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Production, characterization and antibacterial activity of prodigiosin pigment produced by *Pseudoalteromonas rubra* BF1A IBRL associated with a marine macroalgae *Enteromorpha* sp.

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ABSTRACT

Aims: Marine bacteria are a great source of natural pigments, which can be used as colouring agent in food, textile, cosmetics and aquaculture industry to overcome the drawbacks poses by the synthetic pigments. The aim of the study is to identify the potential bio pigment producer, determine the antimicrobial susceptibilities, and characterize the pigment produced.
Methodology and results: In this study, the surface attached marine bacteria isolated from the surface of seaweed, *Enteromorpha* sp. has been identified as *Pseudoalteromonas rubra* BF1A IBRL through the molecular identification step. This species produced intracellular and extracellular red pigment with antibacterial activity. The susceptible bacteria include *Bacillus subtilis*, *Bacillus cereus*, Methicillin Resistant *Staphylococcus aureus*, *Staphylococcus aureus* and also *Acinetobacter anitratus* with inhibition zone ranges from 7.33 to 10.33 mm, whereas Minimum inhibitory concentration (MIC) values ranges from 0.055 to 8.88 mg/mL. The UV/vis analysis indicated that the maximal absorbance of ISO and DE pigment extract were at 531 and 534 nm, respectively. Based on the antimicrobial activity, the extracellular extract poses greater antibacterial activity, thus was selected as the potential pigment extract and were further evaluated. The Thin Layer Chromatography (TLC) profile of the DE extract showed one major band under visible light ((Rf = 0.87) and the bioautography analysis of the pigmented band showed positive activity against both Gram-positive and Gram-negative bacteria. The pigment in DE extract was identified as prodigiosin based on the spectroscopic properties, presumptive test and HPLC analysis.
Conclusion, significance and impact of study: This study highlights the dual benefits of the *P. rubra* BF1A IBRL pigment extract, which exhibited both tinctorial and pharmacology benefits, thus it can be act as colouring agent with own preservative value in food, textile, or cosmetics industries.

Keywords: Marine bacteria, *Pseudoalteromonas rubra*, prodigiosin, antimicrobial activity, bio pigment

INTRODUCTION

The currently employed synthetic pigment as colouring agent has poses many disadvantages on human and environments (Alihosseini *et al.*, 2008), imposing the need to explore for natural pigments from a biological source which includes higher plants (Al-Refai *et al.*, 2010), lower plants (Warkoyo and Saati, 2011), and microorganisms (Franks *et al.*, 2005). Microorganisms are the most valuable sources of natural pigment compared to plants because of the ease of propagation in laboratory condition, thus preventing the damage of the vulnerable ecosystem during the sampling. Among the microorganisms, the marine bacteria are the gifted sources since they are major colonizer of the untapped marine environment and that the pigmentation is a vital characteristic of marine bacteria for adaptation mechanism against the photo-oxidation from the sunlight (Goecke *et al.*, 2010). Besides, many bioactive compounds including the pigments produced by the marine bacteria especially the surface attached epiphytic bacteria is known to exhibits antimicrobial property in regard to the symbiotic relationship between the bacteria and its host as described by Goecke *et al.* (2010).

Pigments as secondary metabolite from marine bacteria have been reported previously, which includes carotenoids from *Rubritalea sabulii* sp. (Yoon *et al.*, 2008) and thambjamine from *Pseudoalteromonas tunicata*

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(Franks *et al.*, 2005). Other than that, pigments that possess antibacterial property also has been extracted previously from marine bacteria which includes prodigiosin from *Vibrio* sp. (Alihosseini *et al.*, 2008), pyocyanine and pyorubin from *Pseudomonas aeruginosa* (Saha *et al.*, 2008), quinines from *Streptomyces* sp. (Maskey *et al.*, 2003), violacein from *Chromobacterium violaceium* (Suleiman *et al.*, 2010), and carotenoids from *Agrobacterium* sp. (Misawa *et al.*, 1995).

Among the marine biopigments, prodigiosin that belongs to red colored pigments poses wide functions after the carotenoids pigments. Prodigiosin, a tripyrole derivative (Sobin and Stahly, 1941) with pyrolypyrromethane skeloten (Gulani *et al.*, 2012) is water insoluable pigment, but readily soluble in organic solvents such as DMSO, alcohol, diethyl ether, ethyl acetate and hexane. Prodigiosin can be synthesized as secondary metabolite by *Pseudoalteromonas* sp. and also other bacteria such as *Serratia* sp. (Samrot *et al.*, 2011; Gulani *et al.*, 2012; Priya *et al.*, 2013), *Vibrio* sp. (D'Aoust and Gerber, 1974; Allihoseini *et al.*, 2008), *Pseudomonas* sp. (Gandhi *et al.*, 1976), and *Hahella* sp. (Park *et al.*, 2012). The functional bioactivity of prodigiosin are antimicrobial activity (Samrot *et al.*, 2011; Gondil *et al.*, 2017), antiploriferate activity of cancer cells (Dalili *et al.*, 2012), antioxidant activity (Arivizhivendhan *et al.*, 2018) algicidal activity (Yang *et al.*, 2013), and immunosuppressor activity (Montaner *et al.*, 2000). *Pseudoalteromonas rubra* is one of the prodigisoin producing marine bacterium, which has been isolated previously from the surface of sponge (Feher *et al.*, 2008), macroalage (Ulva lactuca), tunicate, mussels (Ivanova *et al.*, 1996; Holmstrom *et al.*, 1998; Egan *et al.*, 2001). While the pigment property of this bacterium was reported previously (Gauthier, 1976; Feher *et al.*, 2008), the antibacterial activity of the pigment produced has been less investigated.

