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Isolation and identification of early marine biofilm-forming bacteria on commercial paint surface

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ABSTRACT

Aims: To investigate early marine biofilm-forming bacterial diversity on immersed antimicrobial-free commercial paint substratum in seawater.
Methodology and results: Total ten bacterial strains were successfully isolated and identified by complete 16S rRNA sequencing. The isolates morphological, biochemical properties, biofilm-forming ability, extracellular polymeric substance (EPS) productivity and components were characterised. The morphological and biochemical characterization of the strains showed strains-specific variation. All isolates were strong biofilm producers with four motile strains being both flat-bottom and air-liquid-interface biofilm producers, while other strains were only air-liquid interface biofilm producer. Based on 16S rRNA, three strains were identified as *Marinomonas communis,* two were *Marinomonas* sp., while the rest were *Alteromonas litorea*, *Alteromonas* sp., *Salinimonas lutimaris*, *Idiomarine baltica* and *Bacillus niabensis*. The amount of EPS that the isolates produced ranged from 1.95 to 2.89 g/L and productivity of EPS was inversely correlated with the cell biomass. Analysis of the extracted EPS using attenuated total reflectance-fourier transform infrared (ATR-FTiR) showed that all isolates EPS contained carbohydrates, nucleic acid, protein, DNA/RNA and lipid.
Conclusion, significance and impact of study: Bacterial diversity in early stages of biofilm on the commercial paint surface was dominated by Gram-negative bacteria from Gammaproteobacteria class. Isolates with superior cell growth showed lowest EPS production. This finding was expected to provide knowledge on distribution of different marine bacterial species in the biofilm on paint coated surfaces which may beneficial to formularize a new antibiofilm paint additive.

Keywords: Biofilm, marine bacteria, commercial paint surface, extracellular polymeric substance

INTRODUCTION

Formation of biofilm on surfaces was thought to be one of the microbial strategies to survive in the new environment. Life in a biofilm confers a broad range of advantages to its members. As reported in literature, biofilm structure providing shelter against environmental pressure (Salta *et al*., 2013), predations (Dang and Lovell, 2000) and antimicrobial compounds (de Carvalho, 2018). Biofilm consists of heterogeneous bacteria enclosed in a highly hydrated matrix called extracellular polymeric substance (EPS) (de Carvalho, 2018). EPS is known as a complex tri-dimensional structure composed of combination macromolecules such as polysaccharides, proteins, nucleic acids, lipids and other polymeric substances (Flemming and Wingender, 2010). EPS has multipurpose functions in biofilm formation. EPS plays a vital role in mediate cells aggregation (Salta *et al*., 2013), facilitate nutrient uptake (Flemming, 2016), provide biofilm mechanical and structural stability (de Carvalho and Fernandes, 2010), quorum sensing and gene transfer (Decho and Gutierrez, 2017).

Although biofilm formation has numerous advantages for bacteria development, but it is detrimental to human and many industrial processes. For example, biofilm on artificial structures in marine environment are harmful even at earliest stages because it may lead to biofouling (Salta *et al*., 2013). The early stages of biofilm formation in marine ecosystem are based on the interactions of free-living marine bacteria with the artificial surface submerged in seawater. When marine bacteria colonized on artificial structures submerged in seawater, it will secrete EPS. This EPS will anchor them to substratum thereby altering the local surface chemistry which later stimulate further growth and the recruitment of microorganisms such as algae spores or invertebrate larvae or other planktonic forms that promote macrofouling (Flemming, 2016; de Carvalho, 2018). Biofouling caused costly economic and

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environmental problems including interference with process performance (Cao *et al.*, 2011), material damage by the microbial attack (Fitridge *et al.*, 2012; Turan *et al.*, 2016), decrease of product quality and quantity (Skovhus *et al.*, 2017), contamination of antifouling chemicals (Wang *et al*, 2017) and shortening the life-time of plant components due to extended cleaning (Schultz *et al.*, 2011).

The primary colonizers on artificial surfaces in marine ecosystem are bacteria. Few marine biofilm studies have reported the pioneering colonization of bacterial biofilms species composition at the early-stage biofilms were dominated by the same major bacteria class in marine ecosystem, Alphaproteobacteria and Gammaproteobacteria (Lee *et al.*, 2008; Dang *et al.* 2008; Salta *et al.*, 2013; Rampadarath *et al.*, 2017). Other phyla such as Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Planctomycetes, β-, δ- and ε-Proteobacteria and Verrucomicrobia are identified as minor phyla in marine biofilm (Webster *et al.*, 2004; Lee *et al.*, 2008, Huggett *et al.*, 2009; Salta *et al.*, 2013; Brian-Jaisson *et al.*, 2014; Rampadarath *et al.*, 2017). The bacterial composition of early-stage biofilms are influences by the physicochemical of the surfaces and environmental variation of the immersion sites (Salta *et al.*, 2013; Dang and Lovell, 2016).

Formation of marine biofilm on paint surfaces will slowly deteriorate the paint surface structure, reduce the coating performance and induce settlement and metamorphosis of macrofoulers such as invertebrates and macroalgae which later lead to biofouling. Therefore, in this present study, surface-attached marine bacteria were isolated from early marine biofilms formed on antimicrobial-free commercial paint coated coupon exposed to filtered seawater collected from Tanjung Balau, Desaru seashore, Kota Tinggi, Malaysia. Later, isolated strains with distinguishable morphology characteristics on culture plate were test for biochemical properties and biofilm-forming ability before identified using 16S rRNA techniques. The EPS productivity of the identified bacterial strains were measured, and the EPS components were characterised. A better understanding of the bacterial community colonizing a commercial paint is of interest for both paint formulators and marine microbiologists as deeper knowledge of these early colonizers is required to design more efficient antibacterial or micro-textured surfaces to protect the structure from deleterious biofouling.

MATERIALS AND METHODS

Sample collection

The seawater was collected 30 m away from Tanjung Balau, Desaru seashore, Kota Tinggi, Malaysia (1.6135° N, 104.2582° E) at 1 m depth. The seawater was immediately transported to the laboratory and stored at 4 °C before filtered with 0.2 µm filter membrane prior to use.

Isolation of bacteria

The experiment was done in triplicate comprising three independent experiments on each coated glass coupons. Glass coupons (38 mm × 26 mm × 2 mm) coated with antimicrobial-free commercial paint were exposed to the filtered seawater in dynamic condition shaken at 120 rpm for 7 days at temperature of 30 °C. After proper incubation, the coated glass coupons were removed and washed with 200 L of phosphate buffer saline (PBS) solution to remove non-adherent bacteria cells. The attached bacteria biofilm formed on the paint coated surfaces were collected by swabbing method using a sterile cotton swab that moistened with PBS and wiping the coupons in two directions; from top to bottom and from left to right (Asséré *et al.*, 2008). Later, the cotton swab was cut and placed in test tube containing 10 mL PBS solution before vigorously vortexes for 10 min to remove thoroughly any remaining bacteria biofilm (Luppens *et al.*, 2002). The dilutions of PBS (from 10^{-1} until 10^{-10} dilution) were used to recover microorganisms from the swabs and 0.1 mL from each dilution factor was transferred on marine agar (MA) using spread plate technique and incubated at 30 °C for 24-48 h. Bacterial colonies showing distinctive colony morphology on the culture plate were restreaked onto fresh plates (1 morphotype per plate) and further incubated for 24-48 h at 30 °C. The restreaking process was continues until pure morphotype colonies were obtained. The pure bacteria culture was stored into glycerol stock at –80 °C prior for characterization.

Biochemical characterization of bacteria

The selected bacterial strains were characterized by using series of biochemical tests. Tests employed for presumptive identification included the tests for motility, catalase, oxidase, urease, methyl red, indole, citrate utilization and starch hydrolysis. The characterization methods of the isolates were following the standard microbiological methods (Cappuccino and Sherman, 2005).

