

IDENTIFICATION OF SPECIES AND PREVENTION OF ACCELERATED LOW WATER CORROSION (ALWC) ON MARINE STRUCTURE

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Abstract

*Accelerated Low-Water Corrosion (ALWC) leads to degradation of maritime steel structure and was grouped as one of microbial induced corrosion (MIC) type. This study was performed to isolate, identify, and characterized the growth of the microbes that has induced ALWC in order to decide for the best treatment using understanding of its potential EPS through biofilm formation. The isolation was done by using rust of the corrode metals collected from the steel structures and harbor walls which was exposed to the sea tides at the Port Kelang, Selangor, Malaysia. The rust collected was spread on Seawater Agar medium. Growth of five different species was recorded after 72 hours. The pure colonies were stained for Gram tests and were identified as *Pseudomonas aeruginosa*, *Ochrobactrum anthropic*, *Pseudomonas luteola*, *Sphingomonas paucimobilis* and *Burkholderia cepacia* using the APi KIT. The formation of the biofilm was studied to confirm the consortia-like-activity in the potential of causing the corrosion. Solid-state ¹³C NMR spectroscopy (SSNMR) of the EPS from consortia biofilm has revealed 3 types of EPS namely EPS1, EPS2 and EPS3 with all three contain different amount of biomolecules such as peptides, carbohydrates and lipids in different ratio. The data proposed a very strong environment and interrelation between the biomolecules of the biofilm formation; suggesting a strong biofilm activity was taking place at the maritime structure. Understanding the EPS and biofilm dynamics of the 5 species will assist in the design of engineered anti-corrosion formulation for the ALWC in the related marine structures. Addition of 10% of nitrifying bacteria has proven to rupture EPS1, EPS2 and EPS3 with more than 50% lost in the total concentration of peptide, carbohydrates and lipids. This result indicated that microbial based techniques can be further studied for the purposes of corrosion prevention.*

Keywords: Accelerated Low Water Corrosion (ALWC); Mechanisms; Bacterial growth; Induced microbial corrosion (MIC)

Introduction

Accelerated low water corrosion (ALWC) is defined as a localized and fast corrosion phenomenon that is typically occurs at or below seawater water level on most of maritime structures. It has been declared to be associated with microbial induced corrosion (MIC) [1]. ALWC has been reported as manageable phenomenon since the process is predictable especially when the commissioning owners have included ALWC corrosion protection on all maritime structures. Unfortunately, there are quite many evidences proved that ALWC is

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somehow happening at rates far in excess of normal through accelerated corrosion of 1.0-2.0mm per year and higher which could lead to far worse primary and secondary structural deterioration, huge conservation costs and increased safety risks. This condition has been identified to be contributed by bacterial activities on the surfaces of those related marine structures known as induced microbial corrosion [2]. Diverse aquatic microorganisms are capable of colonizing surfaces of various kinds, leading to the formation of biofilms and to the development of specialized processes within these structures [3, 4]. Corrosion is basically relate and caused by lots of complex processes enhanced by different kind of microorganisms activities; either as in a combination of bacteria performing *bacterial consortia* corrosion or might as well be a perfect one single microbe activities (Nik Raikhan, unpublished). According to *N. Nardy* and *J.A. van Veen* [2], bacterial activities in AWLC are a state of performing different electrochemical reactions and secreting proteins and metabolites that can have secondary effects to the metals. Therefore it is vital to understand that the information on the identity and role of microbial communities are related to the microbial induced corrosion. It is important to highlight that corrosion inhibition in different materials and in different environments is scarce. Surface colonization by different consortia and the production of the shielding such as biofilm matrix is giving arise to the ALWC in which the factors associated are greater with environment factors such as temperature and the right pH. The biofilm matrix of the related bacterial systems and the development within it of specific microenvironments is said to bring arise into a promotion of extracellular enzyme that most potentially harm lots of maritime structures [5]. Recent investigations of marine particle-associated microbial communities revealed a wealth of information about certain common characteristics of microbial particle colonizers [1]. Most damaging corrosion takes place in the presence of a multispecies biofilm. In such biofilms the interactions between different species may induce a cascade of biochemical reactions in the oxic and anoxic parts of the biofilm and exacerbate corrosion [6].

