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Influence of Culture Conditions on Production of Natural Antibacterial Red Pigment Produced by *Serratia marcescens* IBRL USM 84

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This study was undertaken to investigate the influence of cultural conditions on the production of antibacterial red pigment by *Serratia marcescens* IBRL USM 84. This isolate exhibited maximal antibacterial red pigment production of 37.17 U/ml which was achieved at 40 hours of cultivation time when cultivated in marine broth with improved conditions, including the addition of 0.2% (w/v) of agar, cultivation temperature at 25°C, initial medium pH of 7, agitated at 150 rpm and 2% inoculum size ($1x10^9$ cells/ml). There was an increment of 164% of red pigment production after improvement compared to before improvement of cultural conditions. The results suggested different cultural conditions have a significant impact on the secondary metabolite production by the bacterium to gain a high yield.

Key words: *Serratia marcescens*; antibiotic; red pigment; marine microorganism.

Increase in the number of multiple drug resistant pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA)[1] and vancomycin-resistant *Staphylococcus aureus* (VRSA)[2,3] are a serious issue in the context of public health, due to its resistance against almost all available antibiotics. Thus, the development of new drugs or alternative therapies in response to the antibiotic resistance is urgently needed.

The diversity of marine microorganisms and the complex living conditions surrounding them have resulted into production of novel and unique secondary metabolite with much stronger bioactivities compared with terrestrial organisms[4]. The marine environment harbors microorganisms such as bacteria and fungi with antagonistic traits[5], and they are potential sources of novel antimicrobials compounds[6]. Furthermore, many marine bacteria have been reported to produce various types of natural pigments with antimicrobial activities[7] such as *Serratia marcescens*[8].

We have isolated a non-clinical isolate of *S. marcescens* IBRL USM 84 from the surface of a marine sponge *Xetospongia testudinaria* and found that the red pigment it's produced possessed antibacterial activity against MRSA[9]. Therefore, this study was carried out to enhance the antibacterial red pigment production by *S. marcescens* IBRL USM 84 by improving cultural condition parameters. To our knowledge this is the first report on the quantification of antibacterial activity produced by *S. marcescens* IBRL USM 84.

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MATERIALS AND METHODS

Microorganisms and culture maintenance

Serratia marcescens IBRL USM 84 was isolated from the surface of a marine sponge *Xetospongia testudinaria* was supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. This bacterial culture was maintained on marine agar (Difco, United State) slant at 25°C for 24 hours aerobically before storing them at 4°C until further used. The subculturing was performed every month to ensure its survival.

MRSA culture was also supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia and maintained on nutrient agar (Merck, Germany) slants and incubated at 37°C for 24 hours before storing them at 4°C until further used. The subculturing was performed every month to ensure its survival.

Medium composition and cultivation conditions

Cultures (S. *marcescens*) were grown in 250 ml Erlenmeyer flasks containing 50 ml of marine broth containing (g/l); peptone 5, yeast extract 1, ferric citrate 0.1, sodium chloride 19.45, magnesium chloride 5.9, magnesium sulphate 3.24, calcium chloride 1.8, potassium chloride 0.55, sodium bicarbonate 0.16, potassium bromide 0.08 and strontium in a trace amount. In order to get a semi-solid agar, 0.2% (w/v) of agar was added in to the broth medium and the pH was adjusted to 7.5. The initial experiment was conducted to determine the optimal incubation or cultivation time that produced the highest antibacterial red pigment production. Therefore, the experiments containing the medium with the addition of 1% (v/v; $1x10^9$ cells/ml) 15 hour old bacterial inoculum agitated at 120 rpm and incubated at 25°C was carried out for 72 hours. The samples were withdrawn at every 8 hourly intervals and were assayed for antibacterial activity and cell growth determination.

Improvement of fermentation process for antibacterial red pigment production

Various process parameters influencing antibacterial red pigment production during submerged fermentation were improved. The strategy followed was to improve each parameter (antibacterial activity and cell growth), independent of the others and subsequently all the improved conditions were employed in all experiments.