Screening of bacterial strains for biofilm forming ability

Test tube assay

This assay was carried out according to modified method from Christensen *et al.* (1982). A total of 100 µL overnight culture were re-cultured in fresh marine broth (MB) and incubated for 48 h at 30 °C. After incubation, the culture on the test tube were decanted and washed with sterile PBS and dried overnight at room temperature. The dried tubes were stained with 0.1% (w/v) crystal violet (CV) and allowed to stand for 15 min at room temperature. The excess stain was removes by washing the tubes with sterile deionized water. The formation of visible film on the wall and bottom of the tubes were recorded to determine the condition of the biofilm formed (air liquid interface or flat-bottom) (Coffey and Anderson, 2014).

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Microtiter plate assay

The assay was performed using method as described by Stepanović *et al.* (2007) with some modification. A total of 100 µL of an overnight isolated strain cultured in MB with concentration ~10^8 CFU/mL were transferred into 96-well flat bottom microtiter plate (Techno Plastic Product (TPP), Switzerland) and incubated at 30 °C in static and dynamic condition (shake 120 rpm) for 7 days. After 7 days, the microtiter plate wells contents were discharged and rinsed gently with sterile PBS to remove non-adherent planktonic cells. They were then exposed to the hot air before allowed to dry overnight in inverted position. Later, the biofilm formed on the wells were fixed by 250 µL of CV (0.1%) for 15 min before rinsed with deionized water and kept for drying overnight. Next, 150 µL of 95% ethanol was added on the wells to dissolve the CV remained in the wells. Finally, 125 µL from the solution were transferred into new sterile microtiter plate prior to optical density measurement at 570 nm (OD570). Sterile MB without bacteria was used as the control for the assay. The interpretation of biofilm production was done according to the criteria by Stepanović *et al.* (2007). Based on the OD reading of the isolated bacteria and optical density of control (ODc) the biofilm formation was categorized as follows; OD ≤ ODc: Not a biofilm produces; ODc < OD ≤ 2 ODc: Weak biofilm producer; 2 ODc < OD ≤ 4 ODc: Moderate biofilm producer and 4 ODc < OD: Strong biofilm producer.

Identification of bacteria strains

Selected strains were identified based on the partial sequence of 16S rRNA analysis. The genomic DNA of each strain was isolated with a Wizard Genomic DNA Purification Kit (Promega, USA). The extracted genomic DNA templates which approximately 1.5 kb long fragment of 16S rRNA gene was amplified by using universal forward primer sets 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 1518R: 5'-AAGGAGGTGATCCANCCRCA-3' (Giovannoni and Rappé, 2000). The PCR product was purified using PCR purification kit (Promega, USA) prior to DNA sequencing. The full-length 16S sequences data then were analyzed by comparative studies for sequence homology using the BLAST (Basic Local Alignment Search Tool) programs from the National Center for Biotechnology Information (NCBI). The partial sequences of the 16S rRNA gene of the isolated bacteria were then submitted to NCBI Genbank.

Bacterial biofilm EPS analysis

Extraction of EPS

Isolated bacterial strains grown overnight on MA were inoculated in MB before pre-incubated at 30 °C for 24 h. A 200 µL from the overnight culture broth was re-inoculated into 50 mL of MB and shaken at 30 °C for 7 days at 120 rpm. The dead cells were separated from the media by centrifugation at 10,000 ×g for 20 min. The separated pellet and the supernatant (EPS) were stored at 4 °C for further analysis (Jiao *et al.*, 2010).

EPS producing ability of selected bacterial strains

Supernatant from the EPS extraction were mixed with 3 volumes of 99% ethanol and allowed to stand at 4 °C for 24 h. After that, the culture broth was re-centrifuged at 10,000 ×g, 4 °C for 20 min. The weight of supernatant containing total EPS and the cell pellet extracted from the isolated bacteria biofilm was measured after drying at 80 °C for 3 days (Kwon *et al*., 2002).

Chemical characterization of EPS

The EPS components were identified based on the presence of functional groups by using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTiR, Nicolet iS10) Spectroscopy (Thermo Fisher Scientific, USA) with spectra rage between 4000 cm^{-1} to 400 cm^{-1}. The dried EPS was ground in mortar before mixed with a full spatula of potassium bromide (KBr). The EPS-KBr mixture was grinded until it became pasty and sticks to the mortar. The EPS-KBr sticky pasty were transfer into 3 mm die set (PIKE Technology, USA) and pressed for 2 min to mould it into a thin and transparent pallet. The pallet then was placed in ATR-FTiR Spectroscopy chamber for analysis.

RESULTS AND DISCUSSION

Isolation and biochemical characterization of bacteria

The bacterial numbers on the three paint coated glass coupons exposed to seawater yielded the bacterial growth averaging 42.3 ± 2.2 × 10^5 CFU/mL after 7 days incubation. From these coupons, a total 40 colonies of bacteria were isolated from the paint coated surfaces, but only ten colonies with distinguishable morphology characteristics on culture plate were selected for purification and identification. These isolates were initially designated as *PS1*, *PS2*, *PS3*, *PS4*, *PS5*, *PS6*, *PS7*, *PS8*, *PS9 and PS10*. Isolated colonies were presented in different shapes and colours on culture plates. The cell shape and Gram staining of the isolates were recorded in Table 1. Majority of isolates generate circular shape colonies except *PS1*, *PS4* and *PS6*. According to van Teeseling *et al.* (2017), the unique morphological characteristics of bacteria was results of adaptation to certain surrounding environmental conditions and it also serves an important biological function. In addition, Young (2007) suggested that the presence of multiple selective forces including nutrient uptake, motility and predation may drive morphological variations in bacterial.

Regarding of pigmentation, four isolates (*PS3*, *PS4*, *PS6* and *PS7*) from ten isolated colonies were non-pigmented colonies with beige (*PS4* and *PS6*) and white (*PS3* and *PS7*) in colour. Except *PS1* has brownish pigment, other isolates have yellowish pigment. Studies

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shown that pigmented bacteria community in marine environment was diverse and widely distributed (Du *et al.*, 2006). Various type of bacteria synthesizes pigment molecules as secondary metabolites (Rao *et al.*, 2017). Production of these bioactive pigment molecules by bacterial appears to mediate by the quorum-sensing mechanism (Grossart *et al.*, 2009). These pigments are not just colour but it plays an important role to increase their survivability (Agogué *et al.*, 2005) by protecting the bacterial cells from injurious due to exposure to light in the UVA-blue and visible spectral ranges (Du *et al.*, 2006).

Table 1: Morphological characterization of early stage marine bacteria biofilm formation on paint coated surfaces-attached bacteria immersed in filtrated seawater for 7 days.

Strains	Colony shape	Pigmentation	Cell shape	Gram staining
PS1	Irregular	Pigmented (Brownish)	Rod	Negative
PS2	Spindle	Pigmented (Yellowish)	Rod	Negative
PS3	Circular	Non-Pigmented (White)	Rod	Negative
PS4	Spindle	Non-Pigmented (Beige)	Rod	Negative
PS5	Circular	Pigmented (Yellowish)	Rod	Negative
PS6	Spindle	Non-Pigmented (Beige)	Rod	Negative
PS7	Circular	Non-Pigmented (White)	Rod	Negative
PS8	Circular	Pigmented (Yellowish)	Rod	Positive
PS9	Circular	Pigmented (Yellowish)	Rod	Negative
PS10	Circular	Pigmented (Yellowish)	Rod	Negative

Interestingly, even though all ten isolates showed different colony morphologies on culture plates, at the cellular level, all isolates are shown to be a rod-shaped cell. Recent studies have suggested that rod-shaped cells help to enhance the ability of marine bacterial to prosper in their adventurous environments. The tendency of rod-shaped cells to align with nearby cells and surfaces (Volfson *et al.*, 2008; Boyer *et al.*, 2011) would implicate its gliding motility in microfluidic channels (Young, 2006; Wu *et al.*, 2013) and enables the cells to swarm together in collectives community (Cho *et al.*, 2007; Copeland and Weibel; 2009). According to Young (2006), rod-shaped bacterial may optimized it structure to overcome fluid shear stress by orient itself to lengthwise to the direction of current. This orientation increasing the bacterial adherence surface area with the internal surface while exposes a smaller circular surface area to liquid flow, hence help the bacteria to withstand greater shear forces.