Earlier, the microbial prodigiosin has been studied for their application to textile industry for coloring benefit (Allihosseini *et al.*, 2008; Gulani *et al.*, 2012), aquaculture industry for antifouling (Priya *et al.*, 2013; Satish Kumar and Aparna, 2014) and also pharmaceutical industry for antineoplastic drug or immunosuppressor agent (Wang *et al.*, 2012). Despite the huge potential of the multifunctional pigment, we found that very limited studies are carried out to apply the prodigiosin pigment obtained from marine bacteria as an antimicrobial agent. Obviously, pigments with antimicrobial activity can worth as coloring agent with self-preservative value to many industries like cosmetics, foods, textile and aquaculture. For example, dyeing the hospital's textile (e.g. blanket, towel, bed sheets, patient apparel, uniforms and gowns) with pigment exhibiting antimicrobial properties would reduce the spread of nosocomial infections (Alihosseini *et al.*, 2008).

In the present paper, we evaluated a marine bacterium for the pigment production ability jointly with its antimicrobial potential. This communication also reported the isolation source, characterization, antimicrobial spectrum and pigment analysis of intracellular and extracellular extract, as well as bioautography study of the separated pigment of the marine bacterium.

MATERIALS AND METHODS

Isolation, characterization and identification of isolate BF1A IBRL

The isolate BF1A IBRL was isolated from the surface of macroalgae, *Enteromorpha* sp. by using serial dilution and spread plate technique according to Radjasa *et al.* (2007) and Nugraheni *et al.* (2010), with slight modification. Sample of Enteromorpha sp. collected from Batu Feringhi, Penang, Malaysia (5°28'0''N 100°15'0''E) was rinsed with sterile seawater to remove any associated debris and bacteria that temporarily attached to the surface waters. The sample surface was swabbed using sterile cotton swab which was then placed in 250 mL conical shake flask containing 50 mL sterile seawater and agitated overnight at 150 rpm and 30 °C. The resultant samples were serially diluted and spread on the surface of Marine agar (MA, Difco, USA). Pure culture of red pigmented isolate was obtained by transferring the colony onto fresh MA plates.

The isolate was characterized based on macroscopic (colony morphology on Marine agar in term of size, form, elevation, margin, and opacity), microscopic (Gram classification, cell structure study using SEM and TEM) and molecular identification approach (16S rRNA sequencing method). The sequence obtained was blast using National Centre for Biotechnology Information (NCBI) and a phylogenetic tree was constructed by neighbour joining method using software Mega 5.2 Explorer. Citrate utilization test was conducted by culturing the isolate BF1A IBRL on Simmons citrate agar (Himedia) at 30 °C for 24-48 h. The positive results indicated by the presence of bacterial colony on the agar accompanied by the blue coloration of the agar, which determined the ability of the strains to utilize citrate as its sole carbon source.

Growth condition and extraction

The single colony of BF1A IBRL was cultivated in Marine broth 2216 (MB, Difco, USA) under submerged fermentation condition for the pigment production. Approximately 2 mL of overnight grown culture in 50 mL MB (starter culture) was transferred into 250 mL shake flask containing 100 mL MB. The inoculums density of the starter culture was ensured to be 1 × 106 CFU/mL which is spectrophotometrically equivalent to optical density of 0.8 (Khanafari *et al.*, 2010). The culture was then incubated at 26 °C with agitation speed of 120 rpm for 24 h. At the end of incubation period the culture was centrifuged at 4000 rpm for 20 min (Fixed angle rotor; Sigma, Model 4K15). The pigments from cells were extracted using iso-propanol (ISO), whereas diethyl ether (DE) was used to extract pigment from cell-free supernatant. The ISO and DE organic phase containing pigments was concentrated under reduced pressure

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using rotary evaporator (Eyela) at 50 °C and allow to dry in fume hood until constant weight obtained.

Disc diffusion assay

The ISO (intracellular) and DE (extracellular) dry extract paste of isolate IBRL BF1A was redisolved in ethanol (to achieved 50 mg/mL of extract concentration) and were evaluated for antimicrobial activity using disc diffusion method (Tong *et al.*, 2011) against 14 test microorganisms. The final concentration of ethanol was 1% (v/v) and the test bacterium includes *B. cereus*, *B. subtilis*, Methyicillin Resistant *S. aureus* (MRSA), *S. aureus*, *P. aeruginosa*, *Escherichia coli*, *A. anitratus* and *Salmonella parathyphi*, *Candida tropicalis*, *C. albicans*, *Rhodothorula* sp., *Aspergillus niger*, *Rhizophus* sp. and *Aspergillus fumigatus*. The pure strains were obtained from the microbial culture collection of Industrial Biotechnology Research Laboratory (IBRL), Universiti Sains Malaysia (USM), Penang, Malaysia.