Physical parameters

Improvement of cultural conditions in shake flask system for a maximal antibacterial red pigment production involves various physical parameters. They were agar concentration (0, 0.1, 0.3, 0.5 and 0.7%, w/v), temperature (20, 25, 30, 35 and 40°C), initial medium pH in the range between pH 5 to 9, inoculum sizes [1 to 9% (v/v) of $1x10^9$ cells/ml] and agitation speed (0, 50, 100, 150 and 200 rpm. All the experiments were performed in triplicate and the values were reported as standard deviations.

Extraction of antibacterial red pigment

The culture broth containing the cells was centrifuged at 5000 g (Sigma 4k15, Sartorius, Germany) for 20 minutes at 4°C. The cell pellet was washed twice with 0.85% (w/v) physiological saline for 30 minutes and recentrifuged at 5000 g for 20 minutes. The washed cell pellet was soaked in 50 ml sterile distilled water for an hour at 4°C to give osmotic shock condition to the cell. In order to break the cell, 10 sterile glass beads (0.4 mm diameter) were added into the cell suspension and vortex vigorously[10]. The suspension was then separated by centrifugation at 5000 g for 20 minutes at 4°C. The supernatant was used for intracellular antibacterial activity assay.

Assay for antibacterial activity

The amount of antibacterial activity secreted by the isolate was measured quantitatively using the method described previously by Lorian [11]. About 0.1 ml of the 24 hour old Methicillin-resistant *Staphylococcus aureus (*MRSA) cell suspension (3–4 x 10^5 cells/ml) was inoculated in 7.9 ml nutrient broth followed by the addition of 2.0 ml of the supernatant obtained from the centrifuged broth of the isolate IBRL USM 84. The mixture was then incubated at 37°C for 14 hours. The degree of inhibition of the test bacteria (MRSA) was determined based on the decrease in the culture turbidity measured spectrophotometrically at 560 nm, compared to control. The control consisted the same material as for the test culture but no addition of the isolate IBRL USM 84 cultivation broth; instead it was replaced by addition of another 2.0 ml of nutrient broth. To facilitate the expression of the potency of the antibacterial activity of the compounds

secreted by the isolate IBRL USM 84, the antibacterial activity was defined as; one unit of the antibacterial activity was defined as the quantity of the antibacterial compounds which resulted the reduction or inhibition of 1.0% on the growth of MRSA in the liquid medium which was used as the test microorganism. The results were expressed as mean value ± standard error of the readings obtained with three replicates for each experiment.

Cell growth determination

The cell growth was determined spectrophotometrically (Spectronic Unicam, Genesys 10UV) at 540 nm and the actual concentration was compared to standard curve prepared prior determination.

Extraction, purification and confirmation of prodigiosin

The red pigment produced by *S. marcescens* IBRL USM 84 was extracted and analyzed by method of Cang *et al*.[12] and Allihoesseni *et al*.[13] (2008). Three milliliter of acetone was added into 1.0 ml of a thick cell suspension and agitated at 150 rpm for 30 minutes at room temperature (30±2ºC). The mixture was centrifuged at 5000 g for 20 minutes. The red colored supernatant was mixed with petroleum ether with the ratio of 1:2 in a separating funnel and shook vigorously until the red pigment was in the petroleum ether. The extracted red colored petroleum ether solution was evaporated using a rotary evaporator under reduced pressure until the dried red pigment obtained and was used for spectral analysis.

Fifty microgram of dried red pigment was dissolved in 10.0 ml of absolute ethanol and in order to get an acidic condition, 1.0 ml of 1.0 M HCl was added into the solution. To get an alkaline solution, 1.0 ml of 1.0 M NaOH was added and to get a neutral condition 1.0 ml of sterile distilled water was added into the solution. A spectral analysis of the pigment in acidic, alkaline and neutral solution was determined using a spectrophotometer (Spectronic Unicam, Genesys 10UV) at 400-600 nm.