Materials and Methods

Reagent, chemicals and pH. All material and chemicals were collected from various sources and quality as listed. Bacteriological peptone (Ultrapure, protein =N x 6.38 ≥ 76.5%), yeast extract (Y1625, Sigma-Aldrich), ammonium sulphate (A3920, ≥99.0%, (NH₄)₂SO₄, Sigma), ammonium phosphate monobasic (79546, ≥98%, Sigma-Aldrich), urea (U5378 powder, NH₂CONH₂, Sigma), ammonium nitrate (A9642 ≥99.0%, NH₄NO₃, Sigma-Aldrich), ammonium carbonate (379999, 99.99%, (NH₄)₂CO₃, Sigma-Aldrich), glycerol (Merck), Pseudomonas agar (Merck), nutrient agar (Merck), nutrient broth (Merck), potassium dihydrogen phosphate (KH₂PO₄, V90004, Vetec™ reagent grade, 99%, Vetec), ammonium chloride (09718, NH₄Cl, BioUltra, ≥99.5%, Sigma), calcium chloride (Sigma-Aldrich).

Isolation of ALWC promoting species, Seawater medium and inoculum preparation. The Seawater agar was prepared using some modifications of ZoBell Marine Agar 226 by *C.E. Zobell* [7]; with medium composition mimics seawater content as listed in Table 1. Seawater broth (SB) was prepared with the same medium composition but with no addition of agar (see Table 1). The shaking rate was set at 150rpm, T = 25°C and time 72 hours. The 24 hours inoculum preparation was set up using Seawater broth (SB) at pH = 7.6±0.2, shaking rate at 150rpm and 25°C [6]. The amount of 8% (v/v) 24 hour cells at 5.0x10⁵cells/mL was used. Isolation was done by using corrode metals collected from steel structures and harbor walls which exposed to the sea tides at the Port Kelang, Selangor, Malaysia. To start the isolation process, we sprinkled the corroded metal dust on the Seawater agar and incubated for 72 hours at 25°C followed by colony identification. The strain was maintained at -20°C in Seawater Agar (SA).

Table 1. Seawater agar (Sa) and Seawater broth (Sb) medium composition

Ingredients	Agar (g)	Broth (g)
Peptone	5.0000	5.0000
Sodium Chloride	19.450	19.4500
Yeast extract	1.0000	1.0000
Sodium Sulphate	3.2400	3.2400
Potassium Chloride	0.5500	0.5500
Sodium Bicarbonate	0.1600	0.1600
Sodium Silicate	0.0040	0.0040
Ammonium Nitrate	0.0016	0.0016
Boric Acid	0.022	0.022
Agar	15.00	-
Total	44.572	29.572

Estimation of Bacterial Cell Mass. The Optical Density (OD) of the culture broth was measured at 540nm periodically using Spectrophotometer and about 2.0mL sample of the culture was withdrawn from the flask and centrifuged at 10,000rpm for 10 minutes for every 3 hours of sampling. The supernatant was poured out and the remaining cell was suspended in distilled water. The cell suspension was centrifuged again using same parameter. The supernatant was again poured out and the remaining cell was washed slowly using of distilled water on the suspension of cells. Then, it was transferred to a falcon tube with small opening, put into a desiccator at room temperature overnight. After 24 hours, the falcon tube containing dry cell was weight. The difference between the final weight and pre-weight of a falcon tube were used to estimate Cell Dry Weight (CDW). This method was adapted and modified using method by *N.H. Nik Raikhan* and *A.R. Khairul Izwan* [8].

Determination of Bacteria Species Using API Kit. An incubation box (tray and lid) was prepared and wet with about 5mL of distilled water into the honey-combed wells of the tray to create a humid atmosphere. The strip was removed from its packaging and placed in the incubation box.

API 20E Kit by using an inoculum loop, 2-3 colonies of the species were taken and placed into a universal bottle containing 5mL of saline solution (sterilized 0.85% sodium chloride). The suspension was homogenized and must be used immediately after preparation. By using a sterilized pipette, the tube and cupule of test CIT, VP and GEL was filled with the bacteria suspension. For other test, only the tube was filled. The tests ADH, LDC, ODC, H₂S and URE were overlaid with sterile mineral oil to create anaerobic condition. The strip was incubated at 27±2°C for 18-24 hours. After incubation period, the strip was read by referring to the Reading Table provided in the manual. If 3 or more tests (GLU test + or -) were positive, the spontaneous reactions were recorded on the result sheets. If the tests positive were less than 3, the strip was re-incubated for a further 24 hours without adding any reagents. For TDA Test, 1 drop of TDA reagent was added. For IND Test, 1 drop of JAMES reagent was added. For VP Test, 1 drop of VP 1 and VP 2 reagents were added and waited for at least 10 minutes for the reaction (API 20E REF 20100/201600).

