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Influence of Culture Conditions and Medium Composition on the Production of Antibacterial Compounds by Marine *Serratia* sp. WPRA3

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This study was undertaken to investigate the influence of culture conditions and medium components on production of antibacterial compounds by *Serratia* sp. WPRA3 (JX020764) which was isolated from marine water of Port Dickson, Malaysia. Biochemical, morphological, and molecular characteristics suggested that the isolate is a new candidate of the *Serratia* sp. The isolate showed strong antimicrobial activity against fungi, Gram-negative and Gram-positive bacteria. This bacterium exhibited optimum antibacterial compounds production at 28°C, pH 7 and 200 rev/min aeration during 72 h of incubation period. Highest antibacterial activity was obtained when sodium chloride (2%), yeast extract (0.5%), and glucose concentration (0.75%) were used as salt, nitrogen, and carbon sources respectively. Different active fractions were obtained by Thin-Layer Chromatography (TLC) and Flash Column Chromatography (FCC) from ethyl acetate crude extracts namely OCE and RCE in different culture conditions, OCE (pH 5, 200 rev/min) and RCE (pH 7/without aeration). In conclusion, the results suggested different culture conditions have a significant impact on the types of secondary metabolites produced by the bacterium.

***Keywords*:** optimization, antibacterial compound, pigmented bacteria, marine bacteria, Serratia

Introduction

According to Leone *et al.* (2007), the main focus of research interests in marine bacteria is due to their ability to produce several biologically active molecules, such as antibiotics, toxins and antitoxins, antitumor and antimicrobial agents. The marine environment in Malaysia comprises a large reservoir of pristine resources for the discovery of bioactive compounds. Malaysia is a littoral country with the coastal area of 42,330 km^2, coastal length of 4,675 km and sea area of 549,500 km^2 (Department of Environment, Malaysia), making this nation rich with unique treasures.

Nowadays the emergence of pathogens with multiple drug resistant properties, notable examples being methicillin-resistance *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) has warranted the need for the discovery of novel antibiotics. The diversity of marine organisms' species and their complex living environment enables them to produce novel and unique secondary metabolite with much stronger bioactivities as compared with their terrestrial counterparts (Schwartzmann *et al.*, 2001). Consequently, the past two decades have witnessed intense efforts aimed at discovering novel drugs from the sea (Anand *et al.*, 2006).

Serratia is a Gram-negative bacteriaum, classified in the large family of Entrobacteriaceae. The genus *Serratia* can be differentiated from other genera by its production of three special enzymes lipase, gelatinase, and DNAase (Giri *et al.*, 2004). Ten species of *Serratia* have been described, of which only *S. plymuthica*, *S. rubidaea* and some biogroups of *S. marcescens* are capable of producing a special secondary metabolite, the red pigment prodigiosin, which is a linear tripyrrole pigment with a low molecular weight (323.4 dalton) and appearing only in the last stages of bacterial growth (Grimont *et al.*, 1977; Bennet and Beutley, 2000). Although prodigiosin has no known defined role in the physiology of producing strains, it has antifungal, antibacterial, and antiprotozoal activities, thus presenting opportunities for its clinical application (Moraes *et al.*, 2009).

Although many types of differential and selective media have been used for the growth of *Serratia* and elucidation of prodigiosin, none of these have been explored for their effects on the production of antimicrobial compounds. Enumeration of regular prodigiosin production has been carried out in nutrient broth, sesame seeds (Giri *et al.*, 2004), maltose broth and peptone glycerol broth (Jonas *et al.*, 1993). Optimum growth of all strains of *Serratia* has been reported to be at pH 9 and a temperature range from 20–37°C (Giri *et al.*, 2004). In this study we used minimal medium to determine optimal nutritional and culture conditions for antibacterial compounds production by the isolate WPRA3.

