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Assessment of cultivation parameters influencing the growth, pigment production and anti-MRSA activity of *Pseudoalteromonas rubra* BF1A IBRL isolated from Malaysian marine environment

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ABSTRACT

Aims: Pigments are coloured substances that exhibit important characteristics to many industries including food, textile, cosmetics, food, pharmaceutical and also aquaculture industry. Naturally derived pigments from marine bacteria do not only exhibit the tinctorial property but are also known to possess broad range of antimicrobial activities. From the industrial point of view, the necessity to obtain suitable culture conditions for maximum yield of cell growth and pigment production is of utmost importance.
Methodology and results: The effect of cultural conditions, including light, pH, temperature, agitation speed and size of inoculum on bioactivity of an epiphytic marine bacteria, *Pseudoalteromonas rubra* BF1A IBRL was studied using shake flask technology. The antimicrobial activity was determined using the Lorian method. As a result, prodigiosin pigment extract obtained from *P. rubra* BF1A IBRL showed inhibitory activity against the MRSA strain. *Pseudoalteromonas rubra* BF1A IBRL produced the highest level of prodigiosin and anti-MRSA activity ($P<0.05$) in Marine broth at initial pH of 7.6 incubated at dark condition at temperature of 26 °C, agitation speed of 120 rpm and 2% (v/v) (1×10^6 CFU/mL) of inoculums size.
Conclusion, significance and impact of study: A high correlation between pigmentation and antibacterial activity were observed anticipating that the pigment has its own antibacterial properties. The above findings supported the fact that epiphytic marine bacteria were fruitful source for pigmented bioactive compounds, and the physical parameters had significantly influence of the pigment production.

Keywords: Pseudoalteromonas rubra; marine bacteria; prodigiosin; antibacterial; red pigment

INTRODUCTION

The ability of naturally derived pigmented compounds to exhibit antimicrobial properties is a well-known phenomenon (Visalakchi and Muthumary, 2009; El-Shouny *et al*., 2011; Rashid *et al*., 2014). Apart from being coloured, the industrial products must be preserved since they are always subjected to pathogenic attack. By applying natural pigments with antimicrobial properties, the use of chemical preservatives may be hindered, which is more natural, safer and economically an advantage. Hence, the naturally derived pigmented compounds with its own antimicrobial properties can bring dual benefits to various industries including food, textile and cosmetic industries. For instance, dyeing of textiles using natural dye exhibiting antimicrobial property will sponsor the production of protective clothes especially to the hospital's fabrics, since the fabrics has reported to be the vector of spreading harmful bacteria (Gupta and Laha, 2007).

Pseudoalteromonas rubra BF1A IBRL is a marine predominant bacterium, that belongs to the class Gammaproteobacteria (Bowman, 2007). This Gram-negative bacterium is first reported by Gauthier (1976), who described its morphological, physiological and biochemical characteristics. The bacterium was originally known as *Alteromonas rubra*, which was then taxonomically re-classified as *P. rubra* by Gauthier *et al.* (1995). *Pseudoalteromonas rubra* is known to produce a red pigment known as prodigiosin, which exhibit pharmaceutically-relevant activities, including antibacterial properties and cytotoxic activities (Feher *et al*., 2008).

Generally, there are different types of prodigiosin produced by various types of bacteria, such as heptyl-

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prodigiosin was syntheiszed by *Pseudovibrio denitrificans* (Sertan-de Guzman *et al*., 2007), undecycloprodigiosin produced by *Streptoverticillium rubrireticuli*, and the cycloprodigiosin can be obtained from *Vibrio gazogenes* (Alihosseini *et al*., 2008). All the types of prodigiosin share the same skeleton (pyrolydipyrrolmethane skeleton) but differ in the alkyl substituents (Chen, 2008), where the length of the side chain is inversely proportional to the antagonistic activity of prodigiosin (Krishna, 2008). It is also interesting to note that a single bacterium is able to produce more than one type of prodigiosin. Lee *et al.* (2011) is the first report showing that *Zooshikella rubidus* S1-1 can synthesize both prodigiosin and cycloprodigiosin simultaneously as two major metabolites in submerged fermentation process.