The Gram staining analysis showed only isolates PS8 was Gram-positive bacteria while others are Gram-negative bacteria (Table 1). Finding from this research was consistent with previous research by Dang and Lovell (2002). Based on Dang and Lovell (2002) research on marine bacteria community attached on glass slides incubated in a salt marsh tidal creek, it has been found that 90% of the isolated bacteria are Gram-negative Proteobacteria with various characteristics. Even though isolated from different surfaces (glass, polycarbonate, polystyrene and steel, polyethylene, polyvinyl chloride) and immersion sites (South Carolina, Hawaii, USA, Sacheon harbour, Korea, Hong Kong, China, North Atlantic, California and France, Mediterranean Sea), several authors also reported similar finding on the domination of Gram-negative bacteria in marine environment (Webster *et al.*, 2004; Jones *et al.*, 2007; Lee *et al.*, 2008; Huggett *et al.*, 2009; Chung *et al.*, 2010; Zettler *et al.*, 2013; Bryant *et al.*, 2016; Pollet *et al.*, 2018). Not surprisingly, the dominancy of Gram-negative bacteria in marine environment was possibly attributed to their ability to thrive in harsh oceanic conditions (Velankar, 1954; Das *et al.*, 2006). This special survival abilities are because of the structural diversity of the cell wall which containing lipopolysaccharide (LPS) (Mandlik *et al.*, 2008). LPS not only helps regulate the permeability of the cell among other functions (Bertani and Ruiz, 2018) but also provide cell integrity and elicit an immune response to the host (Anwar and Choi, 2014). Moreover, ability to adapt and tolerate to broad range of temperature or hydrostatic pressure and fluctuations in salinity may also contribute to the extremely high (90%) Gram-negative bacteria diversity in marine environment (Nocker *et al.*, 2004; Romano *et al.*, 2016).

To investigate the biochemical properties of the strains, the isolates were subjected to a series of biochemical tests. The results of biochemical tests are presented in Table 2 respectively.

Based on biochemical analysis (Table 2), only PS5 strain showed negative result for catalase test indicated that other remaining isolated strains have ability to degrade toxic hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) with the presence of catalase enzyme. Meanwhile, only PS9 was found to secreted exoenzyme amylase which cleaves the starch into di- and monosaccharides given positive result to starch hydrolysis test (Cappuccino and Sherman, 2005). The positive result on urease test show that only PS3 strain produced urease and able to hydrolyses urea to ammonia and carbon dioxide. Among the isolated strains, only 2 strains, *PS5* and *PS6* produce tryptophanase, an enzyme that used to convert tryptophan to indole hence given positive results on indole test. Most of the isolates except *PS7* and *PS8* are able to utilize the citrate as a source of carbon and produced alkaline carbonates and bicarbonates as end-products (Ferdous *et al.*, 2013)

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Table 2: Summary of biochemical test results for all isolates.

Strains	Motility test	Catalase test	Oxidase test	Urease test	Methyl red test	Indole test	Citrate utilization test	Starch hydrolysis test
PS1	-	+	-	-	-	-	+	-
PS2	-	+	+	-	-	-	+	-
PS3	-	+	-	+	+	-	+	-
PS4	-	+	-	-	+	-	+	-
PS5	+	-	+	-	-	+	+	-
PS6	-	+	-	-	+	+	+	-
PS7	-	+	+	-	+	-	-	-
PS8	+	+	-	-	+	-	-	-
PS9	+	+	+	-	-	-	+	+
PS10	+	+	+	-	-	-	+	-

+, positive reaction; -, negative reaction

which resulted positive results on citrate test.

Bacterial motility has a profound impact on the colonization of surfaces. In order to adhere on the surface, bacteria move towards the surface with variety of mechanisms, but the most studied form of bacterial motility involves the assembly and rotation of propeller-like flagella (Jarrell and McBride, 2008). From the motility test results, it is found that only four strains; *PS5*, *PS8*, *PS9* and *PS10* possess motility ability. It is suggested that the other non-motile isolated bacteria able to attach on the surface and formed biofilm due to drag force from the shaking condition during incubation period. It is well known that the shaking of broth cultures improves aeration, oxygen and better access to nutrients through increased mixing and more homogeneous cell distribution provide more favorable growth conditions for bacteria (Juergensmeyer *et al.*, 2007; Klöckner and Büchs, 2012). In addition, greater oxygen exposure through dynamic condition not only enabling non-motile bacteria metabolism to continue at an advanced rate, but also an advantage for motile cells as they would not have to expend energy on motility.

Screening for biofilm-forming bacteria

Isolated strains were further screening for biofilm-forming ability using test tube assay and microtiter plate assay. Analysis of the test tube assay shows that all four motile isolated strains; *PS5*, *PS8* and *PS9*, *PS10* were able to produce biofilm on both air liquid interface and flat-bottom of the test tube. Other non-motile strains (*PS1*, *PS2*, *PS3*, *PS4*, *PS6* and *PS7*) can only produce biofilm on air liquid interface (Table 3). This is in agreement to O'Toole (2011) who mentioned that motile bacteria generally tend to adhere at the air-liquid interface and/or bottoms of the wells, while non-motile microbes typically adhere to the bottom of the wells. In a favourable niche, oxygen was abundance on air and nutrients in solution at the bottom. Colonization of bacteria on air-liquid interface as floating biofilm occur when attachment at the meniscus to solid surfaces may be limited. The ability to form air-liquid interface biofilms can confer significant fitness advantages in marine environment as the strains has the ability to survive in inconsistent seawater level.

Table 3: Air-liquid interface and flat-bottom biofilm-formation ability using test tube assay for the 10 isolated strains in this study.

Air-liquid interface	Flat-bottom
PS5	*PS1*
PS8	*PS2*
PS9	*PS3*
PS10	*PS4*
	PS5
	PS6
	PS7
	PS8
	PS9
	PS10

Mostly the microtiter plate assay was performed under standard static conditions and little is known about the effect of hydrodynamics and flow pattern to the assay. In this study, two different cultivation conditions: static and dynamic condition were performed to investigate the effect of cultivation conditions on marine biofilm formation in microtiter plate assay. Figure 1 summarizes the mean OD value of 10 isolated strains after 7 days recorded in the microtiter plate assay under static and dynamic conditions. In static condition, the OD reading of the isolates were in the range of 0.208 to 3.040 with the highest OD recorded was from strain *PS10* and the lowest was from strain *PS3*. Meanwhile in dynamic condition, highest OD reading recorded is 2.814 (*PS4*) and the lowest OD is 1.340 (*PS9*). This screening revealed that the biofilm formation ability was very variable according to the bacterial strains and also the cultivation condition.

Based on the OD reading, comparative analysis to determine the ability of the isolated strains to produce biofilm under static and dynamic conditions was performed by categorizing the bacteria according to the criteria of Stepanović *et al.* (2007) as shown in Table 4. Three strains, strain *PS1, PS9* and *PS10* displayed

