates in the present study, MAB24-SW1, a *B. velezensis* isolate exhibited the highest level of antimicrobial activity against all pathogenic aquaculture indicators. *Bacillus velezensis* isolated from wheat anthers has been reported to possesses antibacterial activity against clinical and plant pathogens due to the synthesis of bioactive compounds such as lantibiotic and ericin, which are unique to this strain (Palazzini *et al*., 2016). Palazzini *et al.* (2016) reported the production of ericin by a *B. velezensis* strain, which demonstrated antagonist activity against *Fusarium graminearum* plant pathogen. Furthermore, genome mining of *B. velezensis* has identified polyketides and non-ribosomal synthetase genes, which are responsible for synthesis of structurally novel bioactive compounds with potential pharmaceutical application (Kadaikunnan *et al*., 2015). There are a limited number of studies that have reported on the antibacterial potential of the *B. velezensis* against aquaculture pathogens, as most studies have focused on its biocontrol properties for agriculture use (Borriss *et al*., 2011; Dunlap *et al*., 2015). Cao *et al*. (2011) reported the isolation of *B. amyloliquefaciens* with antibacterial activity against aquaculture *A. hydrophila* isolate The close relatedness of *B. velezensis* to *B. amyloliquefaciens* (Wang et al., 2008) and results obtained in this study demonstrate the potential of the isolate to control disease outbreaks in aquaculture settings.

The misuse of antibiotics has given rise to the emergence of antibiotic resistant bacteria in the aquaculture industries (Huang *et al*., 2015). To overcome the continuous emergence of antibiotic resistance pathogens due to abuse of antibiotics in aquaculture, an alternative to antibiotics is urgently needed for disease prevention and treatment. (Tan *et al*., 2016). Probiotics could be a promising alternative to antibiotics in aquaculture. Benefits of using probiotics include improved immune response, growth and water quality (Dharmaraj and Rajendren, 2014). *Bacillus* spp. and actinomycetes have shown great potential as probiotics due to their ability to produce inhibitory compounds (Prieto *et al*., 2014). The seaweed-associated bacteria in this study were predominantly *Bacillus* and *Streptomyces* spp. The production of a variety of wide-spectrum antagonistic and antimicrobial chemical compounds by *Streptomyces* can be valuable as probiotics in aquaculture (Tan *et al*., 2016). *Bacillus* and *Streptomyces* species identified in this study thus have potential use as probiotics in aquaculture. Furthermore, the activity demonstrated by isolates as a result of production of various antagonistic compounds (e.g. anti-biofilm, anti-quorum sensing and antibacterial) suggests that the use of these isolates could influence the growth of aquaculture pathogens.

Silvia *et al*. (2015) evaluated and reported the positive effect of probiotic *B. amyloliquefaciens* on the growth performance and intestinal morphometry in Nile tilapia (*Oreochromis niloticus*) reared in cages. The action of seaweed-associated bacterial could potentially regulate microflora by outcompeting pathogenic organisms thus enhancing the growth performance of livestock. Probiotics which produce siderophores are reported to limit the bioavailability of iron which is essential for growth and biofilm formation (Tan *et al*, 2016).

Biofilm-associated bacteria are a very serious problem in many infections because they show an innate resistance to antibiotics. Biofilms form on a variety of surfaces including medical implants, water systems and living tissue (Pandey *et al*., 2014). The attachment and growth of these biofilms serve as reservoirs for opportunistic bacteria, which leach out into the aquaculture systems (Natrah *et al*., 2011). Steps to control biofilm formation and dispersion of already formed biofilms are important for the development of commercially viable aquaculture industries (Defoirdt, 2014). Procedures which included frequent cleaning and sanitization of tanks and water systems, prove not to be feasible as biofilm formation resumes soon after, thus more practical methods are required (Defoirdt *et al*., 2011). When bacteria adhere to a surface, growth occurs rapidly and during the first few hours the adhesion is reversible (Singh and Nakayama, 2015). Hence, preventing bacterial adhesion at the preliminary stage itself can reduce the risk of biofilm formation.

Although several reports describing antibacterial activity of seaweed-associated bacteria are available in literature, studies on the anti-biofilm potential of these isolates are very limited (Ben Ali *et al*., 2012). To provide a strategy for biofilm prevention, control, and eradication, extracts from 30 potential quorum quenching seaweed-bacterial isolates were screened against aquaculture indicators, which utilize AHL-autoinducers as part of their virulence and survival strategies (Reuter *et al*., 2015). Seaweed-bacterial extracts inhibited initial adherence $\geq 50\%$ when a concentration of 5 mg/ml was tested. The extracts demonstrated the best effect against *V. parahaemolyticus,* followed by *Y. ruckeri* and *A. hydrophila*. Although the interaction of extracts with bacterial cells is not fully understood, the inhibition effect against initial adherence indicate that they could possibly be disrupting the first attachment stages which include communication, motility, attachment and colonization (Chu and Mclean, 2016). Quorum sensing is involved in regulation of virulence of pathogens in aquaculture. Virulence factors of the opportunistic pathogen include biofolm formation, extracellular toxin and siderophore production (Zhoa *et al*., 2015*).* Due to the close association between QS system and virulence expression, disruption of QS is implicated in decreasing the pathogenicity of strains (Pande *et al.,* 2013). *Vibrio* species, such as *V. cholerae, V. vulnificus*

and *V. anguillarum* utilize autoinducer AI-2, a furanosyl borate diester for QS-mediated virulence expression (Zhoa *et al*., 2015). Thus, it may be possible that the extracts degraded AI-2 and down-regulated the expression of QS signals which are necessary for biofilm formation and expression of virulence factors. Teasdale *et al*. (2011) isolated three *Bacillus* spp. from green seaweed, which demonstrated to QS inhibition against *V. harveyi* BB120. The present data also indicate that extracts could be exerting anti-biofilm activity at different stages of biofilm formation depending on the target bacterium. It is possible that crude extracts contain several different anti-biofilm molecules such as acylases, lactonases, oxidoreductases and/or small QSI compounds with complementary activities (Zhang *et al*., 2015). Nithya and Pandian (2010) also reported on the activity of a marine isolate, which effectively dispersed mature biofilm of *Vibrio* spp., while Rodrigues *et al*. (2015) reported on the activity of a marine *Pseudoalteromonas* sp. 3J6 which inhibited and dispersed biofilm of *V. tapetis*.

Furthermore, extracts were tested for their ability to disperse mature biofilms. The survival of pathogenic organisms even after treatment with antimicrobial agents is enhanced if the cells exist in biofilms rather than as planktonic cells. Pronounced activity of the extracts was observed against the mature biofilm of *A. hydrophila and V. parahaemolyticus.* Response to treatment was also observed to be indicator-dependent. *Aeromonas hydrophila* causes septicaemia in fish and serious damage to the aquaculture industry (Defoirdt, 2016). Quorum sensing signals N-butanoyl-L-homoserine lactone (C4-HSL) and N-hexanoyl-L-homoserine lactone (C6-HSL) are utilized by *A. hydrophila* to establish virulence. Degradation of QS signals of *A. hydrophila* would yield an anti-virulence effect against the strain (Zhoa *et al*., 2015). It is envisaged that the activity of tested extracts could be correlated to the production of quorum quenching enzymes such as acylases and lactonases. *Streptomyces* sp. make use of acylases PvdQ and AhlM, respectively, which are active against degradation of AHL chains longer than and shorter than 8 carbons (Safari *et al*., 2014). Tan *et al*. (2016) reported the anti-biofilm activity demonstrated by *Streptomyces* A66 through the degradation of the QS factor N-AHSL (N-acylated homoserine lactone). Many *Bacillus* species are efficient producers of bioactive compounds and therefore, are dominant colonizers of seaweed surfaces. *Bacillus* spp. synthesize lactonases, such as AiiA, which hydrolyze the ester bond of the AHL molecules yielding N-acyl-homoserine (Tang *et al*., 2013). As QS is a density-dependent system, degradation of AHL reduces their concentration and this leads to the inactivation of QS and subsequently biofilm formation. Chu *et al*. (2014) reported the AHL-degrading *Bacillus* sp. QSI-1, which was able to increase the survival zebrafish *Danio rerio* infected with *A. hydrophila* YJ-1, by decreasing biofilm formation (77.3%) and protease production (83.9%).

Biofilm-inhibiting activity of the bacterial extracts observed in the current study without any antibacterial activity may have an impact in controlling biofilm-mediated infections. Interfering with biofilms is expected to overcome resistance because the effect is not lethal to the bacterium and would only restore sensitivity to antibiotics or allow clearance by the host immune system. Furthermore, utilization of seaweed-associated bacteria as potential probiotic microorganisms to control disease in aquaculture is encouraging, as they demonstrate potential to disrupt the QS systems of pathogens. Extensive trials are needed to establish whether these seaweed-associated bacteria would be effective in disease prevention and growth enhancement of aquaculture animals. A better understanding is needed on the exact mode of action of seaweed-associated bacteria. Hence, further research could focus more on molecular techniques to elucidate the possible underlying mechanisms whereby these isolates would operate in aquaculture settings.

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CHAPTER FOUR

QUORUM SENSING INHIBITORY POTENTIAL OF SEAWEED-ASSOCIATED BACTERIAL EXTRACTS AGAINST GRAM-POSITIVE QUORUM SENSING REGULATORY SYSTEMS.

Abstract

Staphylococcus aureus and *Enterococcus faecalis* are amongst the most frequent causes of a wide range of hospital- and community-acquired infections. The emergence of drug-resistant Gram-positive organisms such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) presents serious challenges due to the limited choice of effective treatments. Since Gram-positive bacteria employ quorum sensing to express their virulence, it can be targeted for the development of anti-virulence agents that can suppress virulence without influencing cell growth. Since seaweed harbor microorganisms, which are capable of producing novel compounds they are a valuable source of potential anti-virulence compounds.