API 20NE Kit by using an inoculum loop, 2-3 colonies of the species were taken and placed into a universal bottle containing 2mL of saline solution (sterilized 0.85% sodium chloride). A suspension with a turbidity equivalent to 0.5 McFarland was prepared. The suspension was homogenized and must be used immediately after preparation. The tests NO₃ to PNPG were performed by distributing the saline suspension into the tubes by using a sterilized pipette. An ampule of API AUX Medium was opened and 200µL of remaining saline solution was added into the ampule. The mixture was homogenized well. The tubes and cupules of tests GLU to PAC was filled with the suspension. Mineral oil was added to the cupules GLU, ADH and URE until a convex meniscus is formed. The incubation box was closed and incubated at 27±2°C for 24 hours. After incubation period, the strip was read by referring to the Reading Table provided in the manual. The spontaneous reactions of GLU, ADH, URE, ESC, GEL and

PNPG were recorded. For NO₃ test, 1 drop of NIT 1 and NIT 2 reagents were added to the NO₃ cupule. After 5 minutes, a positive reaction to be recorded on the result sheet. If the result was negative, 2-3 mg of Zn reagent was added to the NO₃ cupule (Api 20 NE REF 20050). The 7 digit profile number was obtained for the 20 tests of the API 20 E Strip. By using database (V4.0), the 7 digit profile number was entered manually and the database will provide the name of the species.

EPS Extraction. 72 hours old culture medium of the cell consortia was centrifuged at 7000rpm for 10min and cell pellets were resuspended in 20mL of PBS buffer containing 2mM Na₃PO₄, 4.0mM NaH₂PO₄, 9.0mM NaCl and 1.0mM KCl at pH = 7.0. Method by *L.H. Asaulenko et al.*, [9] was used. Then the equivalent volume of cation exchange resin (DOWEXTM Marathon C, Sigma) which was previously treated with PBS buffer for 1h was added and extraction was performed for 4 h at 4°C with stirring at 100rpm. Then, the aqueous phase was filtered through a Simax glass filter funnel. After that, the filter was washed with 10mL of PBS buffer to obtain EPS extract trapped within the DOWEXTM beads. Next, the EPS containing microbiomecule extract was centrifuged at 6000rpm for 5 min, filtered through a 0.2µm pore size membrane filter and dialyzed. In order to obtain EPS from biofilm cells these were detached by ultrasound treatment of coupons in 14.5mM NaCl solution by a non-destructive regime (22kHz) as described *L.H. Asaulenko et al.*, [10]. The resulting cell suspension was treated as described for the planktonic cells. EPS extraction was performed in triplicates. All samples were freeze-dried and then dissolved in deionized water. EPS samples were used for the protein determination and amino acid composition analyses.

Chemical composition analysis. Fourier transform infrared (FTIR) spectroscopy was used to quantify the types of EPS in the biofilm. Dried EPS samples were precipitated with ethanol by adding 3 volumes of 100% cold ethanol and were further incubated on ice for 2 hours. The precipitates were later centrifuged at 17,500g (15min at 4°C) and were dried in an oven at 55°C for 24 hours. FTIR spectroscopy was performed with a PE Spectrum GX FTIR system in attenuated total reflection (ATR) mode using a Split Pea (Harrick Scientific Corporation, United States). The total protein content was measured using 12% trichloroacetic acid (TCA) added to the EPS solution, followed by incubation on ice for 30min before centrifugation at 15,000g for 15min. The TCA precipitates were washed thrice using 12mL acetone before it is resuspended in 2.0mL of 2-N-morpholinoethanesulfonic acid (MES) buffer at pH = 5.0. The protein content was measured using the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the calibration standard [10]. For the total carbohydrate content, measurement was done a modified phenol-sulfuric acid method with glucose standards [11]. The absorbance at 490nm was read with a spectrophotometric plate reader

Solid-state NMR analysis. Solid-state ¹³C nuclear magnetic resonance (NMR) spectra were acquired with a Varian-Chemagetics Infinity 300 nuclear magnetic resonance spectrometer with static field of the superconducting magnet used as 7.05T, and the resonance frequencies of ¹H and ¹³C were 300 and 75mHz, respectively. Cross polarization (CP) (¹H-¹³C) employed an amplitude ramp in the ¹³C channel, and magic angle sample spinning (MAS) was performed to optimize the signal. Methods by *K.P. Mackie* [11] have been modified.