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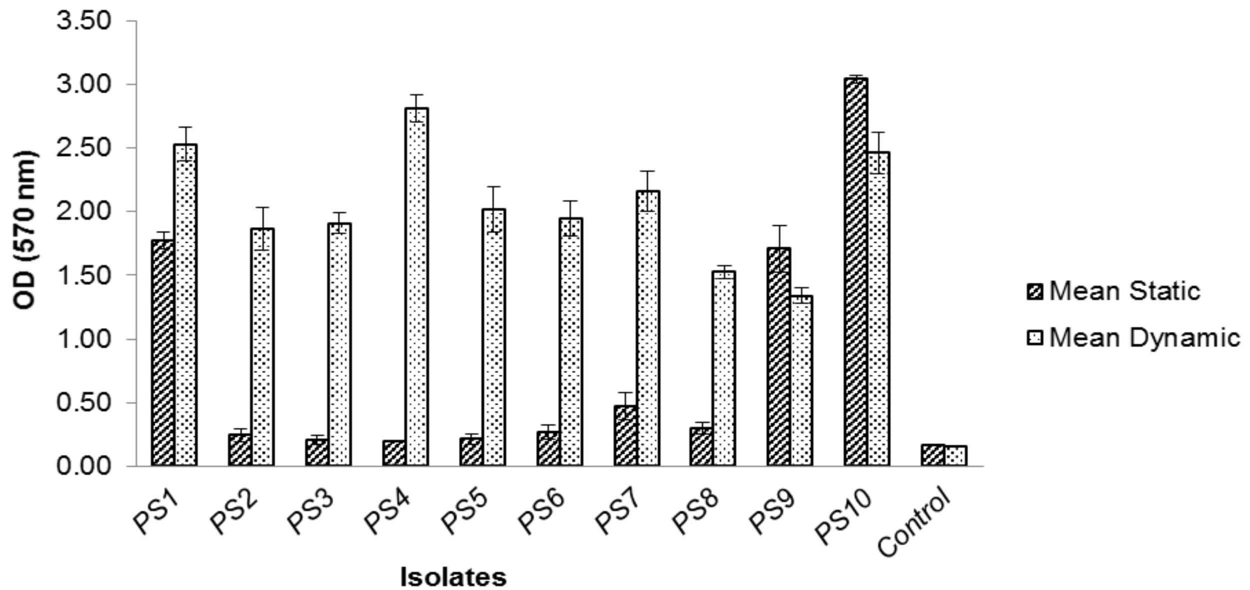

Figure 1: Mean OD (570 nm) of 10 isolated strains after static and dynamic incubation in microtiter plate for 7 days. Sterile MB without bacteria was used as the control for the assay.

similar biofilm-forming potential under both static and dynamic conditions, which are strong biofilm producer. Other strains, *PS2*, *PS3*, *PS4*, *PS6* and *PS8* were determined as non-biofilm producer in static condition but changed to strong biofilm producer in dynamic condition. Both *PS5* and *PS7* which are weak biofilm producer in static condition tend to form strong biofilm under dynamic condition. The discrepancies result on increment in marine bacterial attachment and biofilm formation under dynamic condition suggested that hydrodynamic have great impact on biofilm formation in term of transfer of oxygen and nutrient. Dynamic cultivation condition increased the air-liquid interface area and promote a relatively high oxygen transfer rate which further encouraged bacterial growth and biofilm formation (Masi *et al.*, 2015; Brück *et al.*, 2020).

Identification of selected strains

The strains have been identified using 16S rRNA and the nucleotides generated from this work were subjected to BLAST analysis. The sequence similarity was analysed through the NCBI nucleotide BLAST with a non-redundant database. It is generally accepted that an unidentified isolate whose 16S rRNA sequence showing similarity greater than 97% can be represented at the species level, while the sequence similarities between 93-97% can represent identity at the genus level (Stackebrandt and Ebers, 2006; Kim *et al.*, 2014). The details of BLAST analysis, percentage of similarity, NCBI accession numbers and the systematic classification of the isolated strains were listed in Table 5. Among 10 strains, three were identified as *Marinomonas communis* (*PS2*, *PS6* and *PS7*), two were *Marinomonas* sp. (*PS3* and *PS4*), while others were *Salinimonas lutimaris* (*PS1*), *Idiomarine baltica* (*PS5*), *Bacillus niabensis* (*PS8*), *Alteromonas litorea* (*PS9*) and *Alteromonas* sp. (*PS10*).

It is found that all isolated strains are belongs to Proteobacteria phylum and Gammaproteobacteria class except for *B. niabensis* (*PS8*). Similar finding reported by Dang and Lovell (2016) on isolates colonizers on polystyrene, where most dominant isolates that identified are members of Proteobacteria phylum and Gammaproteobacteria class, consisting of Alteromonadales and Oceanospirillales groups. In line to this finding, Rampadarath *et al.* (2017) also reported on

Table 4: Biofilm-forming capability of isolated strains under different conditions (static and dynamic) categorized based on Stepanović *et al.* (2007) characterization.

Bacteria strain	Biofilm producer	
	Static	Dynamic
PS1	Strong	Strong
PS2	None	Strong
PS3	None	Strong
PS4	None	Strong
PS5	Weak	Strong
PS6	None	Strong
PS7	Weak	Strong
PS8	None	Strong
PS9	Strong	Strong
PS10	Strong	Strong

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Table 5: Details of BLAST analysis, percentage of similarity, NCBI accession numbers and systematic classification of the isolated strains.

Strain	BLAST results	Percentage of similarity (%)	NCBI Genbank accession number	Systematic classification	
				Phylum	Class
PS1	*Salinimonas lutimaris*	93	KY363645	Proteobacteria	Gammaproteobacteria
PS2	*Marinomonas communis*	94	KY368113	Proteobacteria	Gammaproteobacteria
PS3	*Marinomonas* sp.	93	KY368114	Proteobacteria	Gammaproteobacteria
PS4	*Marinomonas* sp.	93	KY385427	Proteobacteria	Gammaproteobacteria
PS5	*Idiomarine baltica*	91	KY392959	Proteobacteria	Gammaproteobacteria
PS6	*Marinomonas communis*	91	KY368115	Proteobacteria	Gammaproteobacteria
PS7	*Marinomonas communis*	91	KY385426	Proteobacteria	Gammaproteobacteria
PS8	*Bacillus niabensis*	98	KY368116	Firmicutes	Bacilli
PS9	*Alteromonas litorea*	99	KY385425	Proteobacteria	Gammaproteobacteria
PS10	*Alteromonas* sp.	93	KY368126	Proteobacteria	Gammaproteobacteria

the predominant of Proteobacteria which members affiliated to Gammaproteobacteria isolated from biofilm grown on microfiber nets exposed to seawater for a period of 24 h in Mauritian (southeast coast of the African continent) coastal water. This identical finding was supported by metagenomic data which suggested that Proteobacteria, a major phylum of Gram-negative bacteria comprise more than half of the marine pelagic microbial communities (Sunagawa *et al.*, 2015). Previous studies by Eilers *et al.* (2000) and Giovannoni and Rappé (2000) shown that culturable marine microbes from seawater fall predominantly within the gamma subclass of the Proteobacteria clade. However, the dominancy of Proteobacteria population that isolated on artificial surface which exposed to seawater (Jones *et al.*, 2007; Huggett *et al.*, 2009; Chung *et al.*, 2010) are also depends on several environmental factors such as nutrient availability (Chiu *et al.*, 2008), temperature (Lau *et al.*, 2005) and the surface condition (Jones *et al.*, 2007; Lee *et al.*, 2008). The dominancy of Gammaproteobacteria class in marine biofilm communities are also associated with carbohydrate metabolism as well as in tolerance of polysaccharide biodegrading which contribute to their survival in marine stressed environment (Rampadarath *et al.*, 2017).

EPS producing ability of selected bacterial strains

Figure 2 shows the data collected from the EPS extraction. Even though the growth medium and condition were identical, data in Figure 2 showed that there are distinct differences in the constituents of identified strains biofilm formed on the surface of the paint coating. The dried cell weight of 10 isolated marine bacteria strains ranged from 0.15 [*M. communis* (*PS2*)] to 0.46 [*Marinomonas* sp. (*PS4*)] g/L and the dried weight of EPS ranged from 1.95 [*Marinomonas* sp. (*PS4*)] to 2.89 [*B. niabensis* (*PS8*)] g/L after 7 days dynamic incubation in MB. The dried weight EPS production ability yield of all isolates were greater than 1.0 g/L, hence all isolates were classified as strong EPS producer (Nwosu *et al.*, 2019). Among isolated strains, *B. niabensis* (*PS8*) is the most potent EPS producer as it produced copious amount of EPS up to 2.89 g/L followed by *S. lutimaris* (*PS1*) with 2.72 g/L and *A. litorea* (*PS9*) with 2.64 g/L.

The EPS productivity are differing from one strain to another. Highest EPS production was recorded from isolate *B. niabensis* (*PS8*) (24.08) while lowest EPS production was from *Marinomonas* sp. (*PS4*) (4.24). The EPS production trend was indirectly proportional to the dried cell weight where strains showing a superior cell growth showed lowest EPS production. Compared to EPS productivity of marine bacteria isolated on glass slides (Kwon *et al.*, 2002), it is revealed that isolated marine bacteria strain on paint surface had higher EPS productivity compared to on glass surface and it is found that strains showing superior cell growth showed lowest EPS production (Kwon *et al.*, 2002).