Statistical analysis

The significant difference of the mean data was analyzed using one way Analysis of variance (ANOVA) and Duncan test with PASW Statistics 18 version.

RESULTS

Antibacterial red pigment production and growth profiles before improvement of physical parameters

Figure 1 shows the isolate of *S. marcescens* IBRL USM 84 which produced red pigment with antibacterial activity. The time course profiles of the antibacterial red pigment production and cell growth is shown in Figure 2. The antibacterial activity was detected significantly at 8 hours (4.41 U/ml) of cultivation and increased gradually and achieved it maximal value of 14.08 U/ml at the 48 hours of cultivation. The activity was then decreasing slowly thereafter until 72 hours of cultivation. This may be due to the depletion of nutrients and accumulation of toxic metabolites in the cultivation medium. Thus, incubation time of 48 hours was used to improve other physical parameters. The growth of *S. marcescens* IBRL USM 84 also followed the same pattern as the antibacterial activity and achieved its maximal growth of 2.10 g/L at the 48 hours of cultivation. There was a slight increased in pH of the medium from 7.5 at the beginning of the cultivation to 9.6 at 72 hours of cultivation showing that the bacterial cells need a slight alkaline condition to produced the antibacterial activity significantly.

Effect of the addition of agar in to the medium

The non-clinical isolate of *S. marcescens* IBRL USM 84 which was isolated from the surface of a marine sponge *Xetospongia testudinaria* was a facultative anaerobic isolate. Therefore it needs a semi-solid agar to grow and to produce its antibacterial red pigment. As shown in Figure 3, the addition of agar at 0.3% (w/v) produced the highest antibacterial red pigment of 16.90 U/ml. The isolate also capable of producing antibacterial red pigment even in the medium without the addition of the agar but the amount was low (5.91 U/ml). The amount of antibacterial red pigment production increased as the amount of agar increased and dropped after achieving its maximal production. However, the cell growth increased drastically from 0 to 0.3% and increased slowly after that until achieving the highest growth production at 0.7% (1.64 g/l). The results revealed that even though the cells of *S. marcescens* IBRL USM 84 able to grow and produced antibacterial

red pigment in the liquid medium (without the addition of agar) but its preferred semi-solid, and the best was at the addition of 0.3% of agar.

Effect of cultivation temperature

Figure 4 shows the effect of temperature on antibacterial red pigment production. There was an increase in the antibacterial red pigment production as well as growth with the increase of cultivation temperature from 20ºC to 25ºC. The optimum temperature was 25°C with the antibacterial red pigment production of 22.24 U/ml. The cell growth achieved its maximal production of 1.40 g/L at 25ºC.

There was changes occurred in the coloration of the cultivation medium. The color of the medium remained red when the cultivation temperature was between 20 - 25ºC. However the color of the medium changed to orange coloration when incubated at higher temperature of 35ºC and 40ºC.

Effect of pH

pH of the cultivation medium plays a very crucial role in the synthesis of secondary metabolites. As shown in Figure 5, the maximum production of antibacterial red pigment was obtained when the pH value was 7.0 and at this pH the antibacterial red pigment produced was 22.51 U/ml. In addition, results showed that the increase or decrease in the pH value of the cultivation medium above or under the optimum pH decreased the antibacterial red pigment production. The cell growth achieved its optimized value at pH 9.0 with 1.64 g/L.

Effect of inoculum size

Inoculum size plays a vital role in induction and production of secondary metabolites as the secondary metabolites are induced in the late stationary phase of the bacterial growth. Figure 6 shows the effect of inoculum sizes on antibacterial

Fig. 1. *Serratia marcescens* IBRL USM 84 produced red pigment with antibacterial activity