Results and Discussion

The accelerated low water corrosion (ALWC) commonly involved seven corrosion zones which are atmospheric zone, splash zone, tidal zone, low water zone, immersed zone, embedded zone and concentrated corrosion in all zones [11]. Figure 1 shows ALWC at Port Klang, Selangor, Malaysia which occur in the tidal zone where metals are alternately submerged in the seawater and exposed to splash zone as tide decrease causing the metal structure of the jetty to submerge during high tides. The tides cause water rises and falls causing

wetting and drying on the exposed metal structure of the jetty. The sample needed for the bacterial isolation has been collected here.

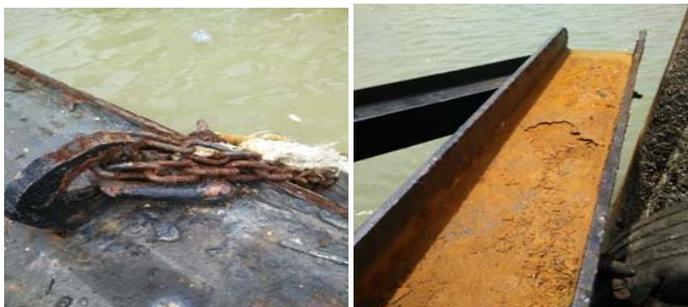


Fig. 1. Accelerated Low Water Corrosion (ALWC) at Port Klang, Malaysia

Isolation. Metal rust collected from the structure in Figure 1 has been spread on agar plate and was incubated at 27°C for 72 hours. Growth was detected with clear significant colors representing 5 potential isolates. The 5 colonies were then cultivated into 5 different petri dishes with artificial sea water as their growth medium. As shown in Figure 2, the 5 types of colonies was further defined as bright yellow colony (BY), creamy white colony (CW), dark pink colony (DP), light pink colony (LP) and creamy colony (C). Species color is used at this level since identification is not yet complete.



Fig. 2. Corroded metal dust on the Seawater agar with early growth of microbial colonies

Identification. Colonies in Figure 3 stained to differentiate the Gram types in order to test with API Kit to determine its species. The Gram negative bacteria were tested with API Kit 20E meanwhile Gram positive bacteria with API Kit 20NE. When the bacteria were stained with crystal violet, some of them were able to retain the crystal violet and some were decolorized by alcohol. The cell walls of Gram positive bacteria have a thick layer of peptidoglycan and content of lipid is lower. Decolorizing the cell caused the thick cell wall to dehydrate and shrink which then closes the pores in the cell wall and prevent the stain from exiting the cell. Thus, ethanol cannot remove the crystal violet-iodine complex that was bound to the thick layer of peptidoglycan of the Gram positive bacteria and appears blue in color. Meanwhile for Gram negative bacteria, the cell walls also take up crystal violet-iodine complex. However, because of the thin layer of peptidoglycan and thick outer lipid layer, crystal violet-iodine complex washed off. When they were exposed to alcohol, the decolorizer dissolved the lipids in the cell walls, allowing the crystal violet-iodine complex to exit out of the cells. When safranin was added, they took the stain and appear red in color [12].

Bright yellow (BL) colonies have been identified as *Pseudomonas aeruginosa* (P.

aeruginosa). This species has therefore named as *Pseudomonas aeruginosa* NR66. Table 2 shows the details. *Pseudomonas* is a large group of free-living bacteria that live primarily in soil, seawater and fresh water. *Pseudomonas aeruginosa* can be found in natural habitat such as in soil, seawater, sewage, and associated with some plants [13].

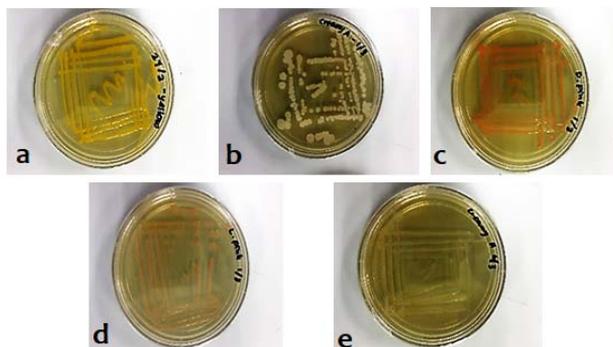


Fig. 3. Pure colonies of ALWC promoting bacteria Colonies of ALWC promoting bacteria: a. Bright yellow colony (BY); b. Creamy white colony (CW); c. Dark pink colony (DP); d. Light pink colony (LP); e. Creamy colony (C)