The inhibitory effects of 60 seaweed-associated bacterial extracts on phospholipase production was assessed using the phospholipase plate assay. To identify quorum sensing inhibitors (QSIs) that target *agr*/*fsr* systems, an *S. aureus agr* reporter strain that carries luciferase and green fluorescence protein genes under the *agr* P3 promoter was utilized, while *E. faecalis fsr* system inhibition was assessed using gelatinase-activity. Inhibition of phospholipase was not detected with all extracts tested. When tested against luminescence and green protein fluorescence (GFP) reduction in the *agr* assay, crude extracts of MAB6-SW1 (*Rhodococcus fascians*), MAB10B-SW1 (*Bacillus* sp), MAB24-SW1 (*Bacillus velezensis*), AB7-SW8 (*Streptomyces* sp.), AB1-SW9 (*Microbacterium maritypicum*) and AB4-SW10 (*Strepyomyces labedae*) were capable of quorum sensing inhibition without causing cell death, with some isolates demonstrating ≥ 50% inhibition of GFP. When tested against *Enterococcus* virulence factor under the control of the *fsr* QS system (gelatinase production), AB2-SW8 (*Streptomyces* spp.) demonstrated wide-spectrum QSI potential against both *fsr* and *agr* systems without causing cell death. Seaweed-associated bacteria serve as potential producers of diverse compounds with potential anti-virulence activity for the treatment of MDR Gram-positive infectious diseases. These bacteria are potential candidates for identifying novel QSI compounds from natural resources.

4.1 Introduction

One of the greatest achievements of modern medicine has been the discovery of antimicrobial agents for the treatment of infectious diseases. Traditional treatment of infectious diseases was previously based on compounds that aim to kill or inhibit bacterial growth (Giannakaki and Miyakis, 2012). A major concern with this approach is the frequently observed development of resistance to antimicrobial compounds. Infections caused by multidrug-resistant (MDR) Gram-positive bacteria represent a major public health concern, not just in terms of morbidity and mortality, but also in terms of increased expenditure on patient management and implementation of infection control measures (Gupta *et al*., 2016).

Staphylococcus aureus and *Enterococcus* spp. are established pathogens in the hospital environment, and their frequent multidrug resistance complicates effective treatment of infections (Vazquez-Guillamet and Kollef, 2014). Methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecalis* strains have emerged, with these clinical isolates being reported and isolated from infections, which have proved difficult to treat (Kalia, 2013). These organisms are now a serious epidemic in healthcare settings and are linked to increased rates of illness particularly in intensive care units and death (Painter *et al*., 2014). A global post-antibiotic era is currently faced with decreased efficiency of antibiotics thus the development of novel therapeutic approaches for the treatment of bacterial infections constitutes an urgent need in research (Defoirdt, 2016).

The ability of these Gram-positive isolates to invade host tissue and cause infections is due to the expression of virulence factors such as toxins, adhesins and immune evasins (Painter *et al*., 2014). Expression of virulence factors is under regulatory control of quorum sensing (QS) systems. Several studies have demonstrated that QS is essential for the establishment of infections by Gram-positive organisms, in particular *S. aureus* and *E. faecalis* (Harris *et al.*, 2013*;* Nielsen *et al.*, 2014*;* Painter *et al.*, 2014*).* Therefore, there is considerable need for the discovery of anti-QS drugs to be utilized in therapeutic settings. In recent years anti-QS has focused on Gram-negative organisms (Padmavathi *et al*., 2014; Torres *et al*., 2016) and there are fewer reports on inhibition of QS as a mechanism to control Gram-positive organisms (Gray *et al*., 2013). This is due to the fact that the enzymes responsible for auto-inducer synthesis, such as ribosomes and peptidases, are commonly essential for the growth and survival of the bacterial cells (Desouky *et al*., 2013).

Compounds able to override bacterial signaling are present in nature. Seaweed-associated bacteria secrete biologically active, beneficial compounds that regulate the morphogenesis of marine organisms and help them survive under varied environmental

conditions such as QS inhibition through metabolite production by the host systems (Egan *et al*., 2013). The nutrient-rich seaweed surface constitutes a competitive environment that induces microorganisms to synthesize bioactives in order to ensure their dominant positions on the host, leading to the biosynthesis of structurally novel compounds (Singh *et al*., 2013). Mansson *et al*. (2011) investigated crude extracts and fractions from a marine *Photobacterium*, which led to the identification of two novel depsipeptides, solonamides A and B, solonamide B, which interfere with *agr* in *S. aureus* 8325-4 and USA300 community-acquired MRSA (CA-MRSA) strain, respectively. Quorum sensing inhibitory action of ambuic acid was observed in *S. aureus* and other Gram-positive bacteria. The compound affected the biosynthesis of cyclic peptide (AIP) thus leading to discovery of ambuic acid as a broad spectrum anti-QS activity (Nakayama *et al*., 2013). Compounds with such abilities are termed anti-virulence drugs as opposed to antibacterial drugs (i.e., most traditional antibiotics). Anti-virulence drugs target key regulatory bacterial systems that govern the expression of virulence factors. An alternative to antibiotic action is attenuation of bacterial virulence such that the organism fails to establish successful infection (Lee *et al*, 2014). The observation that quorum sensing is linked to virulence factor production and biofilm formation suggests that many virulent organisms could potentially be rendered non-pathogenic by inhibition of their QS systems (Kalia, 2013). Research into QS, and inhibition thereof, may provide a means of treating many common and damaging chronic infections without the use of growth-inhibitory agents, such as antibiotics, preservatives, and disinfectants, that unavoidably select for resistant organisms (Quintana *et al*., 2015). This current study places emphasis on anti-QS ability of seaweed-associated bacterial extracts as therapeutic means to combat MDR Gram-positive infections.

4.2 Materials and methods

4.2.1 Bacterial isolates

Ninety-six bacterial strains were previously isolated from ten South African intertidal seaweeds (*Amphiroa bowerbankii* Harvey, *Cheilosporum cultratum* Areschoug (Harvey), *Codium duthieae* (P. Silva), *Codium* spp., *Gelidium pteridifolium* (R.E, Norris, Hommersand & Fredericq), *Gracilaria* spp., *Jania verrucosa* (Lamourous), *Laurencia brongiartii* (J. Agarah), *Ulva rigida* (C. Agarah) collected along the coastline of Durban, South Africa. Based on preliminary antimicrobial and anti-biofilm results, 60 bacterial isolates were selected for fermentation and ethyl acetate extraction of secondary metabolites.

4.2.2 Fermentation and ethyl acetate extraction

Isolates were pre-cultured in 5 ml of International *Streptomyces* Project media 2 (ISP2; Shirling and Gottlieb 1966) broth for 2 d, and then inoculated in 250 ml of ISP2 broth and incubated with shaking for 7 d at 30 °C. Bacterial cells were pelleted at 9500 rpm for 10 min to collect supernatants. An equal volume (1:1) of ethyl acetate was added to each cell-free supernatant followed by agitation for 1 h at 30 °C. The ethyl acetate layer was collected and then subjected to a second extraction (1:1 volume ethyl acetate) with agitation of flasks for 4 h after which the ethyl acetate layer was collected. Ethyl acetate was evaporated in a rotary evaporator (Ilmvac, ROdist digital 230V 50/60Hz) and each crude extract obtained was weighed (Nithya *et al*., 2011). Thereafter, crude extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml.

4.2.3 Detection of anti-quorum sensing activity

4.2.3.1 Phospholipase assay

Phospholipase plate assay was conducted with some minor modifications (Kouker and Jaegar, 1987). Olive oil (1%) and Rhodamine B (0.001% w/v) was used as a substrate. Rhodamine B was dissolved in distilled water and sterilized by filtration. Growth medium contained (per liter): 8 g nutrient broth; 4 g sodium chloride and 10 g agar. The medium was adjusted to a pH 7.0 autoclaved and cooled to 60 °C, following which 31.25 ml of 1% olive oil and 10 ml 0.001% w/v of Rhodamine B solution were added with vigorous stirring and emulsified by mixing for 1 min. The medium was allowed to stand for 10 min at 60 °C to reduce foaming.

Eighteen-hour cultures of methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA) were grown in trypticase soy agar (TSA) with the presence of extracts (0.5, 0.75 and 1 mg/ml). Cells were pelleted by centrifugation at 10,000 rpm for 10 min and the cell-free supernatant harvested and filtered through a 0.2 µm filter, and then added to the 6 mm punched holes made in the phospholipid agar plate. Following an incubation period of 24 h, plates were exposed to UV irradiation and photographed. Orange halos were indicative of lytic activity of a quorum sensing inhibition (QSI) negative extract. Supernatants from QSI-positive cultures exhibited limited to no lytic effects and no orange halo (Kouker and Jaeger, 1987).

4.2.3 *Staphylococcus aureus agr* inhibition assay

Staphylococcus aureus agr reporter strain 8325-4 (pSB20035) (kindly provided by Prof. Paul Williams, University of Nottingham) and *S. aureus* ATCC 12600^{T} were cultured overnight in LB broth at 37 ˚C with gentle agitation (Desouky *et al*., 2013). An overnight culture of *S.*

aureus 8325-4 (pSB20035) and *S. aureus* ATCC 12600^T were diluted 1:50 into 200 µl of fresh LB broth, containing 5 µl of the culture extract to be tested then the mixture was dispensed into flat clear bottom, white 96-well microtiter plate. Microplates were agitated at 120 rpm at 37 ˚C. *S. aureus* 8325-4 (pSB20035) and *S. aureus* ATCC 12600^T were used as positive and negative controls, respectively (Fig. 4.1).

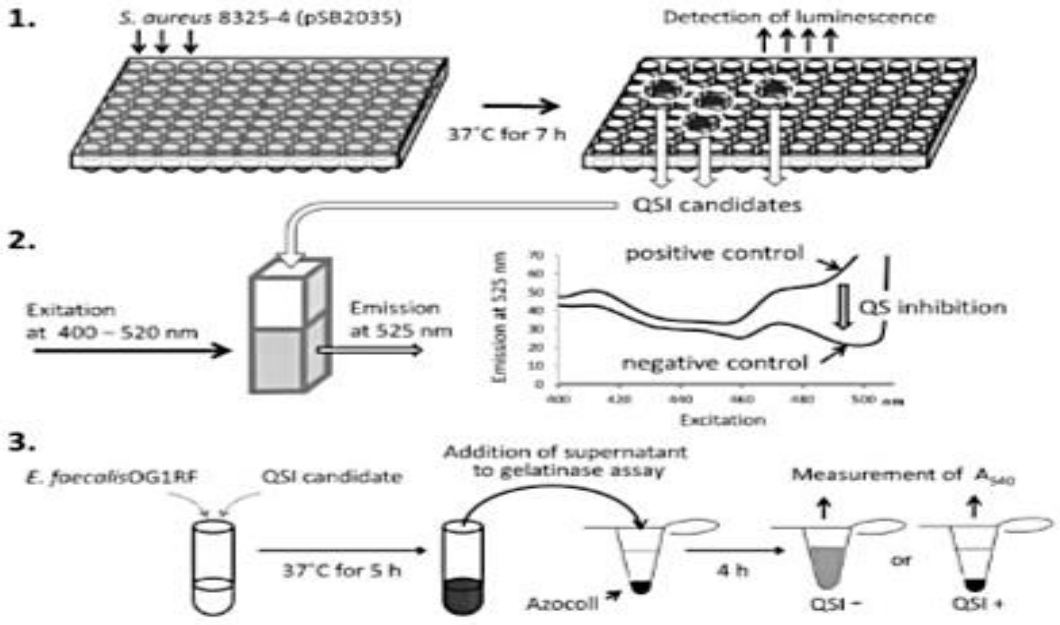

Figure 4.1: Schematic representation of the Three-Step high throughput system for *agr/fsr* QS inhibitors. Step 1: *Staphylococcus aureus* 8325-4 (pSB2035) is incubated with extracts in 96-well microtitre plate its growth and luminescence are monitored. Step 2: Bacterial cells are harvested from the positive wells and fluorescence is measured after washing. Step 3: *Enterococcus faecalis* OG1RF is cultured with positive samples from step 2, and the gelatinase activity in the culture supernatant is measured by azocoll assay (Desouky *et al*., 2013).