The prodigiosin pigment is earlier found to only occur in terrestrial *Serratia* sp., where much attention has been focused in the antibacterial activity of prodigiosin synthesized by this bacterium (Siva *et al*., 2011; Bharmal *et al*., 2012; Gulani *et al*., 2012). Consequently, the bioactivity of *P. rubra* BF1A IBRL is less studied. Till date, most of the existing studies on *P. rubra* were reported on the chemical profiling of the metabolites produced, without establishing the extent of the metabolite's bioactivity. The little information available regarding the antibacterial activity of prodigiosin from *P. rubra* imposing the needs for thorough studies in term of the quantification and also the optimization process.

It is well known that MRSA is the most problematic and nosocomial pathogen in public health, which exhibited resistance towards different types of synthetics antibiotics (Darabpour *et al*., 2011). Prodigiosin isolated from *Serratia marcescens* is known to possess anti-MRSA activity (Samrot *et al*., 2011). Therefore, the present investigation focused on the time course profile of growth, prodigiosin production and anti-MRSA activity of *P. rubra* BF1A IBRL, as well as the optimization process of several cultural conditions, to further enhance the pigment production and the anti-MRSA activity. A shake flask system was implemented to produce prodigiosin by the marine pigmented bacterium, *P. rubra* BF1A IBRL.

MATERIALS AND METHODS

Bacterial strain, medium, and seed culture

The pigmented marine bacterium, *P. rubra* BF1A IBRL was isolated from the surface of macroalgae, *Enteromorpha* sp., collected from Batu Feringhi, Pulau Pinang, Malaysia (5°28'0''N 100°15'0''E). It was maintained on Marine agar (MA; Difco, USA) at 25 °C and subcultured regularly at every 2 weeks time to ensure its viability. Marine broth (MB; Difco, USA) was used as the cultivation medium for the pigment production. The seed culture was prepared by transferring several colonies of *P. rubra* BF1A IBRL grown in MA into 250 mL of Erlenmeyer flask containing 50 mL of MB. The culture was incubated at 26 °C for 24 h at 120 rpm of agitation speed.

Profile study of isolate BF1A IBRL

The 250 mL Erlenmeyer flasks containing 100 mL of MB were inoculated with 2% (v/v) (1×10^6 CFU/mL) of seed culture, and incubated at 26 °C with agitation speed of 120 rpm for 72 h. Sampling was done for every 8 h of incubation period by harvesting three flask of culture (triplicates), where the growth, antibacterial activity and prodigiosin pigment production was determined.

Optimization process

For the optimization process of the prodigiosin production, several cultural conditions were studied independently including lighting conditions (dark and light incubation condition), initial medium pH, which ranged from 5 to 9, incubation temperature (20, 26, 30, 37, 40, and 45 °C), agitation rate (0, 50, 120, 150, 200, and 250 rpm), and inoculums sizes [0.5 to 20% (v/v) of 1×10^6 CFU/mL]. The incubation period is 24 h.

The optimum parameter from each experiment was utilized in the subsequent assay unless otherwise stated. All the assays were carried out in triplicates. For the optimization of agitation speed, the cell structure and arrangement of *P. rubra* BF1A IBRL in various agitation speeds were observed using light microscope after performing the Gram staining method (Chandra and Mani, 2011).

Growth determination, extraction and quantification of prodigiosin (Gravimetric analysis)

For every harvesting period, the growth was determined by recording the optical density of the culture using spectrophotometer (Darabpour *et al*., 2012a). Firstly, the supernatant and cell pellet were separated by centrifugation (4000 rpm for 15 min). The supernatant was collected and extracted using ethyl acetate (EtOAc) (1:1). The EtOAc extract of extracellular pigment was taken up with diethyl ether (DE) by solvent-solvent partitioning system and spectral analysis was carried out using UV/Vis spectrophotometer (Spectronic Unicam, Genesys 10_UV) to determine the absorbance of the pigment at 534 nm of wavelength. The prodigiosin yield was determined by using a standard correlation graph between absorbance (A) and concentration of standard prodigiosin pigment (Sigma, Aldrich), which was expressed in µg/mL (Kim *et al*., 2007; Wang *et al*., 2012)