P. aeruginosa is one of the aerobic slime-forming bacteria that are ubiquitous in marine environment that corrode steel structures. *P. aeruginosa* is a dominant bacterium in marine environments and one of the aerobic SFBs, which form a biofilm layer on steel surface The chemical reaction of the biofilm layer with steel and the formation of differential aeration cells create conditions on steel that initiate and accelerate the corrosion process. *P. aeruginosa* can reduce ferric to ferrous iron, thus exposing steel to further oxidation since ferrous iron compounds are more soluble and the protective ferric iron layer is solubilized by this process. This type of bacterium in its biofilm state could be detrimental to steels and cause severe corrosion damages. It forms a heterogeneous biofilm layer on the steel surface and causes the formation of differential aeration cells, which induce localized corrosion iron is an essential nutrient for *P. aeruginosa* [14].

Table 2. Identification of bright yellow (BL) colonies

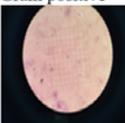
Characteristic		Identification				
 Gram positive Sticky bright yellow colony (BY) on Seawater agar		Identified as				
		<i>Pseudomonas aeruginosa</i> NR66				
Significant taxa	% ID	T	Tests against			
<i>Pseudomonas aeruginosa</i>	98		ONPG	0%	URE 25%	TDA 0%
			OF/O	98%	NO2	12%

Table 3 shows results of identification of species AWLC creamy color (C). Creamy colonies have been identified as *Ochrobactrum anthropic*. Certain types of microbial biomass can retain high quantities of metals by biosorption, which depends on the affinity between the binding sites on the molecular structure of the cellular wall and the metallic species or its ionic forms [15]. Biosorption is the ability of microbial biomass to collect heavy metals from wastewater through physico-chemical pathways of uptake or metabolically mediated [16]. *Ochrobactrum anthropic* able to bind and accumulate metal ions in the form of superficial

mucilage layer. *O. anthropic* is a good adsorbing medium for metal ions and had high adsorption yields for the treatment of wastewater containing copper, cadmium, and chromium [17]. Table 4 exhibits the result of identification of the creamy white colonies. This colony was successfully identified as *Pseudomonas luteola*. *Pseudomonas luteola* has been further classified as *Pseudomonas luteola* NR66. Common species of *Pseudomonas luteola* can absorb certain heavy metals such as Cr(VI) and Al(III). Both ions are found in industrial wastewaters. These metals are specifically targeted by *P. luteola* strain TEM05. Under relatively acidic conditions, pH = 4 for Cr(VI) and 5 for Al(III). Experiments indicated a maximum adsorption capacity of 55.2mg·g⁻¹ for Al(III) and 3.0mg·g⁻¹ for Cr(VI) [18]. *P. luteola* strain TEM05 can also produce exopolysaccharide (EPS) that is utilized in the adsorption of nickel and copper. For the adsorption of Ni and Cu to occur, the strain must be immobilized in a calcium alginate beads. With this enhancement, maximum adsorption capacities range from 45.87-50.81mg·g⁻¹ and 52.91-61.73mg·g⁻¹, respectively [19].

Table 3. Identification of creamy color (C) colonies

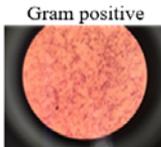
Characteristic		Identification	
 <p>Gram positive</p>	<p>Creamy colony (C) on Seawater agar</p>		<p>Identified as</p> <p><i>Ochrobactrum anthropi</i></p>
<p>Significant taxa</p> <p><i>Ochrobactrum anthropi</i></p>	<p>% ID</p> <p>89</p>	<p>T</p>	<p>Tests against</p> <p>ONPG 15%</p> <p>TDA 1%</p> <p>GEL 0%</p> <p>URE 25%</p>

Table 4. Identification of creamy white colony (CW)

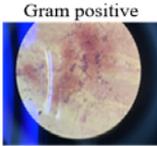
Characteristic		Identification	
 <p>Gram positive</p>	<p>Creamy white colony (CW) on Seawater agar</p>		<p>Identified as</p> <p><i>Pseudomonas luteola</i> NR66</p>
<p>Significant taxa</p> <p><i>Pseudomonas luteola</i></p>	<p>% ID</p> <p>88%</p>	<p>T</p>	<p>Tests against</p> <p>TDA 0%</p> <p>McC 91%</p> <p>ARA 85%</p> <p>GEL 13%</p> <p>ARA %</p> <p>OF/O 94%</p>