Briefly, the test inoculums of approximately 105 CFU/mL for bacteria, 4 × 10^5 CFU/mL for yeasts and 4 × 10^5 spores/mL for fungi were swabbed using sterile cotton swab onto surface of Mueller Hinton Agar (Hi-Media; for bacteria) and Sabouraud Dextrose Agar (Hi-Media; for yeasts and fungi). The sterile Whatman antibiotic disc with 6 mm diameter was loaded with 20 µL of test extract. The positive control contained a standard antibiotic that is Chloramphenicol (30 µg/disc) for antibacterial evaluation test, whereas Ketoconazole (30 µg/disc) for antiyeast and antifungal tests. The negative control is the solvent used to dissolve the extract. The discs were impregnated onto the plate agar that was seeded with test microorganisms previously. For bacteria, the plates were incubated at 37 °C for 24 h, whereas for yeast and fungi, the incubation condition was at 30 °C for 48 h. After incubation period, assessment of antimicrobial activity was done by measuring the size of inhibition zone formed around the disc. The results were expressed as mean value ± standard deviation of the inhibition zone obtained with three replicates for each experiment. The disc diffusion results of intracellular and extracellular extract were compared. One-way Analysis of Variance (ANOVA) were considered as significant at $p < 0.05$. Statistical analysis was carried out using SPSS version 16.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determination

The susceptible test microorganisms in disc diffusion assay were used as test microorganisms for determining the MIC and MBC values of both intracellular and extracellular extract using microdilution broth method. The broth microdilution method was done according to Wiegand *et al.* (2008) using Mueller Hinton broth medium (MHB; Hi-Media). Two-fold dilution was performed to the extracts with sterile medium and 50 µL of the extract was distributed into each well of microtiter plate. Separately, 50 µL of MHB containing approximately 1 × 105 CFU/mL of bacterial cells was added into each well, which makes total volume of 100 µL. Chloramphenicol (Sigma-Aldrich) was used as reference drug to serve as positive control whereas negative control well containing 1% ethanol with inoculums added. After 24 h of incubation time at 37 °C, 30 µL of 0.2 mg/mL p-iodonitroetrazolium violet salt (INT) (Sigma) as growth indicator was added to each well and was further incubated for 30 min. The colour change of INT from pale yellow to purple indicated the microbial growth. The MIC was recorded as lowest extract concentration showing no colour change. The antibacterial effect of the extract was categorized as weak, moderate or strong according to MIC interpretation described by Rattanachaikunsopon and Phumkhachorn, (2009). Following MIC determination, MBC was determined by sub-culturing one loopful of the suspension from wells that showing no apparent growth on Nutrient Agar (NA; Hi Media) and subsequent incubation at 37 °C for 24 h. The MBC was recorded as lowest concentration of extract showing no growth on NA. The MIC and MBC values of intracellular and extracellular were compared by using One-way Analysis of Variance (ANOVA) and were considered as significant at $p < 0.05$. Statistical analysis was carried out using SPSS version 16.

Characterization and identification of major pigment produced by isolate BF1A IBRL

For UV/vis spectrophotometer analysis and presumptive test

The ISO and DE extract containing intracellular and extracellular extract, respectively was concentrated using rotary evaporator and the extract were scanned in UV spectrophotometer (Genesys; Thermoscientific). The absorbance spectrum was measured in the series of wavelength ranging from 300 to 600 nm to obtain the wavelength of maximum absorption and was compared with spectrum range of authentic sample of standard prodigiosin (Sigma-Aldrich). (Bharmal *et al.*, 2012; Pradeep *et al.*, 2013).

Presumptive test for prodigiosin has been carried out according to method described by Gulani *et al.* (2012), with slight modification. Approximately 2 mL of each extract of *P. rubra* BF1A IBRL was taken into three test tubes. The content of one of the test tubes was acidified with a drop of hydrochloride acid (2 M) while the second tube was alkalinized with a drop of sodium hydroxide solution (2 M). A red or pink colour in the acidified solution and a yellow colour in alkaline solution indicated a positive presumptive test for prodigiosin (Gulani *et al.*, 2012). The third tube was served as colour control.

Separation of active compounds by Thin Layer Chromatography (TLC), Bio-autography assay and identification of prodigiosin by HPLC

From the screening results, extracellular pigment was more efficient in term of antimicrobial activity and pigmentation strength compared to intracellular pigments,

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hence the extracellular pigments will be focused in the next step of analysis.

The DE extract of prodigiosin of *P. rubra* BF1A IBRL was spotted on TLC plate (Silica gel plate; 60 F254, MERCK) (11 × 2 cm) and developed under saturated condition using acetone:hexane (1:1). The chromatograms were viewed under visible and ultraviolet (UV) light (254 and 366 nm).

The bioautography assay was performed by agar overlay method, described Ejikeme *et al.* (2010), with slight modification. The developed chromatogram was placed under UV lamp for 30 min to allow the evaporation of solvent and at the same time to sterile the chromatogram plate, which was then placed on the surface of Mueller Hinton agar plate. The same molten agar medium (10 mL) was seeded with 1 mL overnight grown test microorganism (adjusted to have 1×10^8 CFU/mL using 0.5 M Mc Farland standard) and poured in the Petri dish containing the chromatogram. The plate was then incubated at 37 °C for 24 h. The antibacterial effect was determined by observing the presence of inhibition zone around the spots on the chromatogram by spraying with 2% (w/v) solution of *p*-iodonitrotetrazolium violet (INT). The microbial growth inhibition appeared as clear zone around the active spot.