Evaluation of antibacterial activity of prodigiosin

The DE partition extract obtained at different harvesting time was then allowed to completely dry until constant weight obtained. The dried extract was then re-disolved in ethanol (0.5 mL) for quantitative antibacterial evaluation test, as described by Lorian (1991). Methycillin-resistant *Staphylococcus aureus* (MRSA) was selected as test bacteria for this experiment. Briefly, 0.1 mL of the extract was added into 9.9 mL Nutrient broth (NB) that contained 10^4 CFU/mL MRSA. The initial optical density of the

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mixture was about 0.294. The mixture was incubated at 37 °C for 18 h. Then, the optical density of the mixture was measured at 620 nm and the antibacterial activity was expressed in U/mL. One unit of antibacterial activity indicated the quantity of the extract that was able to reduce or inhibit 1% of the MRSA growth in the liquid medium (Lorian, 1999). NB seeded with 10^4 CFU/mL of MRSA and 0.5 mL ethanol (without addition of extract) was prepared as a control.

Bioautography assay

The DE partition extract was first run on TLC silica plate using the solvent system acetone:hexane (5:5). The developed chromatogram was then placed on the solidified MHA agar plate and overlaid with with 10 mL of molten MHA agar seeded with 1mL of MRSA (adjusted to contain 1×10^8 CFU/mL using 0.5 McFarland standard). The plate was then incubated at 37 °C for 24 h. The cell viability on the chromatogram was further determined using MTT assay (calorimetric assay) by spraying the surface with 2 mg/mL solution of INT (2-(4-iodophenyl)-3-(4-nitophenyl)-5-phenyl tetrazolium chloride). Zone of inhibition were observed as clear zones against a purple background (purple coloration indicating the cell viability) (Ejikeme and Josiah, 2010).

Statistical analysis

The significant differences of the mean data were analyzed using One Way Analysis of Variance (ANOVA) following post-hoc test and were considered as significant at $P<0.05$. Statistical analysis was carried out using SPSS version 16. All the experiments were performed in triplicate and the values were reported as standard deviation.

RESULTS AND DISCUSSION

Time course profiles of isolate *P. rubra* BF1A IBRL for growth, pigment production and antibacterial activity

Profile study of growth, pigment production and antibacterial activity of isolate *P. rubra* BF1A IBRL (Figure 1) revealed that the pigment production and antibacterial activity of the isolate were parallel, where the highest reading for both was achieved at 24 h of incubation time. This observation strongly indicates that the antibacterial activity of the isolate could be contributed by the bacterial pigment. However further analysis needs to be done to confirm this fact.

The obtained results indicate that several changes occur to the growth, pigment production and antibacterial property of *P. rubra* BF1A IBRL as the time of incubation prolonged. After 24 h of incubation time, the colour intensity slowly decreased. The level of prodigiosin production by *P. rubra* BF1A IBRL started to increase dramatically at middle log phase of growth (8 h) and the maximum production of prodigiosin (0.284±0.02 µg/mL) occurred at 24 h of cultivation period (late exponential phase). Similarly, the maximum prodigiosin pigment production by *Serratia* sp and *Vibrio* sp. was also achieved at 24 h of cultivation time (Alihosseini *et al*., 2008; Bharmal *et al*., 2012).

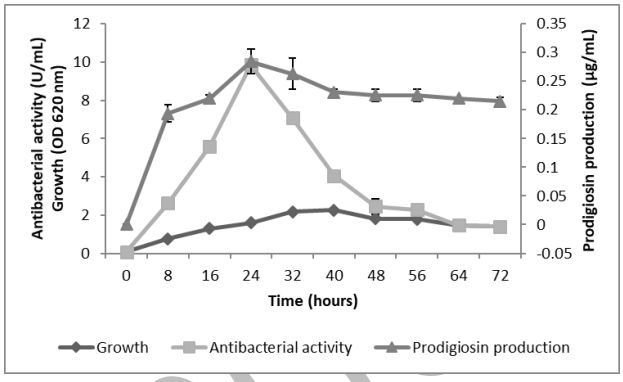

Figure 1: Time course profile of growth, pigment production and anti-MRSA activity of diethyl ether extract of *P. rubra* BF1A IBRL.