Growth readings and luminescence were taken after 7 h incubation using a microtitre plate reader (Promega Glomax Multi+ Detection System). The OD_{600} and luminescence were taken after 7 h incubation. If the OD was less than 50% of the positive control, the sample was judged to have growth inhibitory activity and removed from the QSI test. The induction level of luciferase was calculated by subtracting the luminescence of the negative control from the positive control *S. aureus* ATCC 12600^T. Extracts that reduced the induction level to less than 50% were considered to be QSI positive. Fluorescence was measured by transferring sample to 1.5 ml microtube and cells harvested by centrifugation at 13000 × *g* for 2 min. The supernatant was discarded and the cells washed with 200 µl of phosphate buffered saline (PBS). After repeating the washing twice, the cells were suspended in PBS and dispensed into each well of black walled microtitre plates. Then the fluorescence was measured using Promega

Glomax Multi+ Detection System at an excitation wavelength of 485 and emission wavelength of 535 nm. The induction level of GFP was calculated by subtracting the F485-F535 value of the negative control. The full induction level was calculated by subtracting the F485-F535 value of the negative control from that of the positive control. The inhibitory effect was evaluated by dividing the induction level by the full induction level, and less than 50% was taken to represent significant inhibition. (Desouky *et al*., 2013).

4.2.4 *Enterococcus faecalis fsr* inhibition assay

Enterococcus faecalis fsr inhibition assay was conducted according to Desouky *et al*. (2013) with some minor modifications. *Enterococcus faecalis* OG1RF was cultured in Todd Hewitt Broth (THB) overnight at 37 ˚C with gentle agitation. Samples to be tested for *fsr* QSI were dispensed into 1.5 ml microtubes. An overnight culture of *E. faecalis* OG1RF was diluted 1:50 in fresh THB and the culture was dispensed into 1.5 ml microtube containing 5 µl of sample. Tubes were incubated for 24 h at 37 ˚C with agitation at 120 rpm. Following incubation, 200 µl of the culture was dispensed into each well of 96-well flat bottom microtiter plate and growth measured at 600 nm optical density (GloMax multi-detection systems-Promega). The rest of the culture in the microtube was centrifuged (Prism-microcentrifuge-Labnet) at 9,100 × *g* for 5 min. Eight hundred microliters of azocoll solution was dispensed into a new 1.5 ml microtube and pre-incubated by shaking at 37 ˚C for 15 min (Desouky *et al*., 2013). Forty microliters of *E. faecalis* OG1RF culture supernatant was collected and added to azocoll suspension, and mixture was incubated with shaking (120 rpm) at 37˚C for 24 h. After 24 h, the azocoll suspension was centrifuged at 20,400 × *g* for 5 min. Two hundred microliters of the supernatant was dispensed into the wells of a 96-well microtitre plate, and the absorbance measured at OD_{560}. The inhibitory effect was evaluated by dividing the induction level by the full induction level and less than 50% was be taken as significant inhibition.

4.3 Results

4.3.1 Screening of phospholipase activity

To evaluate whether extracts could inhibit extracellular phospholipase production, a plate-based assay was conducted using Rhodomine B as an indicator. Using MRSA ATCC 43300 without treatment as a reference, the inhibitory effects of the seaweed-associated bacterial extracts on phospholipase production was observed. At all concentrations tested (0.5, 0.75 and 1 mg/ml), orange halos which are indicative of phospholipase activity were observed. Although

concentration was varied the orange halos did not decrease with increasing concentration and could be clearly observed after 24 h incubation (Fig 4.2).

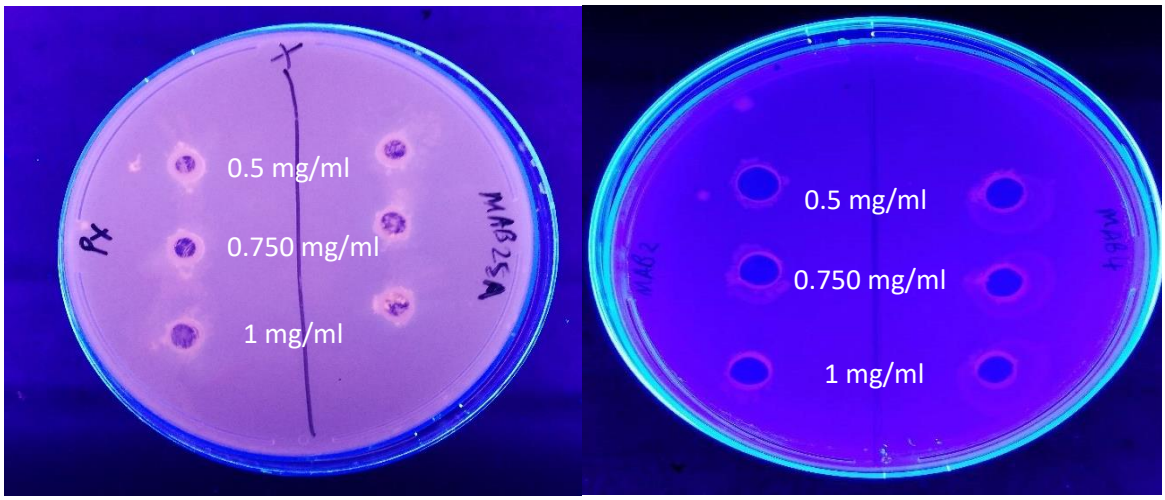

Figure 4.2: Phospholipase plate assay demonstrating lipase activity in the presences of crude seaweed-associated bacterial extracts **A**) *S. aureus* ATCC 43300 control and MAB25A-SW1 demonstrating presence of lytic activity **B**) presences of orange halos after treatment with MAB2-SW1 and MAB4-SW1.

4.3.2 Screening inhibitors targeting *agr* and *fsr* QS systems

To screen seaweed-associated bacterial extracts targeting the *agr* and *fsr* QS systems, a three-step system was utilized. Prior to analysis, the growth inhibitory effect of extracts was assessed in order to ascertain that QS inhibition was not due to growth inhibitory effect. None of the 60 extracts demonstrated a growth inhibitory effect. At the first and second steps, *S. aureus* 8325-4 encoding luminescence and GFP genes under the QS control was used. A total of 60 seaweed-associated bacterial extracts were tested, of which 27% (16/60) decreased the luciferase level to < 50% of the positive control (Table 4.1). Those extracts were taken further to validate the QS inhibitory potential by reflecting the promoter activity through GFP in the second step. Extracts from MAB6-SW1 (*Rhodococcus fascians*), MAB10B-SW1 (*Bacillus* spp.), MAB24-SW1 (*Bacillus velezensis*), AB7-SW8 (*Streptomyces* sp.), AB1-SW9 (*Microbacterium maritypicum*) and AB4-SW10 (*Streptomyces labedae*) maintained their inhibitory activity (Fig 4.2). The bacterial extracts were further subjected to the third step which tested the ability to inhibit the biosynthesis of gelatinase by *E. faecalis* OGIRF. Only the bacterial extract from AB2-SW8 (*Streptomyces* spp.) decreased induction of gelatinase to less than 50%. The results are summarized in Table 4.2.

Table 4.1: Effect of seaweed-associated bacterial extracts targeting the *agr* and *fsr* regulatory systems.

Step	**Screening Method**	**# of positive samples**	**% of positive samples**
1st	Inhibition of *agr* system reflected by luciferase (<50%)	16	27
2nd	Inhibition of *agr* system reflected by GFP (<50%)	6	10
3rd	Inhibition of gelatinase induction through the *fsr* system (<50%)	1	2