For HPLC analysis, the DE crude extract paste was first chromatographed over a silica gel column (47 × 2 cm) eluted with acetone:hexane (1:1) to yield 8 fractions with different colours. The fraction with high colour intensity was selected for further purification using preparative TLC. Several spots of the fraction were applied on TLC plate. After air drying, the plate was developed using acetone:hexane (1:1). The active spot with the Rf of 0.87 were scrapped off carefully from the plate. The scratched sample was dissolved in HPLC grade iso-propanol and centrifuged at 4000 rpm for 5 min in order to remove the silica. The supernatant was collected, filtered from 0.22 µm filter (Millipore, Whatman) (Rajauria and Abu-Ghanam, 2013). The preparative-TLC purified prodigiosin were analysed using HPLC (Waters. 2489 System), which was carried out in an reverse phased C18 column (5 µm) and Waters 2489 UV/Visible detector coupled with Breeze software. The mobile phase was mixture of methanol and 0.1 % (v/v) of trifluoroacetic acid (8:2, v/v) and the sample injection volume is was 20 µL. The mobile phase was flowed at 1.0 mL/min for 10 min and the detection wavelength was 325 nm. The identity of the compound was confirmed with the commercial sample of prodigiosin.

RESULTS AND DISCUSSION

Description, characterization and identification of the marine bacterium

In this study, a marine bacterium with great potential in production of pigment with antimicrobial activity was isolated from a surface of macroalgae. This confirms the fact that the surface of higher marine organisms was colonized by many potential antagonistic bacteria. Similarly, Zheng *et al.* (2005) and Radjasa *et al.* (2009) also had isolated various antimicrobial compounds from macroalgae associated bacteria. It is well known that the bacteria that were isolated from biotic sources have potential in producing various bioactive compounds compared to abiotic source (Darabpour *et al.*, 2011; Hamid *et al.*, 2013) because of the symbiotic relationship that occurred between the bacteria and host. In this symbiotic relationship, the host will provide nutrients such as vitamins, polysaccharides and fatty acids, and in returns the bacteria will secrete amino acid, antibiotics and toxins which improve defence system of its host. Instead macroalgae, other biotic sources that has been explored for isolation of antibiotic-producing *Pseudoalteromonas* were sponge (Feher *et al.*, 2008), tunicates (Holmstrom *et al.*, 1998), and also mussels (Ivanova *et al.*, 1996).

Macroscopically, the colonies of isolate BF1A IBRL were moderate in size (1 to 2 mm in diameter), red, with convex elevation, entire margin, smooth surface and opaque in opacity when grown in MA for 3 days (Figure 1A). Whereas, microscopically isolate BF1A IBRL is a Gram-negative bacterium, rods in shaped, appeared individually (Figure 1B) and motile by means of single flagellum (Figure 1C). Cross-sectioned cells under TEM were shown in Figure 1D revealing the cell wall.

The isolated bacterium was identified as *P. rubra* BF1A IBRL by using genotypic (16S rRNA sequencing) and phenotypic method (biochemical test). Partial 16S gene of 754 bp was obtained after sequencing with forward primer. The sequence of *P. rubra* BF1A IBRL was deposited in the GenBank and was assigned accession no. AB858988. Gene sequencing analysis of its genome showed 98% similarity with *P. rubra* (FJ457184.1) and *P. viridis* (AB681561.1). Similarly, the phylogenetic analysis of 16S rRNA sequence (Figure 2) also showed that the strain BF1A IBRL was grouped in the same clade with both *P. rubra* and *P. viridis*. To further confirm the species of the strain, particular phenotypic characteristic (biochemical test), that is the utilization of citrate have been evaluated. Citrate utilization test is one of the phenotypic characterizations that are able to discriminate the two closely related species that are *P. rubra* and *P. viridis*. Yu *et al.* (2014) reported that *P. viridis* showed positive reaction on citrate utilization test. In contrast, isolate BF1A IBRL gives negative result to the citrate utilization test which confirms that the isolate more closely related to *P. rubra*.

The bacterium that belongs to family Alkaligenaceae (Austin, 1988), indeed are ubiquitous in marine environment and the potential antibacterial activity of the bacteria that belongs to *Pseudoalteromonas* genus is well documented (Cetina *et al.*, 2010). Vyne (2011) had stated that many species of *Pseudoalteromonas* shares a very high 16S rRNA gene sequence identity to each other, which was the explanation why both *P. rubra* and *P. viridis* were grouped in same clade of phylogenetic tree in this study. This phenomenon has also been reported by Isnansetyo *et al.* (2009) and Darabpour *et al.* (2011).