In contrast, the highest production of prodigiosin by *Zooshikella rubidus* occurred after cellular multification ceased (Lee *et al*., 2011), whereas isolate *Serratia rubidae* produced maximal prodigiosin at the stationary phase (Siva *et al*., 2012). This testifies that the time course profile of prodigiosin pigment varies depending the producing source. It is interesting to note that the algal bacterium, *P. rubra* BF1A IBRL shows inhibition against the MRSA (8.25±0.03 U/mL) within 24 h and this raises possibilities that the bacterium can be source of antibacterial compounds to control the pathogenic bacteria. MRSA is considered as an important cause of hospital and community acquired infections, which has acquired the chromosomal gene *mec* A that encodes methicillin-inducible PBP with decreased affinity for methicillin (Darabpour *et al*., 2012b). The significant antagonistic activity of *Pseudoalteromonas* sp. against the MRSA has been reported previously (Feher *et al*., 2010) and the mode of action involved is cell membrane permeabilization (Isnansetyo and Kamei, 2003).

Light and darkness condition

Figure 2 shows the effect of light condition towards the growth, pigment production and antibacterial property of *P. rubra* BF1A IBRL. The results indicated that there was no significant effect ($P>0.05$) on prodigiosin production and growth yield of *P. rubra* BF1A IBRL upon incubation either under light or dark condition. However, the antibacterial activity of the bacterium is remarkably high in the culture incubated at dark condition (7.11 ± 0.003 U/mL) compared to light condition (5.05 ± 0.001 U/mL) ($P<0.05$), providing a justification to select the dark condition as optimum condition. This result coincides with the findings by Velmurugan *et al*. (2009) who also reported that the incubation in darkness had increased the bioactivity of five fungi including *Monascus purpureus,*

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Malays. J. Microbiol. Vol xx(x) 20xx, pp. xxx-xxx
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Isaria farinose, Emericella nidulans, Fusarium verticillioides and *Penicillium purpurogenum.* Besides that, Someya *et al*. (2014) also reported that the prodigiosin pigment can be negatively affected by light during the incubation period.

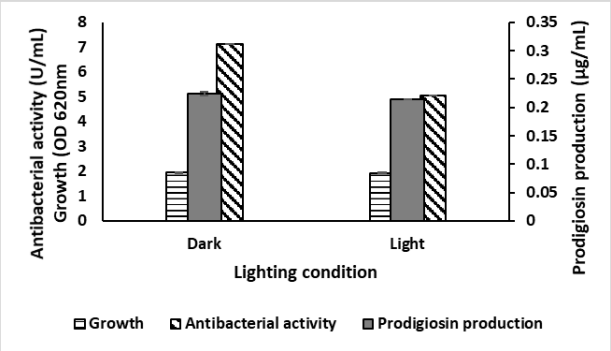

Figure 2: Effect of light on prodigiosin production, antibacterial activity and growth of *P. rubra* BF1A IBRL.

Influence of pH

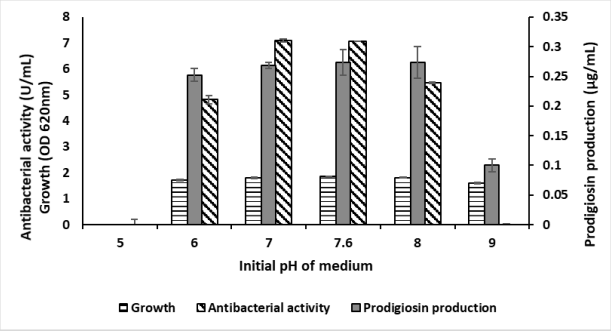

Figure 3: Effect of various pH on growth, prodigiosin production, and antibacterial activity of *P. rubra* BF1A IBRL.