Materials and Methods

Isolation and identification of a marine pigmented bacterium

A red pigmented bacterium designated as WPRA3 was isolated from marine water of Port Dickson, Negri Sembilan,

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Malaysia. The isolate was routinely grown in Marine Agar (MA; Difco). Identification of isolate was done by 16S rRNA sequence analysis. PCR amplification of the 16S rRNA gene was performed using two oligonucleotide primers, forward 5′-CTCCTACGGGAGGCAGCAG-3′ and reverse 5′-WAT TACCGCGGCKGCTG-3′. The PCR programme was set using heat thermal minicycler (MJ Research, USA) as follows: Initial denaturation was carried out for 5 min at 94°C. It was followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1.5 min with a further 10 min extension at 72°C, using UniversAll^{TM} tissue PCR kit. PCR product was purified using QIAquick PCR Purification Kit (QIAGEN, Germany) according to user's instructions manual. The resultant sequence (1,500 bp) of strain WPRA3 was compared with those in GenBank using the program BLASTn (Altschul *et al.*, 1997). The phylogenetic tree of related sequences based on BLAST sequence similarity was constructed using the *MEGA* version 4 program (Tamura *et al.*, 2007) after multiple alignments of data by CLUSTAL W (Thompson *et al.*, 1994) for assignment of close relationships at species level. Nucleotide sequence similarities and DNA gaps were calculated and edited using BIOEDIT software (Hall, 1999). Bootstrap analysis (1,000 resamplings) was used to evaluate the topology of neighbour-joining tree (Felsenstein, 1985). Morphological and biochemical tests were also determined according to Bergey's Manual using an isolated colony from MA.

Test microorganisms

The microbial species, Gram-positive [*Bacillus subtilis* ATCC 11774, *Staphylococcus aureus* ATCC 11632, methicillin-resistant *Staphylococcus aureus* (MRSA)] and Gram-negative (*Vibrio parahaemolyticus* ATCC 17802, *Pseudomonas aeruginosa* ATCC 27853) were used in this study. They were maintained on Nutrient Agar (NA; Difco) slants at 4°C.

Optimization of media and culture condition

Media composition: Inorganic salt medium composed of (g/L): $(NH_4)_2SO_4$ (2.64), KH_2PO_4 (2.38), $MgSO_4{\cdot}7H_2O$ (1.00), $CuSO_4{\cdot}5H_2O$ (0.0064), $FeSO_4{\cdot}7H_2O$ (0.0011), $MnCl_2{\cdot}4H_2O$ (0.0079), $ZnSO_4{\cdot}7H_2O$ (0.0015) was used as the minimal medium (Pridham and Gottlieb, 1948) at an adjusted pH of 7.
Inoculum preparation: Strain WPRA3 was cultivated in 100 ml minimal medium supplemented with 3% NaCl, 1% glucose and 1% yeast extract as carbon and nitrogen sources respectively and incubated at 28°C for 24 h at 200 rev/min in an incubator shaker (INFORS AG CH-4103) and then diluted with minimal medium to give an initial optical density of 1 at a wavelength of 600 nm. Five milliliter of this culture was used as bacterial culture.
Effect of NaCl concentration: Fifty milliliter of minimal medium was dispensed each into 250 ml Erlenmeyer flasks and sterilized. Pre-sterilized yeast extract (1%) and glucose (1%) were added as nitrogen and carbon sources into the minimal medium just prior to inoculation. Five milliliter of the seed culture of strain WPRA3 was inoculated into each Erlenmeyer flask. The effect of salinity on bioactive metabolite production was studied in different concentration of NaCl ranging from 0% to 5%.
Effect of different temperature, initial pH, and levels of aeration: The effect of different culture conditions such as different temperature (20, 22, 24, 26, 28, 30, and 32°C), initial pH (4–11) and aeration (0, 50, 100, 150, and 200 rev/min) on the production of antimicrobial compounds was studied separately by inoculating 5 ml of the seed culture into minimal medium supplemented with optimum NaCl concentration, glucose (1%) and yeast extract (1%).
Effect of different concentrations of glucose and yeast extract: The effect of different glucose and yeast extract concentration ranging from 0% to 3% on the antimicrobial compound production was studied separately by inoculating 5 ml of the seed culture of strain WPRA3 into minimal medium while other parameters were kept at optimum levels.
Effect of incubation period: The effect of incubation period (1–3 days) on the antimicrobial compound production was studied by keeping other parameters at optimum levels.
Estimation of bioactive metabolite production: Manual shaking thrice with equal volume of ethyl acetate (EA) (1:1) in a separating funnel was used for the extraction bioactive metabolite produced by the isolate in optimum culture conditions. The solvent layer was collected and then evaporated in a rotary evaporator to obtain crude extract. Production of antibacterial compounds was determined using disk diffusion method described by Bauer *et al.* (1966) against *S. aureus*, MRSA, *V. parahaemolyticus* and *B. subtilis*.