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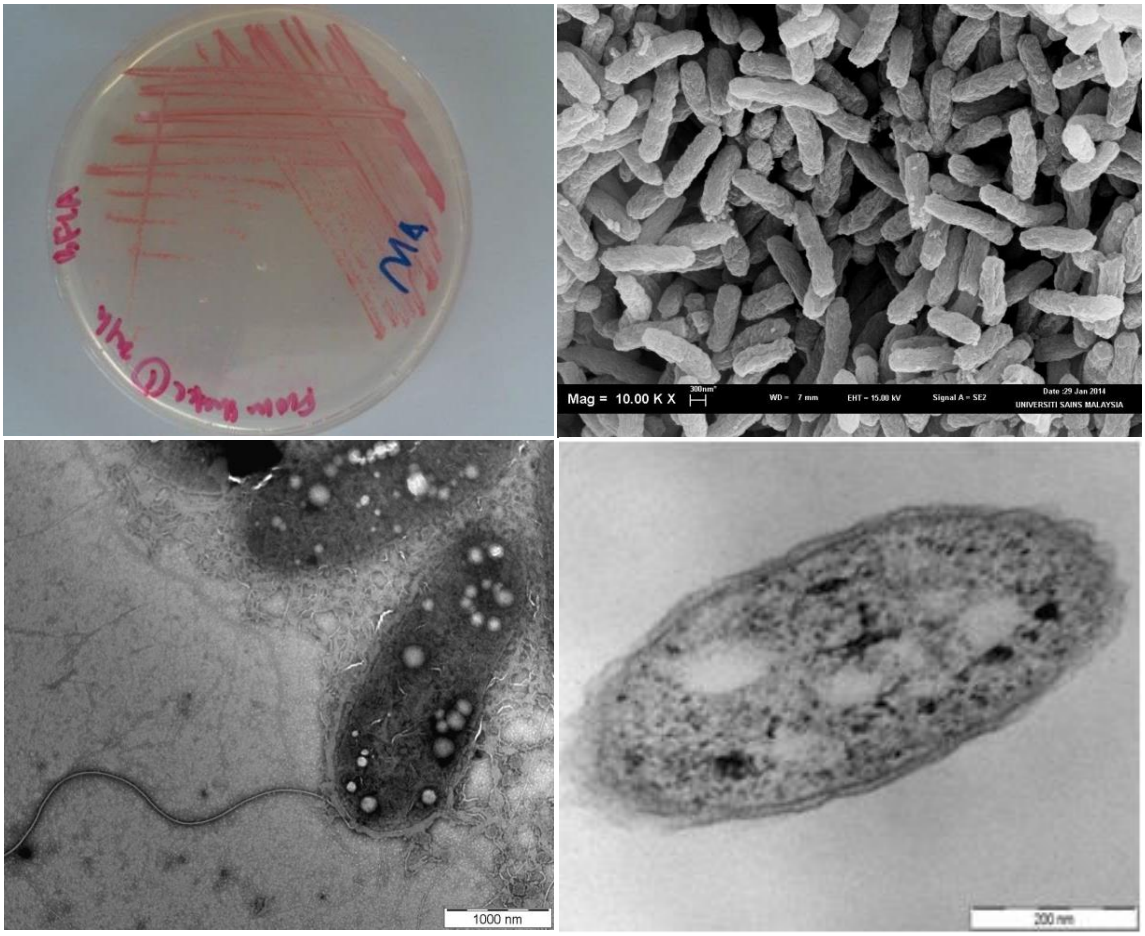

Figure 1: Macro and micro-graph of isolate BF1A IBRL. (A) colony appearance on Marine Broth (MB) plate; (B) SEM micrograph (Magnification 10,000×); (C) negatively stained cell using TEM (Magnification 15,000×); and (D) cross-section cell using TEM (Magnification 31,500×).

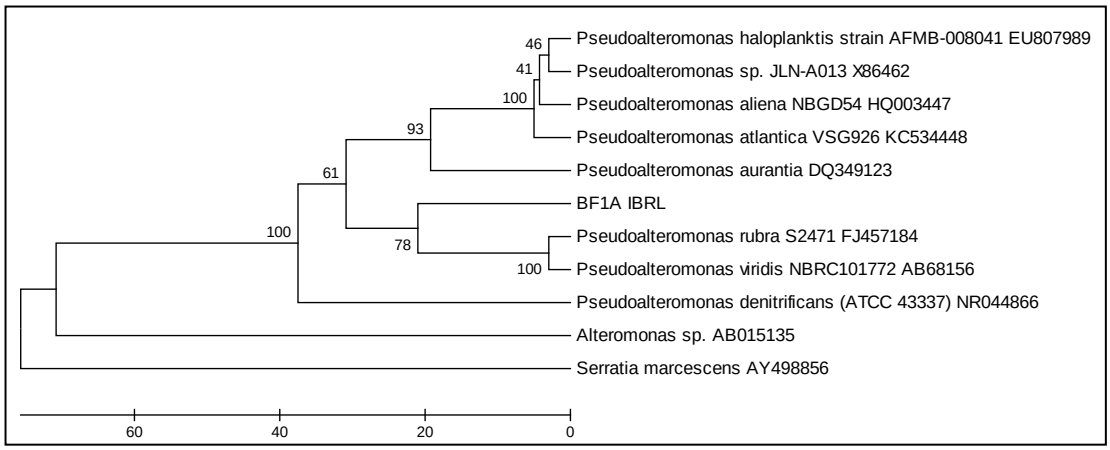

Figure 2: Phylogenetic tree based on bacterial 16S rRNA gene sequence of the isolate BF1A IBRL strain and other species of genus *Pseudoalteromonas*.

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Antibacterial activity of *P. rubra* BF1A IBRL against bacteria, fungi and yeasts

Table 1 shows the mean value comparison of zone of inhibition obtained for intracellular and extracellular extract. Significantly ($p < 0.05$), extracellular extract poses greater antibacterial activity compared to intracellular extract. However, for the both intracellular and extracellular extract, the inhibition zones obtained among the test bacteria were not varied significantly ($p > 0.05$). Both extracts were anti-bacillus, anti-staphylococci and also anti-acinetobacter.