The composition and quantity of EPS production are depending on the type of microorganisms (Kavita *et al.*, 2011), age of the biofilms (Zhang *et al.*, 2010) and response to other environmental stresses under which the biofilms exist (Villeneuve *et al.*, 2011). The environmental stresses conditions are including availability of nutrients, different levels of oxygen and nitrogen, osmotic pressure, temperature, pH, salinity, defence against other competing microorganisms or predator, presence of toxins and high levels of heavy metals (Shukla and Dave, 2018). The production of EPS also related to the physiological stage of the biofilms (Sabater *et al.*, 2007). Most marine bacteria produce maximum EPSs either in stationary or exponential phase (Shukla and Dave, 2018). In contrast, according to research conducted by

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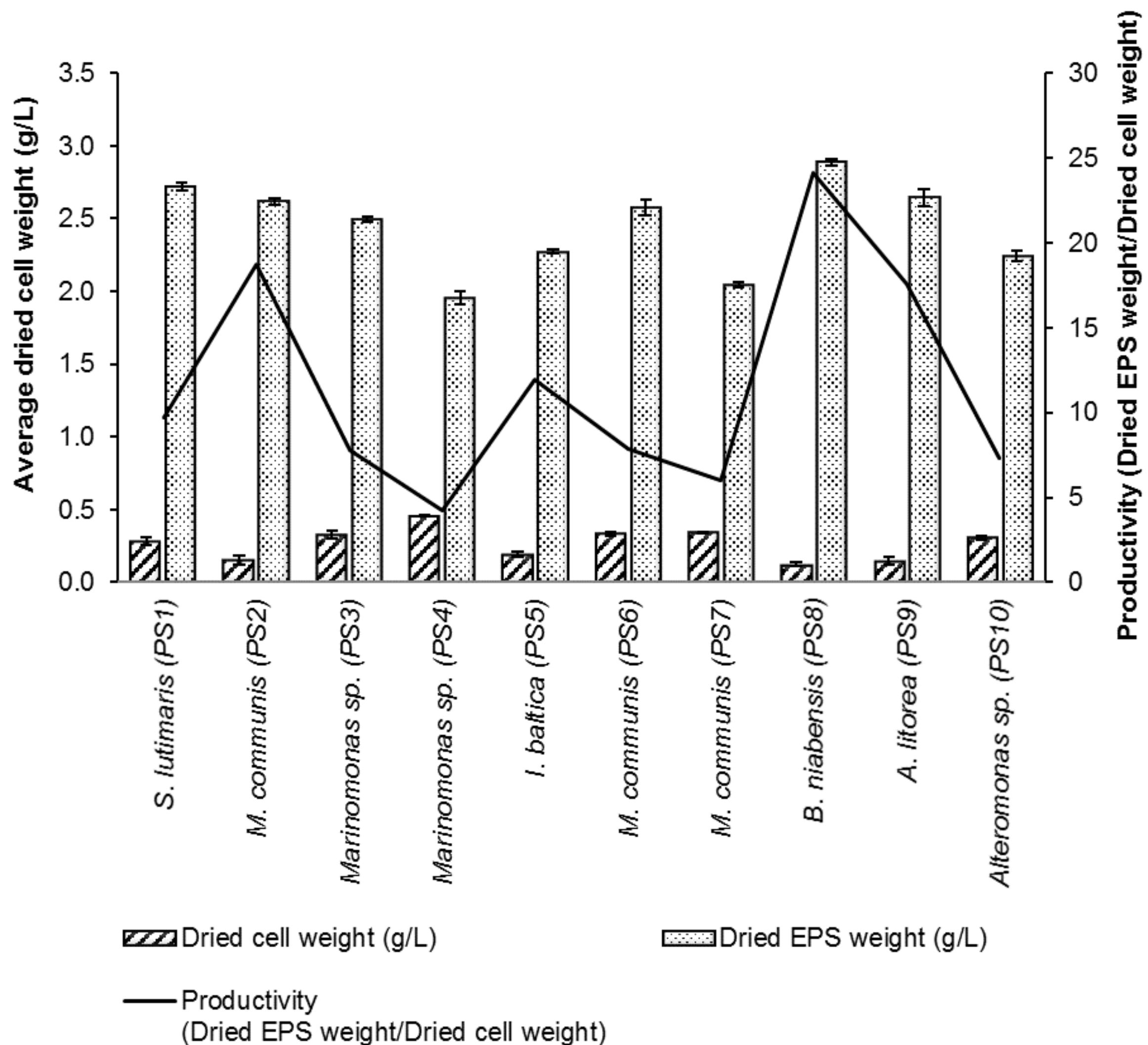

Figure 2: Comparison of biomass, dried EPS and productivity of EPS by isolated marine bacteria.

Zhang *et al.* (2010), it has been shown that the highest productivity of EPS is observed during early stages of biofilm formation. Another research by Jiao *et al.* (2010) found that quantity of EPS of acidophilic microbial biofilm increased more than twice during maturation stage compared to mid-developmental stage (approximately 340 mg and 150 mg of dry weight of EPS per g).

Chemical characterization of EPS

FTIR spectra of EPS extracted from identified strains are presented in Figure 3. The COOH (1600 cm^{-1} to 1725 cm^{-1}) and –OH (2800 cm^{-1} to 3600 cm^{-1}) functional groups detected in the samples indicated that the samples were bacterial cell EPSs (Ortega-Morales *et al.*, 2007). The presence of other similar peaks corresponding to functional groups like –COC–, PO_4^-, $–NH_2^-$ and $–CH_3^-$, $–CH_2^-$ in the EPS spectra of identified strains revealed the presence of carbohydrates, nucleic acid, protein, DNA/RNA and lipid was mentioned in Table 6. The differences in both shape and absorbance intensity, indicating that there was variation in the composition quantity and quality of each individual component.

The FTIR analysis of the EPS composition (Figure 3) showed similar results as reported by Kumar *et al.* (2011). Although Kumar *et al.* (2011) isolated marine bacteria from different surface (glass slides) exposed to coastal area of Tuticorin in South India, the presence of main composition components; carbohydrate, nucleic acid, DNA/RNA, protein and lipid in the EPS are still similar. However, relative concentrations of each components were different among the bacterial strains.

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Table 6: Assignments of FTiR spectrum principal infrared vibrational signals functional group of a bacterial cell EPS.

Region	FTiR spectrum wavelength (cm^{-1})	Functional group assignment	References
Lipid	3000-2830	C–H stretching of $–CH_3$ and $>CH_2$	Alvarez-Ordóñez *et al.*, 2011; Mangwani *et al.*, 2012
Protein	1730-1590	Amide I, C=O different conformation	Long *et al.,* 2009; Alvarez-Ordóñez *et al.*, 2011
	1590-1490	Amide II, N–H, C–N and C (=O) NH–R	Long *et al.*, 2009; Mangwani *et al.*, 2012
Nucleic acid	1280-1190	P=O band	Alvarez-Ordóñez *et al.*, 2011
DNA/ RNA	1490-1000	Amide III	Alvarez-Ordóñez *et al.*, 2011
Carbohydrate	2900-3300	C–OH, C–O–C from polysaccharides	Mangwani *et al.*, 2012