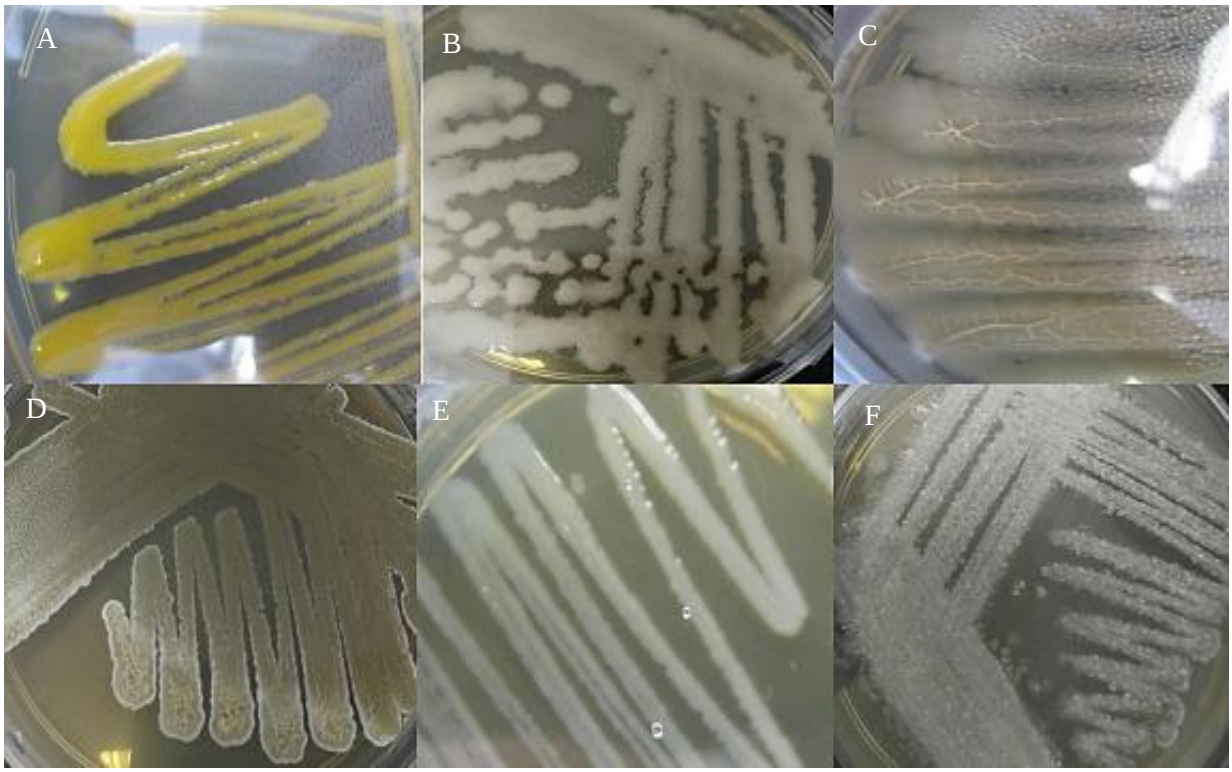

Figure 4.3: Seaweed-associated bacteria (**A**) MAB6-SW1 - *Rhodococcus fascians*, (**B**) MAB10B-SW1 - *Bacillus* spp., (**C**) MAB24-SW1 - *B. velezensis*, (**D**) AB7-SW8 - *Streptomyces* spp., (**E**) AB1-SW9 - *Microbacterium maritypicum* and (**F**) AB4-SW10 - *Streptomyces labedae*, which demonstrated inhibition of *agr* system reflected by green fluorescence protein.

Table 4.2: Quorum sensing inhibition activity of screened seaweed-associated bacterial extracts

	Expression level in the presence of extract		
Extract	**Luciferase[*] (*agr*)**	**GFP[a] (*agr*)**	**Gelatinase[#] (*fsr*)**
MAB2-SW1	44.20	95.42	75.13
MAB6-SW1	62.93	48.86	116.91
MAB10B-SW1	90.77	43.51	66.52
MAB10C-SW1	41.06	65.25	103.07
MAB24-SW1	33.55	14.63	77.25
AB5-SW2	46.96	95.45	-[-]
AB6-SW2	45.99	109.64	-
AB12-SW2	43.23	79.44	107.75
AB3-SW5	40.20	119.98	-
AB6-SW5	42.39	143.78	70.23
AB7-SW5	26.49	72.25	85.32
AB8-SW5	38.14	83.82	65.31
AB3-SW6	47.67	73.04	-
AB1-SW8	49.73	84.62	78.94
AB2-SW8	32.10	76.87	43.01
AB7-SW8	41.04	42.86	61.01
AB9-SW8	46.55	90.33	79.43
AB1-SW9	28.53	48.28	130.89
AB4-SW10	52.02	41.75	100.92

-Results unavailable. [*]The expression of luciferase and GFP was controlled by the *agr* system in reporter strain *S. aureus* 8325-4 (pSB2035). [#]The expression of gelatinase was controlled by the *fsr* system in indicator strain *E. faecalis* OG1RF.

4.4 Discussion

The rise of mortality and morbidity rates due to previously treatable infectious has created a serious epidemic in the health care and food sectors. The ability of microorganisms such as *S. aureus*, *P. aeruginosa* and *E. faecalis* to evolve mechanisms of antimicrobial resistance and poor management of infections has been the main contributor (Bhardwaj *et al*., 2013). Therapeutic options are limited to the use of antibiotics, which, also contributes to antibiotic resistance. As treatment options run dry, there is an urgent need for discovery of alternative options (LaSarre and Federle, 2013). The discovery of QS systems and their role in virulence has now shifted the focus of research to anti-virulence compounds, which may be useful alternatives to antibiotics (Painter *et al*., 2014).

Research into QS inhibition has focused more on Gram-negative pathogens, and less on Gram-positive due to the fact that most pathogenic organisms are Gram-negative (Zhao *et al*., 2015; Padmavathi *et al*. 2014). This has led to an increase in drug resistant Gram-positive pathogens with limited treatment options (Kalia, 2013). *Stapylococcus aureus* causes of a wide range of nosocomial and community-acquired infections (Shojima and Nakayama, 2014). Stapylocooccal QS is encoded by the *agr* locus and is responsible for the production of δ-

hemolysin, lipase, proteases and enterotoxins, which contribute to its virulence (Nakayama *et al*., 2013). Thus the inhibition of QS is seen as a viable option to decrease the pathogenicity of Gram-positive pathogens (Singh *et al*., 2016).

The lack of phospholipase inhibition in this study could be linked to the low concentration of extract, which was utilized. However, upon further screening of seaweed-associated bacterial extracts, 10% displayed QSI against the *S. aureus* reporter strain. This could suggest that the assay is not very sensitive or the presence of potential AIP-interfering molecules in the crude extracts. Inhibition of auto-inducer biosynthesis is regarded as the first preference as it is the starting point in QS signal circuit. Two enzymes, AgrB and SpsB are known to be involved in the biosynthesis of AIP (Singh *et al*., 2016). These are processing enzymes with protease activity and degradation is expected to block QS systems. However, enzymatic inhibition has a growth inhibitory effect, which is a contributory effect to the raise of MDR and therefore has to disregarded (Kalia, 2013; Singh *et al*., 2016). This explains the difficulty in obtaining natural QSI compounds and the low data hits which were obtained in this study. Blockage of AgrC-AgrA two component regulatory system and utilization of AIP antagonists would, therefore, be the preferred QSI method (Singh *et al*., 2016).

To the best of our knowledge, there are no reports on seaweed-associated bacterial extracts, which inhibit Gram-positive QS, making this study amongst the first to report on QSI of Gram-posistive organisms. On screening of 60 seaweed-associated bacterial extracts, six appeared to have QSI activity against *S. aureus* reporter strain as reflected by GFP inhibition. These results reflect those of Desouky *et al*. (2013) who reported the screening of 906 soil and marine actinomycetes culture extracts. Extracts decreased the luciferase level in the *agr* reporter strain to < 10% of their negative control, the second screening of 20 samples subjected to GFP assay yielded only16 samples which inhibited the expression of GFP (Desouky *et al*., 2013). Further screening of the 16 samples against gelatinase induction in *E. faecalis* identified only four culture extracts (608, 609, Y51, and Y67), which demonstrated QS inhibitory activity against the *agr* and *fsr* systems without growth inhibitory activity. Igarashi *et al*. (2015) identified avellanin C from *Hamigera ingelheimensis* as an inhibitor of QS in *S. aureus.* Avellanin C decreased luminescence emission from the reporter strain with an IC_{50} value of 4.4 μM. Arthoamide from *Arthrobacter* sp. inhibited luminescence production of reporter strain *S. aureus,* which indicated inhibition of *agr* signaling pathway (Igarashi *et al.*, 2015). Another compound polyhydroxyanthraquinone isolated from *Penicillium restrictum* inhibited QS signaling of all four groups of *S. aureus* (Daly *et al*. 2015). Quorum sensing inhibition of *S. aureus* could potentially decrease staphylococcal virulence by hindering the production of

certain virulence factors without hampering growth thus potentially avoiding selective pressures for drug-resistance (Chen and McClane, 2012). This makes it an interesting target in anti-virulence theraphy.

Quorum sensing inhibition targeting *fsr* system of *E. faecalis* has also received attention for its potential ability to decrease the pathogenicity of the strain (Singh and Nakayama, 2015). *Enterococcus faecalis* often causes opportunistic infections such as bacteremia, endocarditis, and urinary tract infections. Similar to *S. aureus,* virulence factors in enterococci are primarily mediated by QS (Thurlow *et al*., 2010). Gelatinase an extracellular metalloprotease that liquefies gelatine and collagen is considered a QS-mediated virulence factor in *E. faecalis* (Singh and Nakayama, 2015). In the current study, only the crude extract of AB2-SW8, identified as a *Streptomyces* sp., inhibited gelatinase production without any significant inhibition on growth suggesting QSI potential of the isolate. Furthermore, the extract also demonstrated QSI against the *S. aureus* reporter strain, which suggests broad-spectrum QSI activity against Gram-positive pathogens. *Streptomyces* sp. have been widely recognized as pharmaceutically important microorganism as they produce diverse range of secondary metabolites (Tan *et al*., 2016). *Streptomyces* sp. are known to inhibit QS of Gram-negative bacteria through the production of acylase, however, this enzyme has no effect on Gram-positive QSI (LaSarrea and Federle, 2013). This suggests that other QS inhibitory compounds are present in the crude extract of AB2-SW8. Nakayama *et al*. (2007) reported QSI potential of siamycin isolated from *Streptomyce*s sp. strain Y33-1, which inhibited the *fsr* QS system through inhibition of the receptor histidine kinase. Additionally, Nakayama *et al*. (2009) also isolated ambuic acid, which demonstrated broad-spectrum Gram-positive QSI. The compound inhibited the biosynthesis of the cyclic peptide quormones of *S. aureus* and the QS-mediated gelatinase production of *E. faecalis*.

Pathogenic bacteria are often resistant to traditional antibiotics, forging the need to develop new strategies to control infectious diseases. Identification of seaweed-associated bacterial extracts demonstrating Gram-positive QS inhibitory activity may undoubtedly prove to be of paramount importance in the future of clinical as well as industrial treatments of bacterial infections. This study showed the potential of seaweed-associated bacteria as a source for compounds targeting the *S. aureus agr* and *E. faecalis fsr* systems as a basis for discovering broad-spectrum anti-virulence compounds that target communication by Gram-positive pathogens mediated by *agr*-type QS systems. Further studies on identification and characterization of specific molecules within the crude extracts and their role in QSI must still be carried out.

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CHAPTER FIVE
DISCUSSION AND CONCLUSION

The screening of marine microorganisms as a potential source of novel bioactive compounds is a promising strategy due to the unique physiochemical properties of the marine environment (Egan *et al*., 2013). In comparison to terrestrial microorganisms, they produce bioactive compounds with uncommon functional entities (Rocha-Martin *et al.*, 2014). Seaweed-associated bacteria offer competitive strategies against other epibiotic/biofilm/biofouling micro- and macro-organisms by synthesis of a variety of chemical interaction compounds; (Defoirdt, 2013). As such they represent a reservoir of novel compounds for the production of bioactive molecules of pharmaceutical interest. This study investigated the potential of seaweed-associated bacteria as a source of novel bioactive compounds with antimicrobial, anti-biofilm and Gram-positive anti-QS activity.