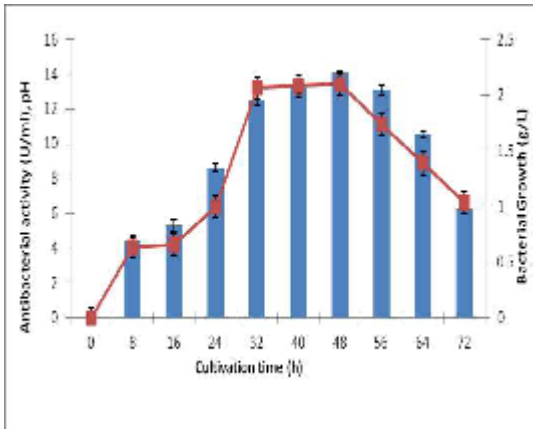

Fig. 2. Profiles of antibacterial red pigment and cell growth production by *Serratia marcescens* IBRL USM 84

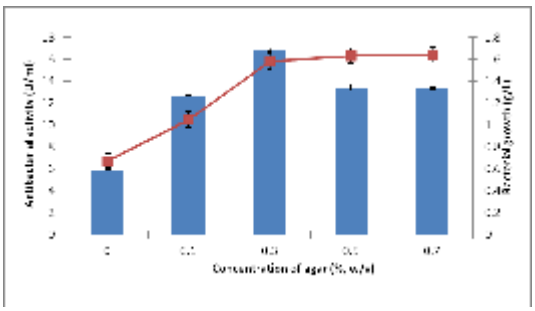

Fig. 3. Effect of agar on antibacterial red pigment and growth production by *S. marcescens* IBRL USM 84

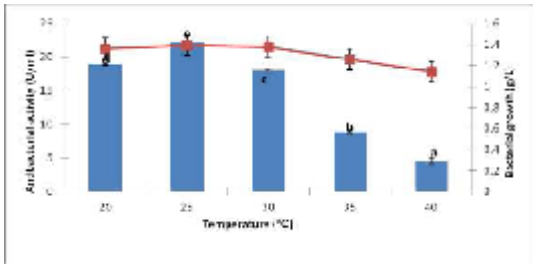

Fig. 4. Effect of temperature on antibacterial red pigment and growth production by *S. marcescens* IBRL USM 84

red pigment production. The optimum inoculum size was found to be at 2% (v/v) $1x10^9$ cell/ml which produced 25.51 U/ml antibacterial red pigment and 1.72 g/l cell growth. Further increased in inoculum size decreased the antibacterial red pigment production. It also noted that beyond the optimum inoculums size decreased the bacterial cell growth.

Effect of agitation speed

Figure 7 shows the effect of agitation speed on the antibacterial red pigment production. The antibacterial red pigment production increased as the agitation speed increased. Maximum antibacterial red pigment production of 28.16 U/mL was achieved at agitation speed of 150 rpm. It was observed that increased in mixing help the microbial synthesis of antibacterial red pigment. However, at higher agitation speeds (beyond 150 rpm) the antibacterial red pigment production dropped. The highest growth of 1.99 g/l also achieved at 150 rpm of agitation speed. Higher or lower agitation speed than the optimum value reduced the growth as well as antibacterial red pigment production.

Antibacterial red pigment production using improved physical parameters

After the improvement of the physical parameters [initial medium pH of 7.0, cultivation temperature of 25ºC, inoculums size of 2% (v/v) $1x10^9$ cell/ml, agitation speed of 150 rpm and 0.3% (w/v) of agar] a time-course profile was carried out for 72 hours. Figure 8 shows the antibacterial red pigment production increased gradually and achieved its maximum production of 37.17 U/ml at the 40 hours of cultivation time. The antibacterial red pigment production started to decline thereafter. The cell growth was also increased gradually and achieved its maximum of about 2.35 g/l at 40 hours of cultivation. There was about 164% of increment in the antibacterial red pigment production after improvement of cultural conditions compared to before improvement of cultural conditions. Furthermore, the results showed that the redder colour of the pigment with the highest activity was achieved when the improved cultural conditions were employed.