Table 5 shows the result of the identification of the dark pink colonies. Dark pink colonies were confirmed to be identified as *Sphingomonas paucimobilis*. *Sphingomonas paucimobilis* is non-fermentative, aerobic, and Gram negative motile bacillus [20]. It is recovered from sea water, sea ice, water, river, and waste water. *Sphingomonas paucimobilis*'s distribution can be explained by their ability to survive and grow at low temperature, low nutrient concentration and in toxic chemical environments [21]. In the latter case, the presence of copper-tolerant film-forming *Sphingomonas paucimobilis*, resulted in a 20-fold increase in the corrosion rate of 90/10 Cu-Ni and Al-bronze alloys. *Sphingomonas paucimobilis* is also found grown on stainless steel [22].

Light pink colonies represent *Burkholderia cepacia*, a Gram negative, motile, aerobic and catalase- and oxidase-positive bacteria [23]. *B.cepacia* is closely related to *Pseudomonas* genus. *Pseudomonas* genus known to be the pioneer colonizers in biofilm formation causing corrosion that is found in marine and other aquatic environment. *B.cepacia* is one of the organisms that are attached on the corroded galvanized steel [24]. *B.cepacia* form biofilms

leading to negative outcomes such as corrosion pits, infections, and fouling depending on where they occur. Bacterial biofilms can only occur if the bacteria successfully attaches to a surface. The biofilms formation proves that *Burkholderia cepacia* attached to the metal surface.

Table 5. Identification of the dark pink species

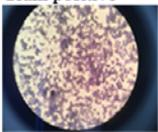
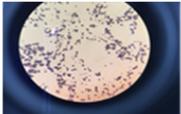
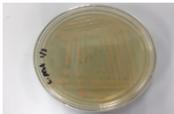
Characteristic		Results Identification				
 <p>Gram positive</p>	Dark pink colony on Seawater agar		Identified as			
			<i>Sphingomonas paucimobilis</i>			
Next taxon	% ID	T	Test Against			
<i>Sphingomonas paucimobilis</i>			NO ₂	GLU	PNPG	ARAa
			10%	%	90%	83%

Table 6. Identification of light pink colony

Characteristic		Results Identification	
 <p>Gram positive</p>	Light pink colony on Seawater agar		Identified as <i>Burkholderia cepacia</i>

The Growth of Identified Accelerated Low Water Corrosion (ALWC) species. The identified bacteria were used to study the growth on artificial sea water broth. The reading was taken every 12 hours since the species have quite a slow growth rate. They grew within approximately 72 hours, thus the reading was taken for 5 days to observe growth rate and exponential growth curve. Microbial growth is affected by nutritional and physical factors mostly such as pH, temperature, shaking rates, metabolites and water content. Nutritional factors are referring to the components or concentration of the growth medium used in the system. Artificial sea water provides the same environment as the sea water. The growth of bacteria can be observed by plotting the cell growth (absorbance) versus the time of incubation which is known as standard curve as Figure 4. Based on Figure 4, the time taken for the *Ochrobactrum anthropi* in the exponential phase was the shortest. This showed that *Ochrobactrum anthropi* has the greatest metabolic activity. It achieved its optimum growth rate and number of bacteria increases exponentially and the replications occur rapidly. It stops replicating at 60th hour and undergoes stationary phase. The nutrients in the medium were used up by the bacteria as it continues to grow rapidly. This caused the accumulation of toxic metabolites, waste materials and the inhibitory compounds with some conditions of the medium and pH changes. Thus, this created unfavourable environment for the growth of bacteria. The rate of reproduction slowed down and the cells undergo replication is equal to the number of cell death. *Ochrobactrum anthropi* undergo death phase at 120th hour. The reducing of nutrients and increase in toxic metabolites, waste materials and the inhibitory compound in the medium caused the bacteria to die. As this happen, the bacteria completely loses its ability to replicate. It began to die and the death rate is rapid at constant rate. The number of dead cells exceeds the live cells.

Pseudomonas aeruginosa NR66 has the shortest life span since it achieved the death phase faster than the other species. Meanwhile the longest life span is *Sphingomonas paucimobilis*, *Burkholderia cepacia*, and *Pseudomonas luteola*. *Pseudomonas aeruginosa* achieved death phase at 120th hour. Meanwhile the death phase for *Sphingomonas*

paucimobilis, *Burkholderia cepacia*, and *Pseudomonas luteola* is at 130th hour.