Majority of seaweed-associated bacterial isolates inhibited one or more of the resistant indicator organisms. The best activity was against the MRSA isolate, however, the inhibitory activity of extracts was more pronounced against *A. salmonicida,* the aquaculture indicator. These results are similar to the findings by Kanagasabhapathy *et al*. (2009) who reported secondary metabolites from seaweed-associated bacteria with pronounced antimicrobial activity against aquaculture indicators as compared to clinical indicators. Epiphytic bacteria isolated from *Padina pavonica* demonstrated broad-spectrum antibacterial activity against clinical and animal pathogens (*A. salmonicidia, A. hydrophila, E. coli, S. aureus, V. proteolyticus*) (Ismail *et al*., 2016). JanakiDevi *et al*. (2013) isolated seaweed-associated bacteria which demonstrated antibacterial activity against *E. coli, K. pneumoniae, P. aeruginosa, Salmonella* sp., *Serratia* sp., *Shigella dysenteriae, V. cholerae, Micrococcus* and *Staphylococcus* spp. Several antibacterial compounds (cosmomycin B, galtamycin B, maltophilins) identified from seaweed-associated bacteria displayed antimicrobial activity against *S. aureus* and *E. coli* (Brana *et al*., 2014).

Isolate MAB24-SW1, identified as *B. velezensis*, which is closely related to *B. amyloliquefaciens,* exhibited the highest level of antimicrobial activity against both clinical and aquaculture indicators in this study. This activity was in part associated with the synthesis of a potential analogue of surfactin. Surfactin is synthesized by multifunctional non-ribosomal peptide sythetase (NRPS) (Płaza *et al*., 2015; Jasim *et al*., 2016). The presence of polyketide synthetase (PKS) and NRPS biosynthetic clusters appear to be widespread amongst *Bacillus* strains isolated from different shallow and deep water marine hosts (Blunt *et al*., 2015).

Nonribosomal peptide synthases are an important group of enzymes, which are responsible for the biosynthesis of well-known secondary metabolites like vancomycin, siderophores, cyanotoxins, biosurfactants, immunosuppressants, anticancer agents, anti-inflammatory agents etc. (Tambadou *et al*. 2015). Surfactin production is reported for *B. subtilis* isolates and it possesses several desirable industrial, pharmaceutical and physicochemical properties, including activity over a wide pH range (Mnif and Ghribi, 2015). Jasim *et al*. (2016) isolated a novel endophytic *Bacillus* sp., which demonstrated antibacterial activity due to the production of NRPS related surfactin. Furthermore, MAB24-SW1 (*B. velezensis*) also demonstrated biofilm inhibitory potential, which indicates that the crude extract contains a mixture of bioactive compounds, which could be working synergistically. Thus further work should be directed at exploring *B. velezensis* as a potential source of novel bioactive agents.

An alternative way to control infection in aquaculture is by applying probiotics (Tan *et al*., 2016), which have QSI potential using seaweed-associated bacterial isolates administered by feeding. Quorum sensing-degrading bacteria could be considered a better alternative to replace application of antibiotics in aquaculture as biocontrol of bacterial fish disease and in reducing the pathogenicity of *Vibrio* species. The production of surfactin could be a desirable property should *B. velezensis* be employed as a probiotic in aquaculture applications. Chen *et al*. (2010) reported the use of recombinant AHL-lactonase from *Bacillus* sp. as a potential probiotic by injecting the isolate in carp infected with pathogenic *A. hydrophila*. They obtained a decrease in the fish mortality rates. Li *et al*. (2014) reported on the effect of probiotic mixture of *B. subtilis* and *B. cereus* in controlling infections caused by *V. alginolyticus*. *Bacillus* spp. to be good candidates as probiotics due to the wide spectrum of bioactive compounds they produce (Li *et al*., 2014). Furthermore, they are known to promote growth of beneficial bacteria in intestinal tract of humans and animals (Pandey *et al*., 2014). The identification of *Streptomyces* and *Bacillus* species in this study, further highlights the potential of isolated seaweed-associated bacteria as a source of probiotics. Studies have showed that feed-stock supplemented with *Streptomyces* protected fish and shrimp from pathogens (Defoirdt, 2016; Tan *et al*., 2016). *Streptomyces* produce broad-spectrum inhibitory compounds involved in the attenuation of biofilm formation, anti-QS activity and anti-virulence activity in *Vibrio* sp. (Tan *et al*., 2016). *Streptomyces* sp. identified in this study demonstrated anti-biofilm inhibitory activity these indicates that crude extracts contain bioactive with potential ant-virulence activity.

In addition to antimicrobial screening, the effect of the extracts on biofilm formation and QS was evaluated. Seaweed-associated bacterial extracts at 5 mg/ml displayed the best

results in the initial adherence assay. Inhibition (≥ 50%) was noted in the following order: *P. aeruginosa, Y. ruckeri, S. aureus, V. parahaemolyticus,* and *A. hydrophila.* The extracts' activity against *P. aeruginosa, Y. ruckeri and V. parahaemolyticus,* might be the result of QS inhibitory compounds that disrupt the QS-mediated virulence expression. Egan *et al*. (2002) demonstrated that seaweed associated *P. tunicata* blocked biofilm formation by the synthesis of pigmented substances that inhibited AHL-dependent transcriptional of *Vibrio cholerae.* Husain and Ahmad (2015) reported that 35 out of 88 actinomycetes inhibited the biofilm formation of *V. harveyi, Vibrio vulnificus*, and *V. anguillarum* without affecting their growth. Seaweed-associated bacterial extracts also inhibited the initial attachment of *P. aeruginosa,* which was promising as *P. aeruginosa* is amongst one of the most difficult pathogens to treat. Secondary metabolites speculated to contain QSI compounds from *Diaphorobacter* sp. and *Delftia* sp. significantly reduced *P. aeruginosa* PAO1 cell adhesion (Christiaen *et al*., 2014).

This study also demonstrated the ability of extracts to disperse mature biofilm, with most extracts dispersing mature biofilm at 10 mg/ml. The order of dispersal (≥ 50% biofilm reduction) was as follows: *V. parahaemolyticus, S. aureus, A. hydrophila, P. aeruginosa, E. tarda* and *Y. ruckeri*. A higher number of extracts disperse mature biofilm of MRSA as compared to those active against MDR *P. aeruginosa* (13%). Extracts had an overall high inhibitory activity against aquaculture indicators demonstrating significant dispersal activity as compared to inhibition of initial adherence.

Most of the isolates obtained in the study were identified as *Bacillus* and *Streptomyces* species. These results are comparable to those of by Burgess *et al*. (2003) who identified majority of *Bacillus* species from varying seaweed sources, i.e. *B. pumilus, B. licheniformis* and *B. subtilis* and observed QS-mediated inhibition, which resulted in decreased fouling. *Bacillus* species are known producers of the QQ enzyme lactonase, which targets and inactivates acylated homoserine lactones required for QS regulation in pathogenic bacteria (Naik *et al*., 2012). Marine *Bacillus* sp. S3 displayed QS inhibition when grown in the presence of the inducer strain *P. aeruginosa* (Dusane *et al*., 2011). Acylase enzymes produced by *Streptomyces* inactivates QS by degrading AHL signals (Hassan *et al*., 2016). Antimicrobial compounds from *Streptomyces* have been extensively studied from an antimicrobial perspective (Tan *et al*., 2016), however less is reported about their QS and biofilm inhibitory compounds (Tan *et al*., 2016). Behenic acid, borrelidin and 1*H*-pyyrole-2-carboxylic acid produced by *Streptomyces coelicoflavus* inhibited QS-mediated virulence factors of *P. aeruginosa* (Hassan *et al*., 2016). Piericidin A1 secreted by *Streptomyces* sp. demonstrated QSI activity against *Chromobacterium violaceum* (Ooka *et al*., 2013). Thus screening seaweed-

associated bacterial these isolates is a promising lead to the discovery of novel QSI compounds for the treatment of infections. However, it must be noted that whilst some extracts had inhibitory activity, others increased biofilm formation. Further investigation into these isolates is required in order to investigate their modes of action.

The Gram-positive *agr* QS system plays an important role in promoting acute and aggressive infection in both animals and humans (Gray *et al*., 2013). Inhibition of *agr* in *S. aureus* has been reported to block ulcer formation and reduce abscess size (Gray *et al*., 2013; Singh *et al*., 2015). The potential anti-virulence activity of seaweed-associated bacterial extracts against Gram-positive QS systems was assessed using reporter strain *S. aureus* 8325–4, which carries plasmid pSB2035 encoding luciferase and GFP genes under *agr*P3 promoter control (Desouky *et al*., 2013). Only 27% (16/60) of extracts performed strongly in this assay, when luminescence inhibition was assessed. Using the confirmatory GFP assay, six extracts displayed inhibitory activity. This could be due to metabolites that hamper *S. aureus* cellular metabolic activity, yielding a false-positive result as *Photorhadbus* luciferase activity depends on a bacterially generated flavinmononucleotide, FMNH2. Hence, the GFP assay directly reflects the promoter activity of *agrP3* was employed (Desouky *et al*., 2013). It was, therefore, unsurprising that very few positive QSI results were obtained when compared to the luciferase assay. Despite the intense effort to discover QSI compounds against Gram-positive organisms, few natural compounds have been reported (Desouky *et al*., 2013, Singh and Nakayama, 2015; Singh *et al*., 2016). This is due to the peptide-based autoinducer utilized by Gram-positive, organisms. Enzymatic (QQ enzymes) activity is non-specific thus enzymes essential for the growth and survival of the bacterial cells are affected (LaSarre and Federle, 2013). This might explain the low Gram-positive QS inhibitory potential of the extracts in this study. Compounds WS9326A and WS9326B isolated from actinomycetes strains inhibited hemolysis production in *S. aureus* strains 8325–4 (type-I AIP) (Desouky *et al*., 2015). Several authors have suggested enzymatic degradation of Gram-negative AHL QS signals as a strategy employed by several organisms, including several *Bacillus, Streptomyces* and *Rhodococcus* species (Reuter *et al*., 2015; Tan *et al*., 2016). However, these enzymes are not effective in QS-mediated systems of Gram-positive organisms (Gray *et al*., 2013). This suggests that seaweed-associated bacteria (*Streptomyces, Bacillus, Rhodococcus*) observed in this study produce other bioactive compounds apart from AHL-degrading enzymes with effect against Gram-positive QS-mediated virulence factors

To obtain an extract, which had wide spectrum of activity, the gelatinase assay was utilized for *E. faecalis* OGIRF exposed to extracts. Extract AB2-SW8 (*Streptomyces* sp.)

displayed inhibition of gelatinase with no effect on cell growth. This suggests that the observed effect may have been due to the inhibition of *fsr* QS-mediated gelatinase induction. Quorum sensing mediated virulence factors in *E. faecalis* include proteases, gelatinase (GelE) which aids in digesting host extracellular matrix proteins and serine protease (SprE), that enable *E. faecalis* to invade host tissues (Gray *et al*., 2013). The methanolic extract from plant *Salvadora persica* demonstrated gelatinase inhibition by targeting *gelE* expression in *E. faecalis* (Rezaei *et al*., 2011). There are a limited number of natural products that have been explored as Gram-positive QS inhibitors (Singh *et al*., 2016). Given the urgent need to treat MDR Gram-positive infections, seaweed-associated bacterial extracts are potential reservoirs of bioactive compounds as they represent a source for natural QS inhibitory compounds. An overall assessment of the QSI assays suggests that while selected extracts demonstrated limited activity against Gram-positive organisms, they displayed potential as QS inhibitory compounds against Gram-negative organisms. This assumption is based on the activity observed in anti-biofilm assays, as QS is important in biofilm formation (Satheesh *et al*., 2016).