Initial pH value of the cultivation media is one of the determinant factors for microbial growth and biosynthesis of secondary metabolites including the pigmented compounds (Visalakchi and Muthumary, 2009; Gulani *et al*., 2012). The results of the effect of initial pH on growth, pigment production and antimicrobial activity of *P. rubra* BF1A IBRL are shown in Figure 3. The amount of red prodigiosin was highest at both pH value of 7.6 and 8.0, which yielded 0.274 ± 0.02 µg/mL each. This is in agreement with results obtained by Palanichamy *et al*. (2011) and Darabpour *et al*. (2012b). Based on the antibacterial activity, the highest yield was obtained at pH 7.6 (7.07±0.01 U/mL). The maximum bioactivity at pH 7.6 can be justified that the marine bacterium *P. rubra* BF1A IBRL requires medium condition that can imitate the pH of seawater which is between 7.2 to 7.6 (Maithili *et al*., 2014). The pH value below 7.0 and above 8.0 did not favour the pigment production and anti-MRSA activity, which indicates that highly acidic and alkaline pH may have inhibitory effect on the pigment production, similar as reported by Krishna (2008). The drastic change of pH may alter or denatured the activity of enzymes that regulates the prodigiosin biosynthesis pathway causing the blocking of the pigment synthesis. According to Solieve *et al*. (2011) prodigiosin biosynthesis pathway was originated from the enzymatic condensation of 2-methyl-3-n-amyl-pyrrole (MAP) and 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC) precursors. Besides, Bharmal *et al*. (2012) reported that the pH value beyond the optimum value can negatively affect the function of proline, which responsible for inducing the prodigiosin biosynthesis process.

Effect of incubation temperature

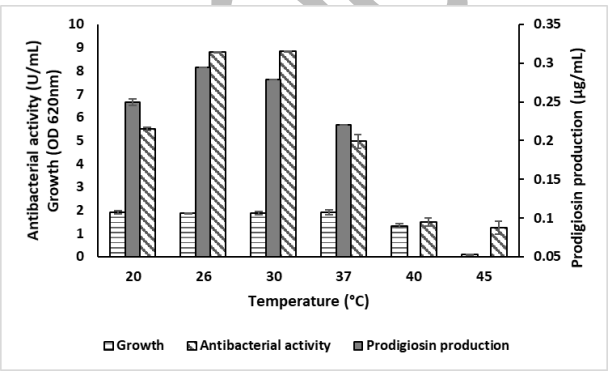

Figure 4: Effect of various temperatures on growth, prodigiosin production, and antibacterial activity of *P. rubra* BF1A IBRL.

Similar to the effect of pH, the incubation temperatures significantly influence the pigment production ability of *P. rubra* BF1A IBRL (Figure 4). In marine broth, the maximal prodigiosin (0.294±0.01 µg/mL) was obtained at a temperature of 26 °C. The anti-MRSA activity was also highest at 26 °C, which yielded 8.8±0.02 U/mL. Thus, the incubation temperature of 26 °C was selected as optimum temperature for *P. rubra* BF1A IBRL. This marine bacterium can grow in a wide range of temperature, however the pigment production occurred over a relatively narrow range of temperatures, which implies that temperature is an important factor that determines the ability of *P. rubra* BF1A IBRL to produce prodigiosin. Similar finding was also reported for bacteria *Serratia marcescens* SU-10, *Serratia rubidaea* and *Serratia* sp. BTWJ8 as they exhibited maximal prodigiosin production when the incubation temperature is between 25 °C to 28 °C (Krishna, 2008; Samrot *et al*., 2011; Siva *et al*., 2011). The growth of *P. rubra* BF1A IBRL in wider range of incubation temperature reflected its ability to adapt to elevated temperature of the marine environment, which symbolized the characteristic of marine bacteria. The production of intended pigment was sorely affected at temperature 37 °C and above. Virtually, a complete block in the prodigiosin synthesis was observed at temperature

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of 35 °C and above for *Serratia marcescens* (Gulani *et al*., 2010).

Effect of agitation speed

Agitation provides aeration to the cultures and also aided in uniform mixing of the medium components (Azlinah, 2010). The data indicates that the organism failed to produce prodigiosin at the agitation speed of 0 rpm and 50 rpm, likewise the expression of the anti-MRSA activity (Figure 5). Contrarily, Darah *et al*. (2014) observed low antibacterial red pigment production upon cultivation of *S. marcescens* IBRL USM 84 at static condition. The amount of prodigiosin (0.3 ± 0.01 µg/mL) and anti-MRSA activity (7.9 ± 0.05 U/mL) is highest when the culture was agitated at 120 rpm of agitation speed. The finding established that the isolate *P. rubra* BF1A IBRL is an aerobic bacterium which requires oxygen to grow and produce metabolites. However, both the pigmentation potency and anti-MRSA activity decreased gradually upon increasing the agitation speed above 120 rpm. Khanafari *et al*. (2010) also reported agitation speed of 120 rpm as an optimum speed for the production of halophilic pigment by a bacterium isolated from Solar Salt Lake.