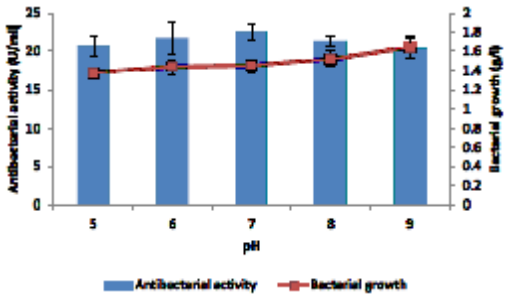

Fig. 5. Effect of pH medium on antibacterial red pigment and growth production by *S. marcescens* IBRL USM 84

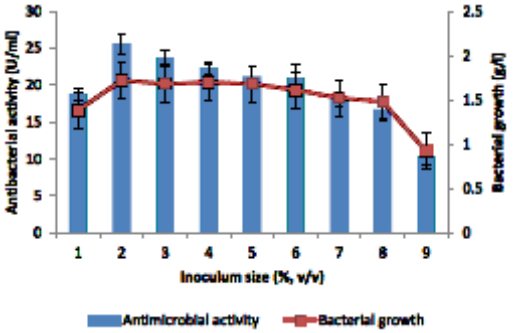

Fig. 6. Effect of inoculum size on antibacterial red pigment and growth production by *S. marcescens* IBRL USM 84

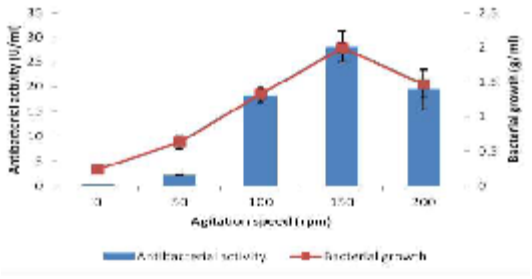

Fig. 7. Effect of agitation speed on antibacterial red pigment and growth production by *S. marcescens* IBRL USM 84

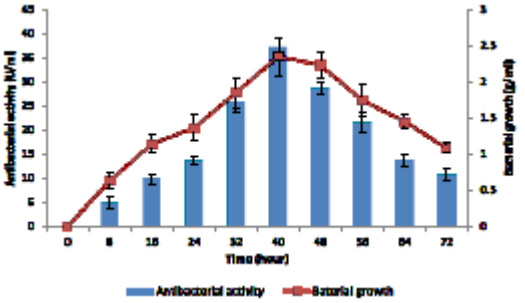

Fig. 8. Profiles of antibacterial red pigment and cell growth production by *Serratia marcescens* IBRL USM 84 after optimization of cultural conditions

DISCUSSION

The production of clinically important microbial pigments especially with antimicrobial (antibacterial) activity is one of the emerging fields of research. *S. marcescens* IBRL USM 84 was studied and found that it produced natural red pigment with antibacterial activity, against MRSA. This non-clinical isolate of *S. marcescens* IBRL USM 84 was isolated from the surface of a local marine sponge *Xetospongia testudinari*. Marine sponges have been reported to produce secondary metabolites which play a decisive ecological role, protecting them against potential invaders, predators or other competitors[14]. They also host variety of heterotrophic microorganisms[15], many of which play a role in sponge nutrition, metabolic transport and defense against predators as well as biofouling[16]. Bacteria growing on the surface of sponges live in a highly competitive environment in which access to space and nutrients are limited[17]. Several potentially therapeutic compounds identified in sponges have striking similarities to metabolites derived from their associated bacteria[18, 19]. Hence, sponge-associated bacteria become a highly potential source for the production of antibiotic compounds.

It is widely accepted that physical and chemical parameters play important role in enhancing microbial secondary metabolite productions. In this study, various physical parameters or also known as cultural conditions were improved in order to obtain maximal production of antibacterial red pigment. pH is important in buffering the medium to enhance the antibacterial red pigment production, whereas the temperature influenced the cell growth. The *S. marcescens* IBRL USM 84 preferred 25°C to grow and it's produced redder colour of pigment with the highest antibacterial activity. The coloration of the medium changed to orange colour when the temperature was more than 25°C and very obvious changed was noticed at 35 and 40°C. Giri *et al*.,[20] and Bharmal *et al*.,[21] reported that non-pigmented *S. maecescens* occurred when the isolate was incubated at 37°C and 38°C respectively. *S. maecescens* IBRL USM 84 need a semi-solid medium to grow and to enhance its antibacterial red pigment production. This condition suggested that the bacterial cells which are highly motile need a substrate to adhere or to attach in order to grow well and producing antibacterial red pigment significantly.