In conclusion, marine substrata are colonized by a variety of marine microorganisms, which are capable of producing novel compounds due to their diverse and often extreme environmental conditions and interactions with their associated marine eukaryotic host organisms (Tang and Zhang, 2014). This study demonstrated that seaweed-associated bacterial isolates synthesize bioactive compounds with antimicrobial, anti-biofilm and anti-virulence activity. Identifying seaweed-associated bacterial as sources of compounds for anti-virulence therapy and as weapon to combat MDR in clinical and aquaculture environments is of paramount importance. The rate of discovery of these anti-QS compounds is still in its early stages, especially anti-QS activity against Gram-positive pathogens. Thus the discovery of potential QSI compounds in this study must be explored further as a weapon to combat MDR pathogens. Future studies willl target isolation and purification of compounds in crude extracts of seaweed-associated bacterial isolates and structure elucidation coupled with determination of their mode of actions. Further screening of extracts against HIV and cancer must also be explored.

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APPENDICES

A1 Colony characteristics of seaweed-associated bacteria

Table A1.1: Colony characteristics of isolates associated with seaweed 1

Isolate	Aerial/pigmentation description	Sporulation: Yes (Y)/No (N)	Substrate description
MAB2-SW1	Rough, white, powdery	Y	Brownish colour
MAB3-SW1	Cream with smooth whitish borders	N	Transparent, pale yellow
MAB4-SW1	Glossy rhizoid white	N	Translucent yellow
MAB4B-SW1	Creamy white	Y	Yellow
MAB5-SW1	Cream with rough wrinkled texture	Y	Dark yellow
MAB7-SW1	Smooth bright yellow	N	Mustard
MAB8-SW1	White	N	Cream
MAB9A-SW1	White, rough	Y	Light yellow
MAB9B-SW1	Peachy-orange, mucoid	N	Peach
MAB10A-SW1	Creamy white	N	Yellow
MAB10B-SW1	Whitish cream glossy surface	N	Cream
MAB10C-SW1	Glossy cream white	N	Mustard
MAB11-SW1	Glistening cream: opaque	N	Dark brown, yellow
MAB12-SW1	Glossy white with smooth borders, translucent	N	Yellow
MAB13-SW1	Green with grey spores	Y	Yellow
MAB14-SW1	Radial colonies, green centre with white rings	Y	Dark mustard, yellow
MAB16-SW1	Glossy cream, whitish border	N	Translucent yellow
MAB17-SW1	Glossy cream white	N	Translucent white
MAB18-SW1	Glistening cream, opaque	N	Yellow
MAB19A-SW1	Glistening yellow, smooth surface	N	Transparent, pale yellow
MAB19B-SW1	Green with white surface	Y	Translucent yellow, brown
MAB20-SW1	Creamy white, mucoid	Y	Light yellow
MAB21-SW1	Cream	N	Translucent
MAB22-SW1	Green powdery	Y	Yellow
MAB23-SW1	Cream smooth surface, butyrous	N	Dark brown, yellow
MAB24-SW1	Cream; wrinkled butyrous surface	N	Cream
MAB25A-SW1	Glistening, smooth cream: transparent	N	Translucent
MAB25B-SW1	Glossy cream, whitish	N	Cloudy yellow
MAB26-SW1	Cream, white borders	N	Dark yellow
MAB27-SW1	Cream	N	Translucent yellow
MAB28-SW1	Pink with white borders	N	Pale Yellow
MAB33-SW1	Cream smooth surface, butyrous	N	Cloudy white
MAB34A-SW1	Creamy white, mucoid.	Y	Light yellow
MAB34B-SW1	Creamy white, mucoid.	N	Light yellow
MAB35-SW1	Cream, butyrous, wavy edges	Y	Light yellow
MAB36-SW1	Cream rhizoid smooth surface	Y	Yellow
MAB37-SW1	Glossy cream with white borders	N	Translucent white
MAB38-SW1	Glossy cream with white borders	N	Cloudy yellow
MAB39A-SW1	Green, brown and black, raised	Y	Dark mustard, yellow

MAB39B-SW1	White, butyrous	N	Cream
MAB39C-SW1	White powdery with rough green surface	Y	Mustard

Table A1.2: Characteristics of isolates associated with seaweed 2

Isolate	Aerial/pigmentation description	Sporulation Yes (Y)/No (N)	Substrate description
AB1-SW2	Cream with pink and purple, powdery	Y	Pale yellow, pink
AB3-SW2	Semi-transparent, cream and some brown, mucoid	Y	Cream
AB4-SW2	White, raised	Y	Light orange
AB5-SW2	Light yellow, smooth	N	Cream
AB6-SW2	Milky white, mucoid	N	Pale yellow, olive green
AB7-SW2	Very bright pink, glossy and butyrous	N	Light, pinky orange
AB8-SW2	Light pink, white, dry, soft	Y	Light pink, mustard
AB9-SW2	Radial colonies, green centre then grey and white rings	Y	Dark olive green
AB9B-SW2	Bright pink, butyrous	N	Light, pinky orange
AB10-SW2	Light pink, white, powdery	Y	Yellow
AB11-SW2	Dark brown with white borders	Y	Dark mustardy yellow
AB12-SW2	Semi-transparent, creamy white	N	Cream
AB27-SW2	Cream, butyrous - turning pink	N	Light Yellow

Table A1.3: Characteristics of isolates associated with seaweed 5

Isolate	Aerial/pigmentation description	Sporulation: Yes (Y)/No (N)	Substrate description
AB1-SW5	White with yellow centres, rough	Y	Greeny yellow, brown
AB2-SW5	White and grey, mucoid and powdery	Y	Dark brown, yellow
AB2B-SW5	Creamy brown with fine green spores	Y	Brown
AB3-SW5	White, grey, powdery	N	Wrinkled, mustardy yellow
AB4-SW5	White with dark patches, powdery	Y	Wrinkled, light yellow
AB6-SW5	White, creamy butyrous	N	Light yellow
AB7-SW5	Milky white, light yellow, transparent, mucoid	N	Transparent, pale yellow
AB8-SW5	Grey circles	Y	Brown

Table A1.4: Characteristics of isolates associated with seaweed 6

Isolate	Aerial/pigmentation description	Sporulation Yes (Y)/No (N)	Substrate description
AB1-SW6	Yellow	Y	Light yellow
AB2-SW6	Translucent yellow and grey	Y	Yellow
AB3-SW6	White, pearly, translucent	N	Cream
AB4-SW6	Grey and white	Y	Cream
AB5-SW6	Milky white, blurred edges, butyrous, pearly	N	Cream
AB6-SW6	Milky white, butyrous, very shiny	Y	Cream

Table A1.5: Characteristics of isolates associated with seaweed 7

Isolate	Aerial/pigmentation description	Sporulation Yes (Y)/No (N)	Substrate description
AB1-SW7	Transparent, some white and grey, powdery	Y	Light yellow
AB2-SW7	Clear, grey and white, powdery	Y	Light yellow

Table A1.6: Characteristics of isolates associated with seaweed 8

Isolate	Aerial/pigmentation description	Sporulation Yes (Y)/No (N)	Substrate description
AB1-SW8	White, matt	U	Cream
AB2-SW8	Olive green with white spores	Y	Dark Yellow
AB3-SW8	White, mucoid	N	Light yellow
AB4-SW8	Mostly white, some beige, powdery	Y	Yellow
AB5-SW8	Darkish brown, white edges, wrinkled	Y	Dark tan, wrinkled
AB6-SW8	White, powdery	Y	Light yellow
AB6B-SW8	White, translucent	N	Cream
AB7-SW8	White, powdery	Y	Wrinkled, mustardy yellow
AB8-SW8	Almost transparent, white	Y	Yellow, wrinkled
AB9-SW8	Beige, white surface and edges	Y	Yellow and pale yellow
AB10-SW8	Dark grey, powdery	Y	White and brown, wrinkled
AB11-SW8	Dark beige, white pigmentation, mucoid	N	Dark mustardy yellow
AB12-SW8	Glistening slight transparent cream	Y	Dark mustardy yellow
AB13-SW8	White	N	Cream
AB14-SW8	Cream with white borders	Y	Cream
AB15-SW8	Cream with white	Y	Light yellow

Table A1.7: Characteristics of isolates associated with seaweed 9

Isolate	Aerial/pigmentation description	Sporulation Yes (Y)/No (N)	Substrate description
AB1-SW9	Opaque white, milky and smooth	N	Pale yellow
AB2-SW9	Opaque, cream, small colonies	N	Pale yellow
AB3-SW9	Opaque, cream, butyrous	N	Pale yellow
AB4-SW9	Yellow, mucoid	N	Yellow
AB5-SW9	Yellow, mucoid	N	Dark yellow

Table A1.8: Characteristics of isolates associated with seaweed 10

Isolate	Aerial/pigmentation description	Sporulation: Yes (Y)/No (N)	Substrate description
AB1-SW10	Grey-beige, rough	Y	Olive green
AB2-SW10	White, rough	Y	Light brown-mustard
AB3-SW10	Light yellow with yellow and white interspersed. Sandy texture.	Y	Yellow
AB4-SW10	Grey with white edges, powdery	Y	Light olive green-brown