Table 1: Results of antibacterial, antiyeast and antifungal activity of intracellular and extracellular extract of *P. rubra* BF1A IBRL.

Test microorganisms	Inhibition zone, mm	
	Intracellular extract (ISO)	Extracellular extract (DE)
Gram-positive pathogens		
B. subtilis	9.00 ± 2.64	10.33 ± 1.53
B. cereus	7.33 ± 0.57	8.67 ± 1.15
S. aureus (MRSA)	8.67 ± 1.15	8.67 ± 0.57
S. aureus	8.00 ± 1.00	8.67 ± 0.57
Gram-negative pathogens		
S. parathypi	-	
P. aeroginosa	-	
E. coli	-	
A. anitratus	7.33 ± 0.57	10.00 ± 0.00
Yeast pathogen		
C. albicans	-	-
C. tropicalis	-	-
Rhodothorula sp.	-	-
Fungi		
A. niger	-	-
A. fumigatus	-	-
Rhizopus sp.	-	-

Results are expressed as mean ± SD.

The inhibition zone was absent for yeasts and fungal microorganisms, conforming that the intracellular and extracellular extract exhibit neither antiyeast nor antifungal activity. The result also implies that the extract (intra and extracellular) have greater antagonistic activity against Gram positive bacteria, in which 100% of the total number of tested Gram-positive bacteria were inhibited, compared to Gram-negative bacteria, where only 25% of the tested bacteria were inhibited. The most susceptible bacterium was the *Bacillus* sp. The resistance of Gram-negative bacteria to the extracts can be attributed by the presence of lipo-polysaccharides layer in the cell wall structure of the bacteria. Similarly, Zheng *et al.* (2005) found that only 4 strains out of 126 epiphytic marine bacteria were able to inhibit the *E. coli*.

The potency of *P. rubra* BF1A IBRL to grow and produce metabolites in Marine broth indicates that the isolate was adapted to saline concentration up to 2% (w/v), and according to Tortora *et al.* (2004), the isolate can be categorised as facultative halophiles. This was in agreeing with the result obtained by Lewis and Corpe (1964) who reported that the optimum salinity for marine bacteria cultivation was between 1 to 3%. Isolate *P. rubra* BF1A IBRL can produce intracellular and extracellular pigments. Accordingly, the antibacterial activity also was scattered inside and outside of cells of *P. rubra* BF1A IBRL upon cultivation in Marine broth under shake flask system (26 °C and 120 rpm within 24 h), suggesting that the antibacterial property could be contributed by the pigment.

Both intracellular and extracellular extract of *P. rubra* BF1A IBRL MIC values of ISO and DE extracts against 5 laboratory strains were summarized in Table 2. The DE extract again showed strongest antibacterial activity compared to ISO extract. The MIC value ranged from 0.055 to 0.11 mg/mL, whereas the MBC value ranged from 0.11 to 0.44 mg/mL for the DE extract. The extract showed lowest MIC value against *S. aureus* and *S. aureus* (MRSA). Then DE extract inhibited the growth of all tested strains to a highest extent, which can be determined to pose strong activity. However, the antagonistic activity poses by the DE extract was lower compared to standard antibiotics, chloramphenicol used in this study.

MIC values were comparable remarkably for both the intracellular and extracellular extract. The quantitative measurement of MIC values indicates that the extracellular extract has higher antibacterial activity compared to intracellular extract ($p < 0.05$). The ability of extracellular pigment extract of *P. rubra* BF1A IBRL to inhibit *S. aureus* and *S. aureus* (MRSA) to a greatest extent is an advantage because of the alarming resistance of the strain towards many antibiotics, which caused by the occurrence of *mec*A gene (gene that encodes resistance to methycillin) in the DNA of MRSA strain (De Olivera *et al.*, 2013).

Moreover, the crude prodigiosin pigment extract from *P. rubra* BF1A IBRL shows lower MIC values (55 µg/mL) against *S. aureus* and *S. aureus* (MRSA) when comparing with crude prodigiosin pigment produced by other bacteria likes *Serratia* sp. as reported by Jafarzade *et al.* (2013), where the MIC values ranges from 83.3 to 200 µg/mL. Presumably, the prodigiosin pigment inhibit the test bacteria by disrupting the cell membrane. According to Isnansetyo and Kamei (2003), the mode of action of an antibacterial agent from *Pseudoalteromonas* sp. is due to the bacterial cell membrane permeabilization.

Characterization and identification of pigment produced by isolate *P. rubra* BF1A IBRL

For UV/vis spectrophotometer analysis and presumptive test

The spectrophotometric analysis of intracellular and extracellular pigments extracts *P. rubra* BF1A IBRL revealed maximum absorption at 531 nm and 534 nm,

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respectively marked similarities with maximum absorption of authentic sample of prodigiosin standard that was at 534 nm (Figure 3). Prodigiosin presumptive test result also gives positive result for prodigiosin, where the ISO and DE fraction turned to pink and yellow after added with acidic and alkaline solution, respectively.