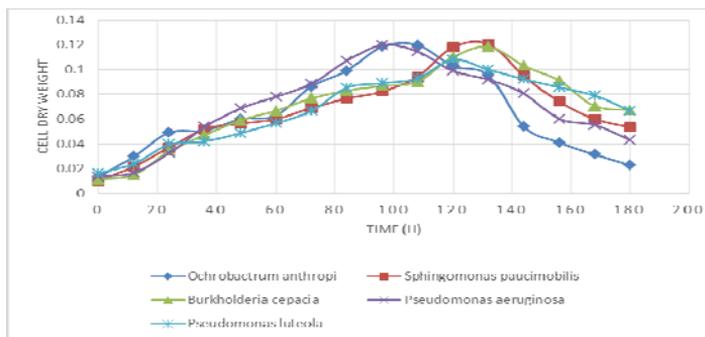


Fig. 4. Cell dry weight (growth rate) of 5 species isolated from ALWC components in Port Klang, Malaysia

Biofilm formation by the consortium of *Pseudomonas aeruginosa*, *Ochrobactrum anthropic*, *Pseudomonas luteola*, *Sphingomonas paucimobilis* and *Burkholderia cepacia*.

While biofilm formation is very common among consortia of marine bacteria, the appearance is not what is aimed especially on marine structures. The 5 isolated species has been confirmed to produce a very stable formation of biofilm with clear thickness. The thickness has been supported by the extracellular polymeric substances (EPS) which is crucial for biofilm structure. Apart from that, the EPS is also confirmed to be the microbial nutrition and proximal stability of habitat in a variety of environments whenever the bacterial consortia are with lacking of nutrients. According to *R.G. Marc* and *C. Lin* [25], exogenous organic substrate and heavy metal ion concentration are crucial factors influencing microbial EPS production and biofilm formation in these systems; which are what seen on corrode marine structures. Silver ions and nanoparticles for example will affect the composition of phototrophic biofilm in operated bioreactors [26] suggesting that in any evidence of the silver nanoparticles appearance, the thickness of the biofilm will be greater. Figure 5 shows the formation of pinkish-brown thick biofilm (sessile cells) in seawater broth after 72 hours. The consortia that gives arise to the biofilm production are *Pseudomonas aeruginosa*, *Ochrobactrum anthropic*, *Pseudomonas luteola*, *Sphingomonas paucimobilis* and *Burkholderia cepacia*.



Fig. 5. Biofilm formation by the consortium of *Pseudomonas aeruginosa*, *Ochrobactrum anthropic*, *Pseudomonas luteola*, *Sphingomonas paucimobilis* and *Burkholderia cepacia*. Very thick formation of EPS in pinkish and light brown color

Solid-state ¹³C NMR spectra (SSNMR) of EPS from the consortia of cells of the biofilms have been performed to reveal out that biofilms are a special complex with at least three types of biomolecules. The data has helped to reveal a very strong environment and interrelation between the lipids, peptide and carbohydrates of the biofilm formation; suggesting a strong biofilm activity was taking place at the maritime structure. Lipids typically resonate in the frequency range from 0 to 40ppm. Secondary alcohols of carbohydrates resonate in a range of frequencies from 60 to 90ppm, whereas the glycosidic carbon of polysaccharides resonates at frequencies between 95 and 106ppm [27]. Peptide region in Figure 6 is referring to region of

peptide bonded carbon shifted to the polysaccharide glycosidic. The fact that these NMR data indicate that EPS1 has proportionally 41-52% more lipid than the EPS2 and EPS3.

This result is consistent with what is observed in the high-frequency C-H region of the FTIR spectra for these biofilms. Apart from that, the Solid-state ¹³C NMR has projected the difference in the secondary alcohol frequency region between 60 to 95ppm of the carbohydrates (Fig. 6). This is believed to be contributed by the distribution of sugar monomers within the polysaccharides. The obvious increase in secondary alcohol intensity relative to the glycosidic carbon intensity in EPS1 is consistent with a change in carbohydrate composition toward larger sugars. There are also some differences in glycosidic resonance (95 to 110ppm) between EPS1, EPS2 and EPS3 where the presence of glycosidic intensity spanning the full range of frequency indicates the potential of having both α and β oxygen monomer linkages.