A2 Antimicrobial potential of seaweed-associated bacteria

Table A2.1: Primary screening of seaweed-associated bacteria against clinical isolates

Bacteria	*E. coli* ATCC 35218	*K. pneumoniae* ATCC 700603	*P. aeruginosa* ATCC 27583	*E. faecalis* ATCC 51299	*S. aureus* ATTCC 43300
S. griseus ATCC 15468	3	7	2	0	0
MAB2-SW1	0	0	0	0	0
MAB3-SW1	0	0	0	0	0
MAB4-SW1	8	0	0	0	0
MAB4-SW1B	0	0	0	0	0
MAB5-SW1	0	0	0	0	0
MAB6-SW1	0	0	0	0	0
MAB7-SW1	7	0	0	17	0
MAB8-SW1	0	0	0	0	0
MAB9A-SW1	0	0	0	0	0
MAB9B-SW1	0	0	0	0	0
MAB10A-SW1	0	TGI	0	0	0
MAB10B-SW1	13	0	0	0	12
MAB10C-SW1	0	0	0	0	6
MAB11-SW1	11	0	0	6	8
MAB12-SW1	0	0	0	0	10
MAB13-SW1	0	0	0	0	0
MAB14-SW1	0	0	0	0	0
MAB16-SW1	0	0	0	0	7
MAB17-SW1	0	0	0	0	8
MAB18-SW1	0	0	0	0	0
MAB19A-SW1	GI	0	0	0	0
MAB19B-SW1	0	0	0	0	0
MAB20-SW1	0	6	0	0	0
MAB21-SW1	0	0	0	0	0
MAB22-SW1	0	0	0	0	12
MAB23-SW1	0	0	0	0	0
MAB24-SW1	0	TGI	0	0	TGI
MAB25A-SW1	TGI	0	0	TGI	11
MAB25B-SW1	0	0	0	0	5
MAB26-SW1	0	0	0	0	0
MAB27-SW1	TGI	0	0	TGI	14
MAB28-SW1	0	0	0	0	0
MAB33-SW1	0	0	0	0	0
MAB34A-SW1	0	0	0	0	2
MAB34B-SW1	0	0	0	0	2
MAB35-SW1	0	0	0	0	0
MAB36-SW1	5	0	0	0	0
MAB37-SW1	0	0	0	0	10

Table continued

MAB38-SW1	0	0	0	0	0
MAB39A-SW1	0	0	0	0	9
MAB39B-SW1	0	0	0	0	0
MAB39C-SW1	0	0	0	0	0
AB1-SW2	0	0	0	8	0
AB3-SW2	0	0	0	0	5
AB4-SW2	0	0	0	0	0
AB5-SW2	0	0	0	0	10
AB6-SW2	0	0	0	0	4
AB7-SW2	0	0	0	0	0
AB8-SW2	0	0	0	0	0
AB9-SW2	0	0	0	0	6
AB9B-SW2	0	0	0	0	0
AB10-SW2	0	7	0	0	0
AB11-SW2	0	0	0	0	9
AB12-SW2	0	10	0	0	5
AB1-SW5	0	0	0	0	0
AB2-SW5	0	0	0	0	0
AB2B-SW5	0	0	0	0	0
AB3-SW5	0	0	0	0	0
AB4-SW5	0	0	0	0	0
AB5-SW5	0	0	0	0	0
AB6-SW5	0	0	0	0	0
AB7-SW5	6	0	0	0	0
AB8-SW5	0	8	0	0	0
AB1-SW6	0	0	0	0	0
AB2-SW6	0	0	0	0	0
AB3-SW6	0	0	0	0	0
AB4-SW6	0	0	0	0	0
AB5-SW6	0	0	6	0	0
AB6-SW6	GI	GI	0	0	0
AB1-SW7	0	0	0	4	0
AB2-SW7	0	0	0	0	0
AB1-SW8	0	0	0	0	10
AB2-SW8	9	0	0	0	0
AB3-SW8	0	0	0	0	0
AB4-SW8	0	0	0	0	0
AB5-SW8	0	0	0	0	0
AB6-SW8	0	0	0	0	0
AB6B-SW8	0	0	0	0	0
AB7-SW8	0	0	12	0	0
AB8-SW8	0	0	0	0	0
AB9-SW8	0	6	0	0	0
AB10-SW8	0	0	0	0	0
AB11-SW8	0	0	0	0	4
AB12-SW8	0	0	0	0	10
AB13-SW8	0	0	0	0	10

Table continued

AB14-SW8	0	0	0	0	0
AB15-SW8	0	0	0	0	0
AB1-SW9	0	0	0	0	12
AB2-SW9	0	0	0	0	6
AB3-SW9	0	0	0	0	0
AB4-SW9	0	0	0	0	0
AB5-SW9	0	0	0	0	0
AB1-SW10	0	13	0	0	0
AB2-SW10	0	0	0	6	10
AB3-SW10	0	0	0	0	0
AB4-SW10	0	0	0	0	0

[*] SW1= *Gracilaria* spp., SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

[#] **Grading:** - = no activity; + = weak activity (zone of inhibition of 1-4 mm); ++ = moderate activity (zone of inhibition of 5-8 mm); +++ = strong activity (zone of inhibition 9-15 mm); ++++ = highly active (zone of inhibition 16-25 mm); TGI = total growth inhibition.

Table A2:2: Primary screening of seaweed-associated bacteria against aquaculture indicators

Seaweed-associated bacteria	***A. hydrophila* ATTC 7966**	***A. salmonicida* ATCC 33658**	***E. tarda* ATCC 15947**	***S. enterica Arizonae* ATCC 13314**	***V. parahaemolyticus* ATCC 17802**	***Y. ruckeri* ATCC 29473**
S. griseus ATCC 15468	0	6	4	10	5	TGI
MAB2-SW1	0	TGI	0	0	0	0
MAB3-SW1	0	TGI	7	0	0	0
MAB4-SW1	0	TGI	TGI	0	0	0
MAB4B-SW1	0	TGI	0	0	0	0
MAB5-SW1	0	0	0	0	0	0
MAB6-SW1	0	0	0	0	0	0
MAB7-SW1	0	TGI	TGI	0	0	0
MAB8-SW1	0	TGI	0	0	0	0
MAB9A-SW1	0	20	0	0	0	0
MAB9B-SW1	0	0	0	0	0	0
MAB10A-SW1	0	0	0	0	0	0
MAB10B-SW1	0	TGI	0	0	0	0
MAB10C-SW1	0	TGI	10	0	6	0
MAB11-SW1	0	TGI	0	0	0	0
MAB12-SW1	0	TGI	4	0	0	0
MAB13-SW1	0	TGI	0	0	0	0
MAB14-SW1	0	0	0	0	0	0
MAB16-SW1	0	0	0	0	11	0
MAB17-SW1	7	TGI	4	0	9	TGI
MAB18-SW1	0	4	0	0	6	0
MAB19A-SW1	0	0	0	0	0	0
MAB 19B-SW1	0	TGI	10	0	0	TGI
MAB20-SW1	0	TGI	5	0	0	0
MAB21-SW1	0	0	0	0	0	0
MAB22-SW1	0	TGI	2	0	0	0
MAB23-SW1	0	0	TGI	0	0	0
MAB24-SW1	3	TGI	5	0	0	0
MAB25A-SW1	0	TGI	6	14	0	15
MAB25B-SW1	0	0	0	0	0	0
MAB26-SW1	0	TGI	5	0	0	0
MAB27-SW1	0	TGI	4	0	0	0
MAB28-SW1	0	TGI	0	0	0	0
MAB33-SW1	0	0	0	0	0	0
MAB34A-SW1	6	TGI	2	0	6	0
MAB34B-SW1	0	TGI	2	0	0	0
MAB35-SW1	0	0	0	0	0	0
MAB36-SW1	0	0	0	0	0	0
MAB37-SW1	0	TGI	9	0	9	16
MAB38-SW1	0	TGI	0	0	0	0
MAB39A-SW1	0	TGI	0	0	0	0
MAB 39B-SW1	0	TGI	0	0	0	0

MAB39C-SW1	0	0	4	TGI	2	0
AB1-SW2	0	TGI	21	0	0	0
AB3-SW2	0	TGI	5	0	0	0
AB4-SW2	0	0	0	0	0	0
AB5-SW2	0	0	0	0	0	0
AB6-SW2	0	TGI	15	0	0	0
AB7-SW2	0	TGI	7	0	0	0
AB8-SW2	0	0	0	0	0	0
AB9-SW2	0	0	0	0	0	0
AB9B-SW2	0	TGI	2	0	0	0
AB10-SW2	0	TGI	2	0	0	0
AB11-SW2	0	TGI	12	0	0	0
AB12-SW2	0	0	0	0	0	0
AB1-SW5	0	TGI	0	0	0	0
AB2-SW5	2	0	TGI	0	0	0
AB2B-SW5	0	TGI	0	0	0	0
AB3-SW5	0	TGI	TGI	0	3	TGI
AB4-SW5	0	TGI	0	0	0	0
AB6-SW5	0	TGI	TGI	4	0	0
AB7-SW5	0	0	0	0	0	0
AB8-SW5	0	TGI	11	0	0	0
AB1-SW6	0	0	0	0	0	0
AB2-SW6	0	0	0	0	0	0
AB3-SW6	0	0	0	0	0	0
AB4-SW6	0	0	0	0	0	0
AB5-SW6	0	TGI	0	0	0	0
AB6-SW6	0	TGI	6	0	0	0
AB1-SW7	0	TGI	0	0	0	0
AB2-SW7	0	0	8	TGI	0	0
AB1-SW8	0	TGI	TGI	0	0	0
AB2-SW8	0	TGI	6	0	0	0
AB3-SW8	0	0	0	0	0	0
AB4-SW8	0	0	0	0	0	0
AB5-SW8	0	TGI	9	0	0	0
AB6-SW8	0	TGI	TGI	0	11	0
AB6B-SW8	0	TGI	2	0	0	2
AB7-SW8	0	TGI	5	0	2	6
AB8-SW8	0	TGI	10	0	12	0
AB9-SW8	0	TGI	TGI	0	TGI	0
AB10-SW8	0	TGI	21	0	0	0
AB11-SW8	TGI	TGI	TGI	TGI	TGI	TGI
AB12-SW8	0	TGI	14	0	0	4
AB13-SW8	0	0	0	6	TGI	TGI
AB14-SW8	0	0	0	0	0	0
AB15-SW8	0	TGI	3	0	0	0
AB1-SW9	0	TGI	TGI	0	0	0
AB2-SW9	0	TGI	4	0	0	0
AB3-SW9	0	TGI	0	0	0	0
AB4-SW9	0	0	0	0	0	0
AB5-SW9	0	TGI	0	0	0	0
AB1-SW10	0	TGI	9	0	0	7
AB2-SW10	0	TGI	2	0	0	0
AB3-SW10	0	TGI	6	0	0	0
AB4-SW10	0	TGI	10	0	0	0

[*] SW1= unidentified red seaweed, SW2 – *Codium* spp., SW5 - *Amphiroa bowerbankii* (Harvey), SW6 - *Laurencia brongniartii* (J. Agarah), SW8 - *Gelidium pteridifolium* (R Norris, Hommersand and Fredericq), SW9 - *Ulva rigida* (C. Agarah), SW10 - *Codium duthieae* (P. Silva).