The slightly different λmax (531 nm for intracellular pigment and 534 nm for extracellular pigment) values can be due to the different solvents used to dissolve the pigments. Previous study reported that prodigiosin can be dissolved in several solvents and its maximum absorption spectrum will be different slightly in different solvent, such as 540 nm in 95% of ethanol (Gandhi *et al.*, 1976) and 535 nm in acidic ethanol (D'Aoust and Gerber., 1974). The pigment from *P. rubra* BF1A IBRL also showed a shoulder peak at 498 nm. This suggesting that the pigment may contained α and β rotamers as stated by Wang *et al.* (2012).

Owing to the colour properties, UV absorption spectra, and presumptive test, the pigments produced by *P. rubra* BF1A IBRL was tentatively characterized as prodigiosin.

Separation of active compounds by Thin Layer Chromatography (TLC), Bio-autography assay and identification of prodigiosin by HPLC

The compounds present in the DE extract were separated using analytical TLC and the results are shown in Table 3. The solvent system of acetone: hexane (1:1) yielded 1 clear spot (dark pink, Rf = 0.87) under visible light. It has observed that the dark pink spot has the brightest colour and biggest size, suggesting that it is the major compound in the fraction. Bioautography analysis revealed that the pigmented spot (Rf = 0.87) has antibacterial property indicated that the pigment has antibacterial property against *B. subtilis*, *B. cereus*, *S. aureus*, *S. aureus* (MRSA), and *A. anitratus* (Table 3). Other spots with R_f values 0.05, 0.1, and 0.93 were also inhibitory against several bacteria, but these spots did not visible under normal light. The pigment in DE extract did not plays synergistic role for the antimicrobial activity, since the separated compound individually were able to inhibit bacteria.

Table 2: Minimum inhibitory concentration (MIC, mg/mL) and Minimum bactericidal concentration (MBC, mg/mL) of crude pigment extract (Intracellular and extracellular) of *P. rubra* BF1A IBRL.

Bacteria	Intracellular		Extracellular		Chloramphenicol	
	MIC	MBC	MIC	MBC	MIC	MBC
B. subtilis	8.88	8.88	0.11	0.11	0.008	0.008
B. cereus	8.88	8.88	0.11	0.22	0.008	0.008
S. aureus (MRSA)	0.28	0.28	0.055	0.44	0.008	0.016
S. aureus	0.28	0.28	0.055	0.44	0.008	0.016
A. anitratus	2.21	4.43	0.11	0.22	0.008	0.016

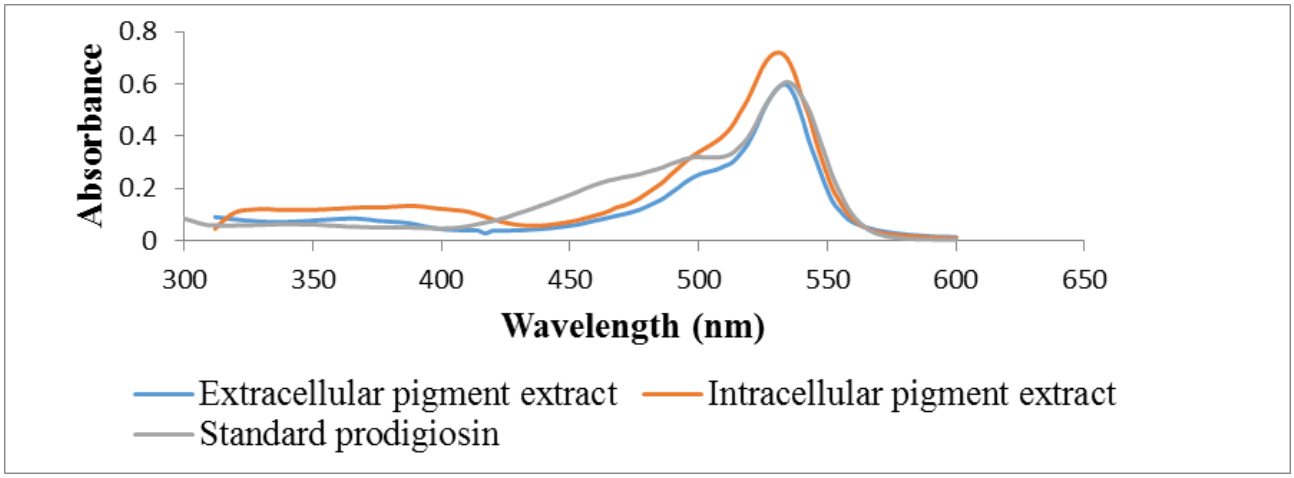

Figure 3: Absorption spectrum of Intracellular (ISO) and extracellular (DE) pigment of *P. rubra* BF1A IBRL in comparison with standard prodigiosin from *Serratia marcescens*.

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Table 3: R_f value and inhibition of growth on bioautography TLC plate.