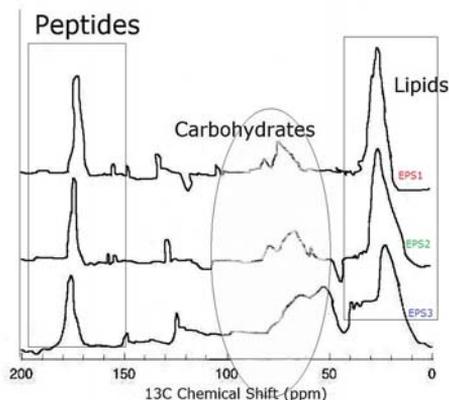


Fig. 6. Solid-state ¹³C NMR spectra (SSNMR) of EPS from biofilm of *Pseudomonas aeruginosa*, *Ochrobacterum anthropic*, *Pseudomonas luteola*, *Sphingomonas paucimobilis* and *Burkholderia cepacia*

Lowering survival rate of consortia cells with anti-biofilm broad spectrum formulation using microbial based techniques. We could never separate bacterial activities in nature because each one of the species survives with strong mechanism that has been developed to support the biofilm formation. The content of the biofilm varies with species but common biomolecules are proteins, lipids, carbohydrates, some bacterial DNA and defined sugars. According to a review paper by *K. Nordy* and *J.A. van Veen* [28], the main types of bacteria associated with the corrosion of iron and steel are sulfate-reducing bacteria (SRB), sulfur-oxidizing bacteria, iron oxidizers, iron reducers, manganese oxidizers and microbes that secrete organic acids and produce extracellular polymeric substances (EPS).

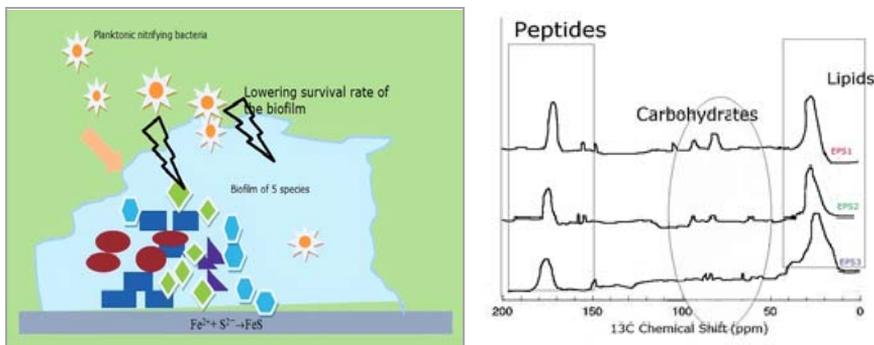


Fig. 7. Potential of using planktonic nitrifying bacteria to reduce the strength of the EPS of the biofilm (left) and Solid-state ¹³C NMR spectra (SSNMR) of EPS from biofilm after addition of 10% of 24 hours culture of nitrifying bacteria (right)

The latter is what is related with the one in this research paper. Adding 10% (v/v) of 24 hours culture of nitrifying bacteria has proven to disturb the stability of the consortia of biofilm of the 5 species identified before. Microbial based technique is hoped to lowering the survival rate of the slimy glycosidic carbon of polysaccharides. Figure 7 shows the potential of using planktonic nitrifying bacteria to reduce the strength of the EPS of the biofilm, thus is hoped to reduce possibility to start a serious corrosion rate in the marine structures. In the same Figure 7(left), result of the ^{13}C NMR spectra (SSNMR) of EPS from biofilm after addition of 10% of culture of nitrifying bacteria has proven to ruptured or at least reduce stability of the tested EPS. All EPS1, EPS2 and EPS3 have shown about more than 50% lost in the total concentration of the microbiomolecules of peptide, carbohydrates and lipids. This result indicated that microbial based techniques can be further studied for the purposes of corrosion prevention.

Conclusion

Five species of bacteria have been identified from the rust of corrode metals, which was later confirmed to be producing very thick slime or biofilm. It shows very strong interrelation between the microbiomolecules of the biofilm formation; suggesting a strong biofilm activity was taking place at the maritime structure. Addition of 10% of nitrifying bacteria has proven to rupture EPS1, EPS2 and EPS3 with more than 50% lost in the total concentration of peptide, carbohydrates and lipids. This result indicated that microbial based techniques can be further studied for the purposes of corrosion prevention.

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