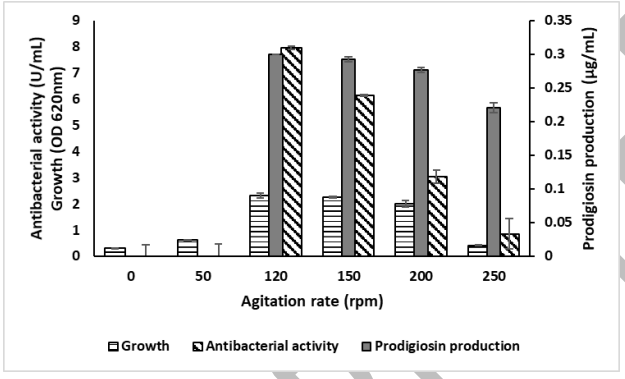

Figure 5: Effect of various agitation rates on growth, prodigiosin production, antibacterial activity of *P. rubra* BF1A IBRL.

This is the first study to report the effect of agitation speed on the bacterial cell arrangement and prodigiosin production by the algae-associated bacterium, *P. rubra* BF1A IBRL. By referring to Figure 6, the cell structure in all the agitation is similar, except for agitation speed of 250 rpm. The arrangement and density of cells were varied under different agitation speeds. Higher cell density was observed at agitation speed of 120 rpm, 150 rpm and 200 rpm, which is parallel to the pigment production ability, since greater prodigiosin yield was obtained at these agitation speed compared to the other speeds. This implies that higher cell density as opposed to loosely pack cells is more effective in the production of prodigiosin pigment, consequently supported the fact that prodigiosin production is cell-growth dependant (Wang *et al*., 2012). Abnormal cell structures of *P. rubra* BF1A IBRL was observed under agitation speed of 250 rpm, possibly due to the cell disruption, which caused by the shearing stress of higher shaking speeds (Bakri *et al*., 2011).

Effect of inoculum size

Figure 7 shows the effect of inoculum size on the growth, pigment production and anti-MRSA activity of *P. rubra* BF1A IBRL. No significant effect of inoculum size was observed for the growth of *P. rubra* BF1A IBRL. However, the bacteria were found to produce more prodigiosin (0.29 ± 0.01 µg/mL) and anti-MRSA activity (8.7 ± 0 .12 U/mL) at 2% (v/v) (1 × 10^6 CFU/mL) of inoculum size compared to other inoculum sizes. This is in agreement with the findings from Goswami *et al*. (2010) and Darah *et al*. (2014). Lower inoculum size may increase the lag phase of the isolates, which indirectly need longer fermentation period to produce higher growth and desired products (Gay *et al*., 1996). However, this fact is contrary with the results obtained in this study whereby the lower inoculums used (0.5% v/v) is also able to give relatively higher amount of growth within 24 h. This is could be due to the use of same type of medium for the preparation of preinoculum. The active microorganism in the preinoculum media would remain active once transferred into other flask containing the same type of medium. Consequently, it can decrease the length of the lag phase.

Detection of bioactive compound by Bioatougraphy assay.

Bioautography was performed in order to check the antibacterial activity of separated compounds on TLC plate. A strong pink band at R^f = 0.87 was discovered under visible light which resemble the R^f value of standard prodigiosin. This spot shows inhibition against *B. subtilis*, *B. cereus*, MRSA, *S. aureus* and *A. anitratus*. This result confirmed that the prodigiosin compound in DE extract possessed its own antibacterial activity.

Effectiveness of prodigiosin from *P. rubra* BF1A IBRL as an anti-MRSA agent

Overall analysis of the present study showed that all the cultural condition tested had significant influence on the production of prodigiosin pigment ($P<0.05$). However, no increments were observed in the prodigiosin yield and anti-MRSA activity after the optimization process, since the preliminary cultivation conditions used before the optimization process is already being the optimized parameters for *P. rubra* BF1A IBRL. There were many other factors that could affect the pigmentation of bacteria which includes the source and types of medium used (Khanafari *et al*., 2006). Furthermore, a thorough understanding of the regulation and pathway of pigment production will render defined process for the enhanced production of the desired pigment (Goswami *et al*., 2010). To the best of our knowledge, there were no researches done to determine the pigment production pathway of *P. rubra*.