Agitation is a crucial factor in enhancing the antibacterial red pigment production. Higher or lower agitation speed than the optimum level reduced the antibacterial red pigment production. This could be due to the higher shear stress when the cells were agitated at higher speeds[22]. Although culture grown on static condition which was not supplied with enough aeration (dissolved oxygen), there was still low antibacterial red pigment production achieved implying that antibacterial red pigment production could also occur at oxygen limited conditions. This result showed that *S. marcescens* IBRL USM 84 is a facultative anaerobic bacterium where they can survive with little oxygen tension. According to Shieh *et al*.[23], there were anaerobic and facultative anaerobic bacteria which produced antibacterial red pigment and this included strains of *Vibrio ruber*[24], *S. marcescens*[8], *Streptomyces coelicolor*[25] *and Streptomyces lividans*[26].

At lower agitation speed than the optimum level resulted in lower antibacterial red pigment production and this condition could be due to the minimum level of dissolved oxygen in the cultivation medium. According to Pansuriya and Singhal[27], incomplete mixing and/or oxygen transfer might be the reason of low production at lower agitation speeds. Thus, mixing is crucial for better oxygen and nutrient transfer rate in microbial secondary metabolite production. The agitation speed is also affected the cell growth. This condition could be due to shear forces derived from higher agitation speed where collision among the cells occurred and damaging them. Darah *et al*.[22] explained that at lower agitation speed, the inadequate mixing of the broth towards the later stages of growth affected the secondary metabolite synthesis, while the drastic dropped in its activity at higher agitation speeds was due to shearing effect on the cells. Darah *et al*.[28] found that changes in the morphology of microorganisms caused by agitation speeds were also influenced secondary metabolite production and growth of the microorganisms.

Bacterial fermentation is a complex process and it is not only depends on the performance and fermentation medium but also

requires the suitable environmental conditions. These factors may affect the antibacterial red pigment production. Fermentation has three obvious phases that are; pre-fermentation phase which is cell growth phase where nutrients are gradually consumed and began to produce secondary metabolites. Then followed by the second phase where a large number of secondary metabolites are produced rapidly, and finally the third phase which is called post-fermentation phase. In this phase there is a slow accumulation of secondary metabolites[29]. During this stage, bacterial cells even produce toxic metabolites. The reasons may due to cell senescence and autolysis where accumulation of final products or other toxic metabolites occurred and eventually the strains produce in a hostile environment.

The antibacterial red pigment produced by *S. marcescens* IBRL USM 84 was previously identified in our study[9]. It was defined and confirmed as prodigiosin based on method described by Cang *et al.*[12] and Alihoesseni *et al.*[13]. *S. marcescens* is a main producer of prodigiosin (belongs to the family of tripyrrole) which possess antibacterial activity[30]. It is appearing only in the later stages of bacterial growth[31]. It has been reported to exhibit antibacterial[30], antifungal[32], algicidal[33], antiprotozoal[34], cytotoxic[35], immunosuppressive[36], anti-tumor[37] and antiproliferative properties[38].

CONCLUSION

There are many cultural condition parameters that influenced red pigment production by isolate S. *marcescens* IBRL USM 84. The maximal antibacterial activity of red pigment production was achieved at 37.17 U/ml with 2.12 g/L cell growth when using marine broth added with 0.3% (w/v) of agar, incubation temperature of 25°C, pH of 7, agitation speed of 150 rpm and 2% (v/v) of $1x10^9$ cells/ml of inoculum size. An antibacterial red pigment produced by the isolate of *S. marcescens* IBRL USM 84 is a secondary metabolite of great interest in medicine due to its multiple properties in the pharmaceutical industry.

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