R_f	Colour in visible light	Susceptible bacteria
0.05	No colour	*B. subtilis*, *B. cereus*, *S. aureus*, *S. aureus* (MRSA) and *A. anitratus*
0.1	No colour	*B. subtilis* and *B. cereus*
0.26	No colour	-
0.37	No colour	*S. aureus*, *S. aureus* (MRSA) and *A. anitratus*
0.42	No colour	-
0.53	No colour	-
0.63	No colour	-
0.68	No colour	-
0.87	Dark pink	*B. subtilis*, *B. cereus*, *S. aureus*, *S. aureus* (MRSA) and *A. anitratus*
0.93	No colour	*B. subtilis*, *B. cereus*, *S. aureus*, *S. aureus* (MRSA) and *A. anitratus*

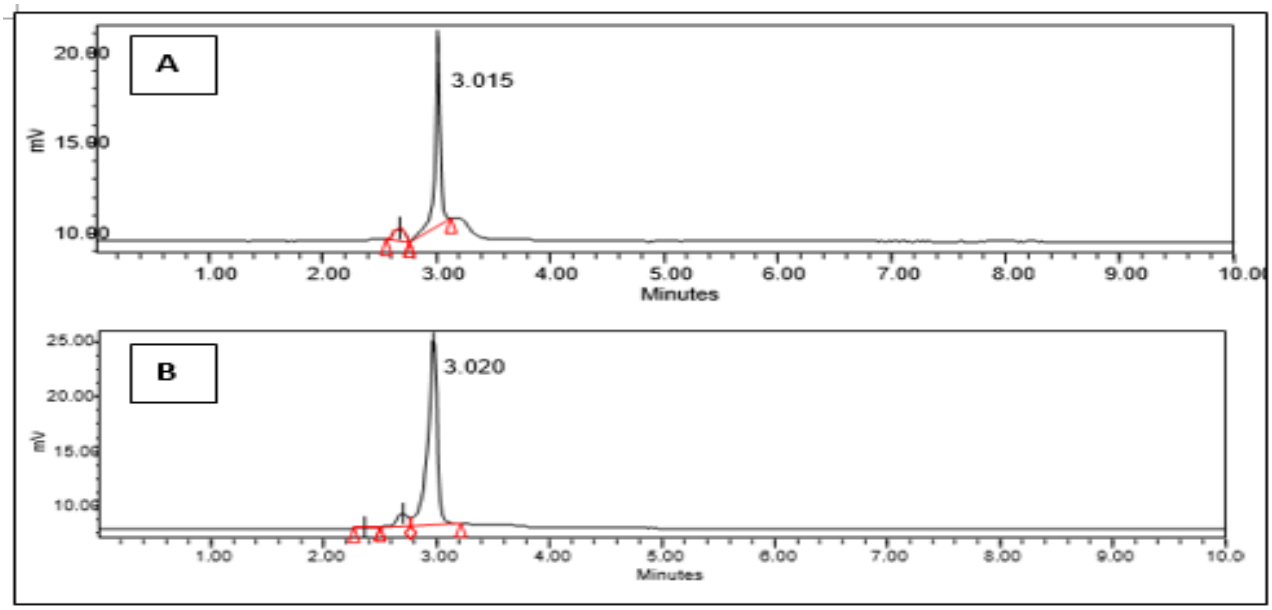

Figure 4: HPLC chromatogram of (A) standard prodigiosin and (B) TLC-prep purified compounds of *P. rubra* BF1A IBRL.

Figure 4 indicated that the pigment was separated by HPLC with 96.1% purity where a single peak at retention time of 3.020 min was obtained. The elution pattern of pigment from *P. rubra* BF1A IBRL are coinciding with the standard prodigiosin, which further confirms that the separated pigment is belongs to prodigiosin.

Prodigiosin is very common in term of its inhibitory effect against wide range of bacteria including pathogens (Jafarzade *et al.*, 2013) and fouling bacteria as well (Priya *et al.*, 2013). Hence, the antibacterial effect from *P. rubra*.

BF1A IBRL might contribute by the pigment but need to be confirmed further by bioautographic method. As stated earlier, *E. coli* and *P. aeruginosa* were resistance towards the *P. rubra* BF1A IBRL extract containing the prodigiosin pigment. Contrarily, prodigiosin isolated from other bacteria, such as *Zooshikella rubidus* (Lee *et al.*, 2011), *Serratia* sp. (Priya *et al.*, 2013) shows inhibition against *E. coli* and *Pseudomonas* sp., respectively. This phenomenon can be affected by several factors such as the extraction target (intracellular, extracellular, or cell bound compounds), the types of solvents and extraction method (Warkoyo and Saati, 2011), types of bacterial species (different types of bacterial species of same genus produced different types with different polarity compounds). Gauthier (1976) had done chromatographic separation of prodigiosin isolated from two different bacteria, which were *Serratia* sp. and *Pseudoalteromonas* sp., and reported that there was heterogeneity in peak elution of prodigiosins obtained from the two bacteria. He further concluded that the pigments from the two bacteria were related but not identical. This supports the hypothesis that prodigiosin appears in different types, and some of the examples are such as 2-substituted prodiginine, 2-(p-hydroxybenzyl) prodigiosin (Feher *et al.*, 2008), cycloprodigiosin, and undecylprodigiosin (Darshan and Manonmani, 2015).

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CONCLUSION

The indigenous prokaryote *P. rubra* BF1A IBRL was isolated from the surface of macroalgae, *Enteromorpha* sp. and was found metabolically active in term of production of pigment with antibacterial property. The red pigment produced by isolate *P. rubra* BF1A IBRL was identified as prodigiosin. This study also demonstrated that the coloured compound produced by *P. rubra* BF1A acted independently from other compounds in extract for the antibacterial effect. In conclusion, the bioactive compounds from *P. rubra* BF1A IBRL was able to create commercial opportunities for the colouring and pharmaceutical values due to their tinctorial property and antibacterial activities.

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