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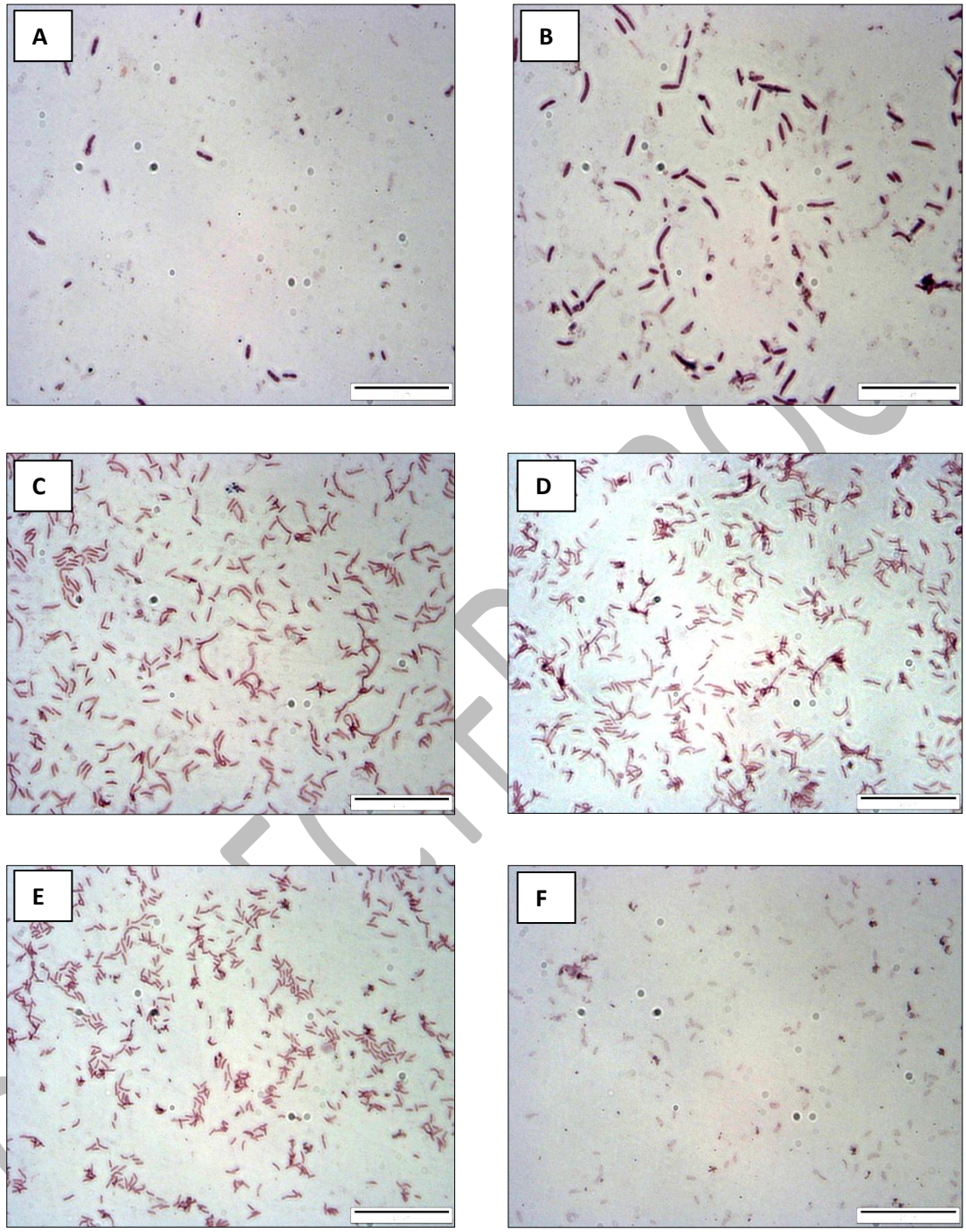

Figure 6: Cell structure and arrangement of *P. rubra* BF1A IBRL as observed with Light microscope (100× at various agitation speeds on 24 h of fermentation. (A) 0 rpm, (B) 50 rpm, (C) 120 rpm, (D) 150 rpm, (E) 200 rpm, and (F) 250 rpm.