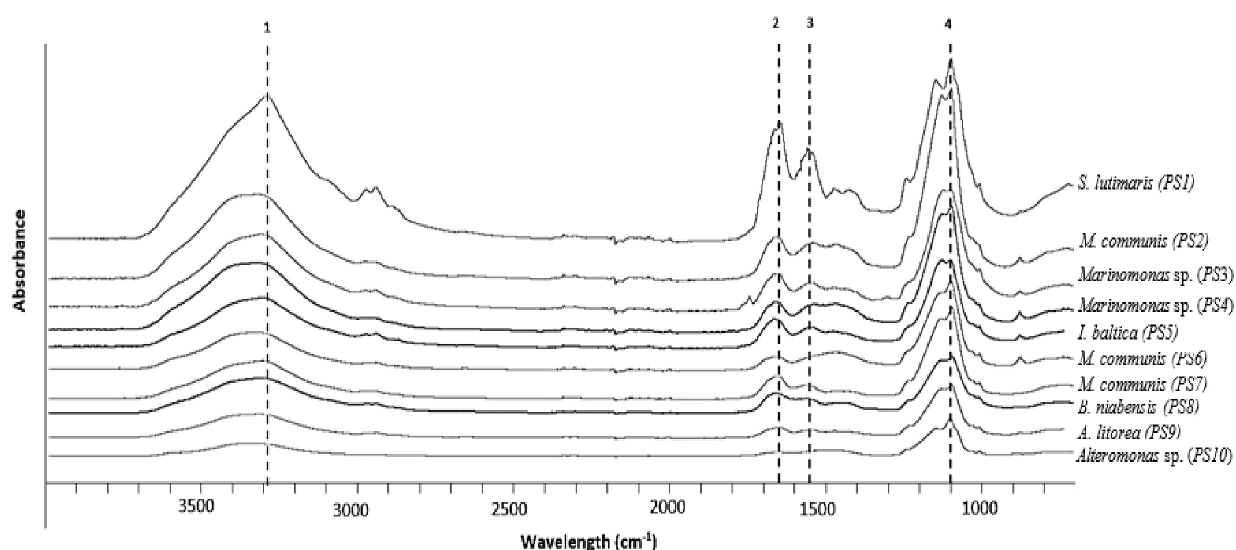

Figure 3: FTiR spectra of EPS from isolated strains biofilms. Peak 1: vibrations result from stretching of the C-OH and C-O-C bond (glycoside bond) in carbohydrate; Peak 2: C-H symmetric stretching of the $-CH_3$ and $>CH_2$ in fatty acids in lipids; Peak 3: C=O bond in the peptide group Amide I in protein; Peak 4: combination of N-H bending and C-N stretching Amide II in proteins; Peak 5: P=O asymmetric and symmetric stretching of DNA, RNA and phospholipids; Peak 6: symmetrical stretching P=O of phosphate groups in nucleic acid.

The composition of EPS produced by the bacteria are mainly influenced by wide range of physical, chemical and biological variables and processes (Velmourougane *et al.*, 2017). Other factors such as the condition of the surface growth (presence or absence of biocide, the type of biocide, the rate of biocide release, the coating matrix type and the surface energy) and the environmental variation conditions (biological, chemical, physical and hydrodynamic) where it had been exposed to also have crucial roles in determining the EPS compositions (Salta *et al.*, 2013; Doghri *et al.*, 2015; Flemming, 2016; Parkar *et al.*, 2017).

Besides major peaks stated in Table 6, some small peaks and vibrations bands also appeared in the FTIR spectra of the identified strains EPS. The small peak in region below 1500 cm^{-1} (the fingerprint region) indicated the presence of sulphated groups in the EPS (Wang *et al.*, 2017). Another small peak observed at 2170 cm^{-1} revealed the presence of free carboxylic functional groups in the EPS samples (Osman *et al.*, 2012). The presence of carboxyl groups in the FTIR spectra of the identified strains EPS indicates that it may serve as the binding site for divalent cations (Bramhachari *et al.*, 2007). The small vibration absorption bands in the region 1219 cm^{-1}

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detected on all strains EPS usually represents the stretching vibration of C–O, alcohol, ester, ether and phenol functional groups (Botelho *et al.*, 2014). Orsod *et al.* (2012) reported absorption of alkenes, ketones, isocyanate and isothiocyanate groups, alcohols, ethers, esters carboxylic acids and phenols groups are commonly detected in the FTIR spectrum of EPS extracted from marine bacteria. The presence of these phenolic and carboxylic functional groups in the EPS spectra may possibly account for antibacterial activity (Anju *et al.*, 2010). A unique peak at 836 cm^{-1} detected in EPS samples from *S. lutimaris* (PS1), *M. communis* (*PS2*), *Marinomonas* sp. (*PS3*), *Marinomonas* sp. (*PS4*), *I. baltica* (*PS5*) to *M. communis* (*PS6*) revealed the presence of α-D glucan in the strains EPS (Wang *et al.*, 2015). The presence of many protein-related amine and amide groups in EPS spectra of identified strains revealed possibility of the strains to produce toxic and may contribute to pathogenesis (Anju *et al.*, 2010).

CONCLUSION

The present study has found that there are marked differences in bacterial diversity and the bacteria EPS composition produced by biofilm communities developed on antimicrobial-free commercial paint surface. Even though the bacterial groups cultured in this study are only represent the attached culturable bacteria population but not microbial community, the data are still beneficial for better exploration and understanding of the physiological characteristics of the biofilm isolates. The knowledge of marine bacterial composition of targeted biofilm layer is important as it will provide fundamental knowledge in the development of effective measures and methods to minimise the formation of biofilm on marine associated surfaces, hence preventing the occurrence of biofouling in marine environment.

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REFERENCES

Alvarez-Ordóñez, A., Mouwen, D. J. M., López, M. and Prieto, M. (2011). Fourier transform infrared spectroscopy as a tool to characterize molecular composition and stress response in foodborne pathogenic bacteria. *Journal of Microbiological Methods* **84(3), 369-378.**

Agogué, H., Joux, F., Obernosterer, I. and Lebaron, P. (2005). Resistance of marine bacterioneuston to solar radiation. *Applied and Environmental Microbiology* **71, 5282-5289.**

Anju, P., Milind, N. and Santosh Kumar, D. (2010). Hemolysin, protease, and EPS producing pathogenic *Aeromonas hydrophila* strain An4 shows antibacterial activity against marine bacterial fish pathogens. *Journal of Marine Biology* **10, 1-9.**

Anwar, M. A. and Choi, S. (2014). Gram-negative marine bacteria: Structural features of lipopolysaccharides and their relevance for economically important diseases. *Marine Drugs* **12(5), 2485-2514.**

Asséré A., Oulahal N. and Carpentier B. (2008). Comparative evaluation of methods for counting surviving biofilm cells adhering to a polyvinyl chloride surface exposed to chlorine or drying. *Journal of Applied Microbiology* **104, 1692-170.**

Bertani, B. and Ruiz, N. (2018). Function and biogenesis of Lipopolysaccharides. *EcoSal Plus* **8(1).**

Botelho, P. S., Maciel, M. I., Bueno, L. A., Maria de Fátima, F. M., Marques, D. N. and Silva, T. M. S. (2014). Characterisation of a new exopolysaccharide obtained from of fermented kefir grains in soymilk. *Carbohydrate Polymers* **107, 1-6.**

Boyer, D., Mather, W., Mondragón-Palomino, O., Orozco-Fuentes, S., Danino, T., Hasty, J. and Tsimring, L. S. (2011). Buckling instability in ordered bacterial colonies. *Physical Biology* **8(2), 026008.**

Bramhachari, P. V., Kavi Kishor, P. B., Ramadevi, R., Kumar, R., Rama Rao, B. and Dubey, S. K. (2007). Isolation and characterization of mocous exopolysaccharide (EPS) produced by *Vibrio furnissii* strain VB0S3. *Journal of Microbiology and Biotechnology* **17(1), 44-51.**

Brian-Jaisson, F., Ortalo-Magné, A., Guentas-Dombrowsky, L., Armougom, F., Blache, Y. and Molmeret, M. (2014). Identification of bacterial strains isolated from the Mediterranean Sea exhibiting different abilities of biofilm formation. *Microbial Ecology* **68(1), 94-110.**

Brück, H. L., Coutte, F., Dhulster, P., Gofflot, S., Jacques P. and Delvigne, F. (2020). Growth dynamics of bacterial populations in a two-compartment biofilm bioreactor designed for continuous surfactin biosynthesis. *Microorganisms* **8(5), 679.**

Bryant, J. A., Clemente, T. M., Viviani, D. A., Fong, A. A., Thomas, K. A., Kemp, P., Karl, D. M., White, A. E. and DeLong, E. F. (2016). Diversity and activity of communities inhabiting plastic debris in the North Pacific Gyre. *mSystems* **1(3), e00024-16.**

Cao, S., Wang, J., Chen, H. and Chen, D. (2011). Progress of marine biofouling and antifouling technologies. *Chinese Science Bulletin* **56, 598-612.**

Cappuccino, J. G. and Sherman, N. (2005). Microbiology A Laboratory Manual. Pearson, New York.