[#] **Grading:** - = no activity; + = weak activity (zone of inhibition of 1-4 mm); ++ = moderate activity (zone of inhibition of 5-8 mm); +++ = strong activity (zone of inhibition 9-15 mm); ++++ = highly active (zone of inhibition 16-25 mm); TGI = total growth inhibition.

A3 Anti-quorum sensing potential of Seaweed-associated bacteria

Table A3.1: The quorum sensing inhibition activity of seaweed associated bacterial extracts monitored by luciferase reporter assay

Extract		Induction	Full induction	Inhibition
S. *aureus* 8325-4	2460.3		774.493	
S. *aureus* 12600	1685.807			
MAB-SW1	2028.16	342.353		44.20
MAB4-SW1	2183.533	497.726		64.26
MAB6-SW1	2173.217	487.410		62.93
MAB10A-SW1	2329.263	643.456		83.08
MAB10C-SW1	2003.863	318.056		41.06
MAB21-SW1	2123.573	437.766		56.52
MAB34A-SW1	2338.87	653.063		84.32
MAB35-SW1	2086.183	400.376		51.69
AB4-SW2	2235.217	549.410		70.93
AB9B-SW2	2160.897	475.090		61.34
AB27-SW2	2349.543	663.736		85.69
AB2-SW5	2269.87	584.063		75.41
AB4-SW5	2453.907	768.100		99.17
AB1-SW6	2147.553	461.746		59.61
S. aureus 8325-4	2074.547		1169.173	
S .aureus 12600	905.3737			
AB3-SW6	1462.757	557.383		47.67
AB5-SW6	1585.457	680.083		58.16
AB1-SW7	1675.803	770.429		65.89
AB2-SW7	1545.803	640.429		54.77
AB6B-SW8	1932.52	1027.146		87.85
AB8-SW8	1622.46	717.086		61.33
AB11-SW8	1502.123	596.749		51.04
AB14-SW8	1623.463	718.089		61.41
AB3-SW9	1733.79	828.416		70.85
AB5-SW9	1669.46	764.086		65.35
AB4-SW9	1674.14	768.766		65.75
AB2-SW10	1699.14	793.766		67.89
AB3-SW10	1640.147	734.773		62.84
AB4-SW10	1928.18	1022.806		87.48

Extract		Induction	Full induction	Inhibition
S. aureus 8324-5	2748.37		517.793	
S. aureus 12600	2230.577			
MAB7-SW1	2700.583	470.006		90.77
MAB10B-SW1	2663.933	433.356		83.69
MAB11-SW1	2537.213	306.636		59.21

MAB12-SW1	2627.26	396.683	76.61
MAB16-SW1	2557.217	326.640	63.08
MAB17-SW1	2738.843	508.266	98.16
MAB2-SW1	2573.817	343.240	66.28
MAB22-SW1	2690.813	460.236	88.88
MAB24-SW1	2404.317	173.740	33.55
MAB25A-SW1	2555.077	324.500	62.66
MAB27-SW1	2572.743	342.166	66.08
MAB37-SW1	2756.43	525.853	101.55
AB1-SW2	2550.077	319.500	61.70
AB5-SW2	2473.757	243.180	46.96
AB6-SW2	2468.743	238.167	45.99
AB10-SW2	2765.777	535.200	103.36
AB11-SW2	2636.757	406.180	78.44
AB12-SW2	2454.47	223.893	43.23
AB3-SW5	2438.75	208.173	40.20
AB6-SW5	2450.073	219.496	42.39
AB7-SW5	2367.743	137.166	26.49
AB8-SW5	2428.073	197.496	38.14
AB6-SW6	2555.743	325.166	62.79
AB1-SW8	2488.117	257.540	49.73
AB2-SW8	2396.813	166.236	32.10
AB7-SW8	2443.107	212.530	41.04
AB9-SW8	2471.65	241.073	46.55
AB12-SW8	2606.983	376.406	72.69
AB1-SW9	2378.317	147.740	28.53
AB1-SW10	2661.94	431.363	83.30
AB4-SW10	2499.973	269.396	52.02

*The expression of luciferase was controlled by the *agr* system in reporter strain *S. aureus* 8324-5 (pSB2025)

Table A3.2: The quorum sensing activity of seaweed-associated bacterial extracts evaluated by green fluorescence protein of reporter strain *S. aureus.*

Extract	Average	Induction	Full induction	Inhibition
S. aureus 8324-5	4283.103		4012.825	
S. aureus 12600	270.278			
MAB2-SW1	4099.647	3829.369		95.428
MAB4-SW1	4323.997	4053.719		101.01
MAB6-SW1	2231.097	1960.819		48.86
MAB0A-SW1	3744.013	3473.735		86.56
MAB10C-SW1	2888.9	2618.622		65.25
MAB21-SW1	4351.907	4081.629		101.71
MAB34A-SW1	4721.413	4451.135		110.92
MAB35-SW1	4393.307	4123.029		102.74
AB4-SW2	4615.47	4345.192		108.28
AB9B-SW2	3980.827	3710.549		92.46
AB27-SW2	3596.917	3326.639		82.90
AB2-SW5	3816.68	3546.402		88.37
AB4-SW5	4321.637	4051.359		100.96
AB1-SW6	3966.81	3696.532		92.11
S. aureus 8325-4	4934.537		4662.765	
S. aureus 12600	271.772			
AB3-SW6	3677.723	3405.951		73.04
AB5-SW6	3501.03	3229.258		69.25
AB1-SW7	5084.89	4813.118		103.22
AB2-SW7	4503.043	4231.271		90.74
AB6B-SW8	3309.993	3038.221		65.15
AB8-SW8	4185.687	3913.915		83.93
AB11-SW8	4592.83	4321.058		92.67
AB14-SW8	4486.52	4214.748		90.39
AB3-SW9	4946.53	4674.758		100.25
AB5-SW9	4101.86	3830.088		82.14
AB4-SW9	4323.59	4051.818		86.89
AB2-SW10	5439.34	5167.568		110.82
AB3-SW10	4253.373	3981.601		85.39
AB4-SW10	2218.563	1946.791		41.75

Table continued

Extract	Average	Induction	Full induction	Inhibition
S. aureus 8324-5	3670.843		893.3063	
S. aureus 12600	2777.537			
MAB7-SW1	3722.32	944.783		105.76
MAB10B-SW1	3166.267	388.7297		43.51
MAB11-SW1	3480.997	703.4597		78.74
MAB12-SW1	3739.66	962.123		107.70
MAB16-SW1	3792.963	1015.426		113.67
MAB17-SW1	3527.59	750.053		83.96
MAB20-SW1	3559.083	781.5463		87.48
MAB22-SW1	3472.863	695.3263		77.83
MAB24-SW1	2908.243	130.7063		14.63
MAB25A-SW1	3918.663	1141.126		127.74
MAB27-SW1	3415.487	637.9497		71.41
MAB37-SW1	3535.07	757.533		84.80
AB1-SW2	3375.36	597.823		66.92
AB5-SW2	3630.27	852.733		95.45
AB6-SW2	3757.023	979.4863		109.64
AB10-SW2	3335.807	558.2697		62.49
AB11-SW2	3590.38	812.843		90.99
AB12-SW2	3487.247	709.7097		79.44
AB3-SW5	3849.36	1071.823		119.98
AB6-SW5	4061.993	1284.456		143.78
AB7-SW5	3423.013	645.4763		72.25
AB8-SW5	3526.32	748.783		83.82
AB6-SW6	3581.487	803.9497		89.99
AB1-SW8	3533.507	755.9697		84.62
AB2-SW8	3464.253	686.7163		76.87
AB7-SW8	3160.427	382.8897		42.86
AB9-SW8	3584.54	807.003		90.33
AB12-SW8	3943.69	1166.153		130.54
AB1-SW9	3208.887	431.3497		48.28
AB1-SW10	3431.19	653.653		73.17

*The expression of GFP was controlled by the *agr* system in reporter strain *S. aureus* 8324-5 (pSB2035).

Table A3.3: The quorum sensing inhibition activity of seaweed-associated bacterial extracts against *fsr* system evaluated by gelatinase activity

Extracts	Average	Full induction	Induction	Inhibition
Negative control	0.084	0.100		
E. faecalis OGIRF	0.184			
MAB2-SW1	0.159		0.075	75.13
MAB6-SW1	0.172		0.087	116.91
MAB10C-SW1	0.187		0.103	103.07
MAB10B-SW1	0.150		0.066	66.52
MAB11-SW1	0.169		0.085	85.72
MAB24-SW1	0.161		0.077	77.25
MAB35-SW1	0.177		0.092	92.83
MAB34A-SW1	0.180		0.095	95.82
AB6-SW2	0.190		0.106	106.75
AB12-SW2	0.191		0.107	107.45
AB3-SW5	0.141		0.057	57.32
AB6-SW5	0.154		0.070	70.23
AB7-SW5	0.169		0.085	85.32
AB8-SW5	0.149		0.065	65.31
AB5-SW6	0.160		0.076	76.45
AB1-SW8	0.163		0.079	78.94
AB2-SW8	0.127		0.043	43.01
AB5-SW8	0.160		0.076	76.53
AB7-SW8	0.145		0.061	61.01
AB9-SW8	0.163		0.079	79.43
AB11-SW8	0.183		0.099	99.39
AB1-SW9	0.214		0.130	130.89
AB3-SW10	0.202		0.118	118.48
AB4-SW10	0.185		0.100	100.92

*The induction of gelatinase was controlled by the *fsr* system of *E. faecalis* OGIRF.