At all the optimization stage, the prodigiosin pigment yield is highly parallel with the anti-MRSA activity, where both the highest pigment yield and highest anti-MRSA activity achieved at the same optimum condition all the time. This highly anticipates that the prodigiosin pigment may responsible for the anti-MRSA activity, imposing the needs for thorough search to further confirm the abovementioned statement. The antibacterial activity of

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prodigiosin pigment present in the crude mixture extract could be confirmed by performing bioassay guided fractionation (e.g. thin layer chromatography and bio-autography assay). The optimization results also demonstrated that direct relationship between growth and pigment production did not exist, corresponding to the findings from Mendez *et al*. (2011). The positive correlation between pigmented compounds and its antibacterial activity has been discovered by earlier studies. Holmstrom *et al*. (2002) and Bruhn *et al*. (2007) reported that the pigmented group of bacteria exhibited broader antibacterial activity compared to the non-pigmented bacteria. From the ecological point of view, the prodigiosin produced by the epiphytic marine bacterium was acted as antimicrobial agent in order to protect its host from the attack of pathogens (Stankovic *et al*., 2014), which could justify the in-vitro antagonistic activity of the prodigiosin against the MRSA.

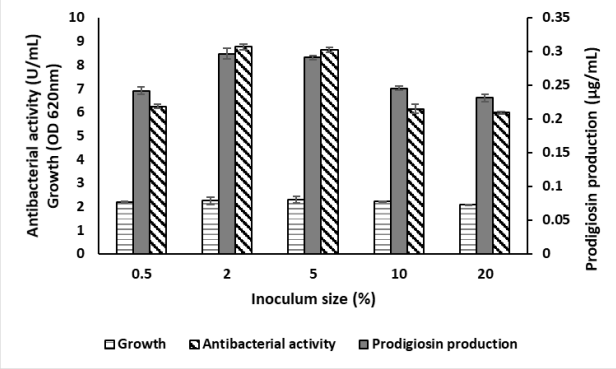

Figure 7: Effect of various inoculum size on growth, prodigiosin production, and antibacterial activity of *P. rubra* BF1A IBRL.

Prodigiosin pigment production of *P. rubra* BF1A IBRL in the submerged batch culture after assessment of all physical parameters was 3.3170 mg/L, which was lower compared to amount of prodigiosin produced by *Zooshikella rubidus* S1-1 (47.8 mg/L) (Lee *et al*., 2011), *Serratia rubidae* (8mg/L) (Siva *et al*., 2011), *Serratia marcescens* (125mg/L) and *Hahella chejuensis* KCTC 2396 (28 mg/L) (Kim *et al*., 2006). Despite the finding that *P. rubra* BF1A IBRL produced scarce amount of pigment, but surprisingly, the pigment amount obtained by this bacterium was several times higher compared to prodigiosin amount produced by *Serratia* sp. BTWJ8, which was only 0.03995 mg/L (Krishna, 2008). In fact, enhancing low pigment productivity is definitely one of the main issues facing by the researchers. Since physical parameters assessment did not improve the productivity of biopigment by *P. rubra* BF1A IBRL, hence further studies are needed in order to get better understanding on the pigment production pathway of this bacterium. To the best of literature review, the biosynthesis pathway of prodigiosin has yet to be performed for *P. rubra*.

CONCLUSION

This study points out that *P. rubra* BF1A IBRL has critical requirement for cultural conditions in order to produce bioactive compounds, where the alteration of several physical parameters has negatively affected the pigment production and antibacterial activity. Most favourable conditions to produce prodigiosin and anti-MRSA activity is in the medium of pH of 7.6, cultivated with 2% (v/v) of inoculums, and incubated at 26 °C under dark condition at the agitation speed of 120 rpm. Although further studies on the bio-guided fractionation are necessary, our findings suggest that the diethyl ether prodigiosin extract from *P. rubra* BF1A IBRL is a great potential pigmented antibiotic against the MRSA strains as it revealed a close association between pigmentation and the anti-MRSA activity. From this study, it can also be inferring that further exploitation to the rich of bacterial communities in Malaysian marine environment could benefit various biotechnological applications.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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