Chiu, J., Zhang, R., Wang, H., Thiyagarajan, V. and Qian, P. (2008). Nutrient effects on intertidal community: From bacteria to invertebrates. *Marine Ecology Progress Series* **358, 41-50.**

Cho, H., Jönsson, H., Campbell, K., Melke, P., Williams, J. W., Jedynak, B., Stevens, A. M., Groisman, A. and Levchenko, A. (2007). Self-

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

organization in high-density bacterial colonies: Efficient crowd control. *PLoS Biology* **5(11), e302.**

Christensen, G. D., Simpson, W. A., Bisno, A. L. and Beachey, E. H. (1982). Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infection and Immunity* **37(1), 318-326.**

Chung, H., Lee, O., Huang, Y., Mok, S. Y., Kolter, R. and Qian, P. Y. (2010). Bacterial community succession and chemical profiles of subtidal biofilms in relation to larval settlement of the polychaete *Hydroides elegans*. *ISME Journal* **4, 817-828.**

Coffey, B. M. and Anderson, G. G. (2014). Biofilm formation in the 96-well microtiter plate. *Methods in Molecular Biology* **1149, 631-641.**

Copeland, M. F. and Weibel, D. B. (2009). Bacterial swarming: A model system for studying dynamic self-assembly. *Soft Matter* **5, 1174-1187.**

Dang, H. and Lovell, C. R. (2002). Numerical dominance and phylotype diversity of marine *Rhodobacter* species during early colonization of submerged surfaces in coastal marine waters as determined by 16S ribosomal DNA sequence analysis and fluorescence in situ hybridization. *Applied and Environmental Microbiology* **68, 496-504.**

Dang, H. and Lovell, C. R. (2000). Bacterial primary colonization and early succession on surfaces marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. *Applied and Environmental Microbiology* **66, 467-475.**

Dang, H. and Lovell, C. R. (2016). Microbial surface colonization and biofilm development in marine environments. *Microbiology and Molecular Biology Reviews* **80(1), 91-138.**

Dang, H., Li, T., Chen, M. and Huang, G. (2008). Cross-ocean distribution of Rhodobacterales bacteria as primary surface colonizers in temperate coastal marine waters. *Applied and Environmental Microbiology* **74, 52-60.**

Das, S., Lyla, P. S. and Ajmal Khan, S. (2006). Marine microbial diversity and ecology: Importance and future perspectives. *Current Science* **10, 90-95.**

de Carvalho, C. C. C. R. and Fernandes, P. (2010). Production of metabolites as bacterial responses to the marine environment. *Marine Drugs* **8, 705-727.**

de Carvalho, C. C. C. R. (2018). Marine biofilms: A successful microbial strategy with economic implications. *Frontiers in Marine Science* **5, 126.**

Decho, A. W. and Gutierrez, T. (2017). Microbial extracellular polymeric substances (EPSs) in ocean systems. *Frontiers in Microbiology* **8, 922.**

Doghri, I., Rodrigues, S., Bazire, A., Dufour, A., Akbar, D., Sopena, V. and Lanneluc, I. (2015). Marine bacteria from the French Atlantic coast displaying high forming-biofilm abilities and different biofilm 3D architectures. *BMC Microbiology* **15, 231.**

Du, H., Jiao, N., Hu, Y. and Zeng, Y. (2006). Diversity and distribution of pigmented heterotrophic bacteria in marine environments. *FEMS Microbiology Ecology* **57(1), 92-105.**

Eilers, H., Pernthaler, J., Glöckner, F. O. and Amann, R. (2000). Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology* **66(7), 3044-3051.**

Fitridge, I., Dempster, T., Guenther, J. and de Nys, R. (2012). The impact and control of biofouling in marine aquaculture: A review. *Biofouling* **28(7), 649-669.**

Flemming, H. C. and Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology* **8, 623-633.**

Flemming, H. (2016). EPS—then and now. *Microorganisms* **4(41).**

Ferdous, T. A., Kabir, S. M. L., Amin, M. M. and Hossain, K. M. M. (2013). Identification and antimicrobial susceptibility of *Salmonella* species isolated from washing and rinsed water of broilers in pluck shops. *Journal of Animal and Veterinary Advances* **5(1), 1-8.**

Giovannoni, S. and Rappé, M. (2000). Evolution, diversity and molecular ecology of marine prokaryotes. Microbial ecology of the oceans. John Wiley & Sons Inc., New York. **pp. 47-84.**

Grossart, H. P., Thorwest, M., Plitzko, I., Brinkhoff, T., Simon, M. and Zeeck, A. (2009). Production of a blue pigment (glaukothalin) by marine *Rheinheimera* spp. *International Journal of Microbiology* **1-7, 2009, Article ID 701735**

Huggett, M., Nedved, B. and Hadfield, M. (2009). Effects of initial surface wettability on biofilm formation and subsequent settlement of *Hydroides elegans*. *Biofouling* **25, 387-399.**

Jarrell, K. F. and McBride, M. J. (2008). The surprisingly diverse ways that bacteria move. *Nature Reviews Microbiology* **6(6), 466-476.**

Jiao, Y., Cody, G. D., Harding, A. K., Wilmes, P., Schrenk, M., Wheeler, K. E., Banfield, J. F. and Thelen, M. P. (2010). Characterization of extracellular polymeric substances from acidophilic microbial biofilms. *Applied and Environmental Microbiology* **76(9), 2916-2922.**

Jones, P. R., Cottrell, M. T., Kirchman, D. L. and Stephan, C. D. (2007). Bacterial Community Structure of Biofilms on Artificial Surfaces in an Estuary. *Microbial Ecology* **53, 153-162.**

Juergensmeyer, M. A., Nelson, E. S. and Juergensmeyer, E. A. (2007). Shaking alone, without concurrent aeration, affects the growth characteristics of *Escherichia coli*. *Letters in Applied Microbiology* **45(2), 179-183.**

Kavita, K., Mishra, A. and Jha, B. (2011). Isolation and physico-chemical characterisation of extracellular polymeric substances produced by the marine bacterium *Vibrio parahaemolyticus*. *Biofouling* **27, 309-317.**

Kim, M., Oh, H. S., Park, S., C. and Chun, J. (2014). Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology* **64, 346-351.**

Klöckner, W. and Büchs, J. (2012). Advances in shaking technologies. *Trends in Biotechnology* **30(6) 307-314.**

Kumar, M. A., Anandapandian, K. T. K. and Parthiban, K. (2011). Production and characterization of exopolysaccharides (EPS) from biofilm forming marine bacterium. *Brazilian Archives of Biology and Technology* **54(4), 259-265.**

Kwon, K. K., Lee, H. S., Jung, S. Y., Yim, J. H., Lee, J. H. and Lee, H. K. (2002). Isolation and identification of biofilm-forming marine bacteria on glass surfaces in Dae-Ho Dike, Korea. *The Journal of Microbiology* **40(4), 260-266.**

Lau, S., Thiyagarajan, V., Cheung, S. and Qian, P. (2005). Roles of bacterial community composition in biofilms as a mediator for larval settlement of three marine invertebrates. *Aquatic Microbial Ecology* **38, 41-51.**

Lee, J. W., Nam, J. H., Kim, Y. H., Lee, K. H. and Lee, D. H. (2008). Bacterial communities in the initial stage of marine biofilm formation on artificial surfaces. *The Journal of Microbiology* **46, 174-182.**

Long, G., Zhu, P., Shen, Y. and Tong, M. (2009). Influence of extracellular polymeric substances (EPS) on deposition kinetics of bacteria. *Environmental Science and Technology* **43(7), 2308-2314.**

Luppens, S. B. I., Reij, M. W., van der Heijden, R. W. L., Rombouts, F. M. and Abee, T. (2002). Development of a standard test to assess the resistance of *Staphylococcus aureus* biofilm cells to disinfectants. *Applied and Environmental Microbiology* **68, 4194-4200.**

Mandlik, A., Swierczynski, A., Das, A. and Ton-That, H. (2008). Pili in Gram-positive bacteria: Assembly, involvement in colonization and biofilm development. *Trends in Microbiology* **16(1), 33-40.**

Mangwani, N., Dash, H.R., Chauhan, A. and Das, S. (2012). Bacterial quorum sensing: functional features and potential applications in biotechnology. Microbial Physiology 22, 215-227.

Masi, E., Ciszak, M., Santopolo, L., Frascella, A., Giovannetti, L., Marchi, E., Viti, C., and Mancuso, S. (2015). Electrical spiking in bacterial biofilms. *Journal of the Royal Society Interface* **12, 20141036.**

Nocker, A., Lepo, E. J. and Snyder, A, R. (2004). Influence of an oyster reef on development of the microbial heterotrophic community of an estuarine biofilm. *Applied and Environmental Microbiology* **70, 6834-6845.**

Nwosu, I. G., Abu, G. O. and Agwa, K. O. (2019). Isolation, screening and characterization of exopolysaccharide producing bacteria. *Microbiology Research Journal International* **29(5), 1-9.**

Orsod, M., Joseph, M. and Fahrul, H. (2012). Characterization of exopolysaccharides produced by *Bacillus cereus* and *Brachybacterium* sp. isolated from Asian Sea Bass (*Lates calcarifer*). *Malaysian Journal of Microbiology* **8(3), 170-174.**

Ortega-Morales, B. O., Santiago-García, J. L., Chan-Bacab, M. J., Moppert, X., Tello, E. M., Fardeau, M. L., Carrero, J. C., Pérez, P. B., González, A. V. and Guezennec, J. (2007). Characterization of extracellular polymers synthesized by tropical intertidal biofilm bacteria. *Journal of Applied Microbiology* **102(1), 254-264. Osman, M. E. A., El-Shouny, W., Talat, R. and El-Zahaby, H. (2012).** Polysaccharides production from some *Pseudomonas syringae* pathovars as affected by different types of culture media. *The Journal of Microbiology, Biotechnology and Food Sciences* **1(5), 1305-1318.**

O'Toole, G. A. (2011). Microtiter dish biofilm formation assay. *Journal of Visualized Experiments* **e2437.**

Parkar, D., Jadhav, R. and Pimpliskar, M. (2017). Marine bacterial extracellular polysaccharides: A review. *Journal of Coastal Life Medicine* **5(1), 29-35.**

Pollet, T., Berdjeb, L., Garnier, C., Durrieu, G., Poupon, C. L., Misson, B. and Briand, J. F. (2018). Prokaryotic community successions and interactions in marine biofilms: The key role of Flavobacteria. *FEMS Microbiology Ecology* **94(6).**

Rampadarath, S., Bandhoa, K., Puchooa, D., Jeewon, R. and Bal, S. (2017). Early bacterial biofilm colonizers in the coastal waters of Mauritius. *Electronic Journal of Biotechnology* **29, 13-21.**

Rao, M. P. N., Min, X. and Jun, L. W. (2017). Fungal and bacterial pigments: Secondary metabolites with wide applications. *Frontiers in Microbiology* **8, 1113.**

Romano, G., Costantinib, M., Sansonea, C., Lauritanoa, C., Ruoccobcd, N., Ianoraa, A. (2016). Marine microorganisms as a promising and sustainable source of bioactive molecules. *Marine Environmental Research* **128, 28-69.**

Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C. and Schmitt-Jansen, M. (2007). Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Analytical and Bioanalytical Chemistry* **387(4), 1425-1434.**

Salta, M., Wharton, J. A., Blache, Y., Stokes, K. R. and Briand, J. F. (2013). Marine biofilms on artificial surfaces: Structure and dynamics. *Environmental Microbiology* **15(11), 2879-2893.**

Schultz, M. P., Bendick, J. A., Holm, E. R. and Hertel, W. M. (2011). Economic impact of biofouling on a naval surface ship. *Biofouling* **27, 87-98.**

Shukla, P. J. and Dave, B. P. (2018). Screening and molecular identification of potential exopolysaccharides (EPS) producing marine bacteria from the Bhavnagar coast, Gujarat. *International Journal of Pharmaceutical Sciences Research* **9(7), 2973-2981.**

Skovhus, T. L., Enning, D. and Lee, J. S. (2017). Microbiologically Influenced Corrosion in the Upstream Oil and Gas Industry. CRC Press, Boca Raton, Florida, United States. **pp. 15-22.**

Stackebrandt, E. and Ebers, J. (2006). Taxonomic parameters revisited: Tarnished gold standards. *Microbiology Today* **33, 152-155.**

Stepanović, S., Vukovic, D., Hola, V., Bonaventura, G. D., Djukic, S., Cirkovic, L. and Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: Overview

of testing conditions and practical's recommendations for assessment of biofilm production by *Staphylococci*. *Journal of Compilation* **115, 891-899.**

Sunagawa, S., Coelho, L. P., Chaffron, S., Kultima, J. R., Labadie, K., Salazar, G. *et al.* (2015). Structure and function of the global ocean microbiome. *Science* **348 (6237), 1261359-1-1261359-9.**

Turan, O., Demirel, Y. K., Day, S. and Tezdogan, T. (2016). Experimental determination of added hydrodynamic resistance caused by marine biofouling on ships. *Transportation Research Procedia* **14, 1649-1658.**

Van Teeseling, M. C. F., de Pedro, M. A. and Cava, F. (2017). Determinants of bacterial morphology: From fundamentals to possibilities for antimicrobial targeting. *Frontiers in Microbiology* **8.**

Velankar, N. K. (1954). Bacteria isolated from seawater and marine mud off Mandapam (Gulf of Mannar and Palk bay). *Indian Journal of Fisheries* **4(1), 208-227.**

Velmourougane, K., Prasanna, R. and Saxena, A. K. (2017). Agriculturally important microbial biofilms: Present status and future prospects. *Journal of Basic Microbiology* **57(7), 548-573.**

Villeneuve, A., Bouchez, A. and Montuelle, B. (2011). *In situ* interactions between the effects of season, current velocity and pollution on a river biofilm. *Freshwater Biology* **56(11), 2245-2259.**

Volfson, D., Cookson, S., Hasty, J. and Tsimring, L. S. (2008). Biomechanical ordering of dense cell populations. *National Academy of Sciences of the United States of America* **105(40), 15346-15351.**

Wang, H., Teng, F., Yang, X., Guo, X., Tu, J., Zhang, C. and Zhang, D. (2017). Preventing microbial biofilms on catheter tubes using ultrasonic guided waves. *Scientific Reports* **7, 616.**

Wang, J., Zhao, X., Tian, Z., Yang, Y. and Yang, Z. (2015). Characterization of an exopolysaccharide produced by *Lactobacillus plantarum* YW11 isolated from Tibet Kefir. *Carbohydrate Polymers* **125, 16-25.**

Webster, N. S., Negri, A. P., Munro, M. M. and Battershill, C. N. (2004). Diverse microbial communities inhabit Antarctic sponges. *Environmental Microbiology* **6, 288-300.**

Wu, J., Wu, X. and Lin, F. (2013). Recent developments in microfluidics-based chemotaxis studies. *Lab on a Chip* **13, 2484-2499.**

Young, K. D. (2007). Bacterial morphology: Why have different shapes? *Current Opinion in Microbiology* **10(6), 596-600.**

Young, K. D. (2006). The selective value of bacterial shape. *Microbiology and Molecular Biology Reviews* **70(3), 660-703.**

Zettler, E. R., Mincer, T. J. and Amaral-Zettler, L. A. (2013). Life in the "plastisphere": Microbial communities on plastic marine debris. *Environmental Science and Technology* **7(13), 7137-7146.**

Zhang, Z. J., Chen, S. H., Wang, S. M. and Luo, H. Y. (2010). Characterization of extracellular polymeric substances from biofilm in the process of starting-up a partial nitrification process under salt stress. *Applied Microbiology and Biotechnology* **89(5), 1563-1571.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538