Partial purification of bioactive metabolite

Partial purifications of crude extracts produced by the strain WPRA3 were carried out up by Flash column chromatography (FCC). Crude extract in red color (RCE) was produced by the isolate WPRA3, which grew in minimal medium with optimum medium compositions without aeration at pH 7 condition. Crude extract in orange color (OCE) was produced by the isolate WPRA3, which grew in same medium compositions as RCE, but different culture conditions (pH 5 and 200 rev/min).
UV absorption: Crude extracts RCE and OCE were dissolved in methanol and scanned using UV mini-1240 spectrophotometer using series of wavelength ranging from 350–600 nm to obtain the wavelength associated with maximum absorption.
Thin Layer Chromatography (TLC): Thin layer chromatography (TLC) of crude extracts were performed on silicagel 60 F_{254} TLC plate (Merck) and developed using different solvent systems to obtain best separation. The compounds were visualized under UV-light to calculate the retention factor (R_f), which was taken as the distance travelled by the compound divided by the distance travelled by the solvent.
Flash Column Chromatography (FCC): Crude extracts RCE and OCE were applied separately to the column (RP-Cartridges Ø 12×150 mm, Buchi, Switzerland) with a flow rate of 3 ml/min. The following solvent systems were used: DCM (1), DCM/MeOH (9:1), DCM/MeOH (8:2), DCM/MeOH (7:3), DCM/MeOH (5:5), and MeOH (1). Additionally, the antibacterial activity of each fraction was investigated against two pathogenic bacteria, MRSA and *V. parahaemolyticus*.

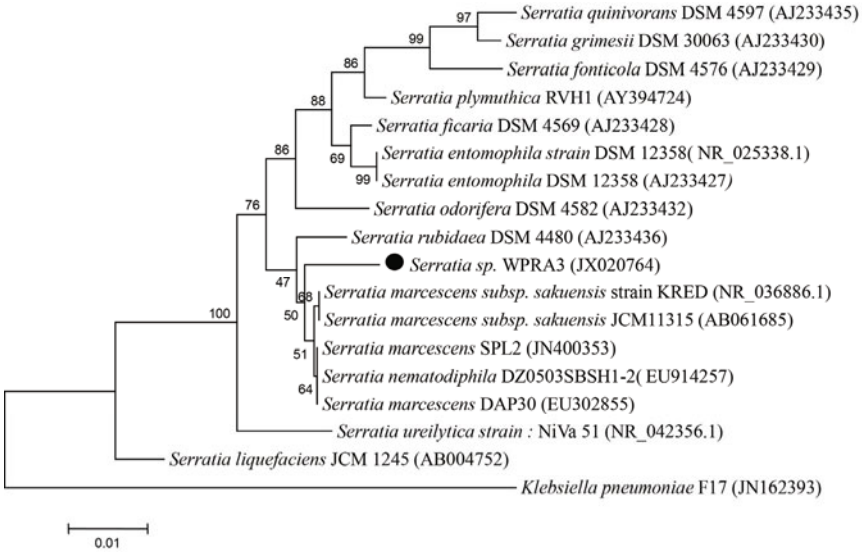

Fig. 1. Phylogenetic relationship of *Serratia* strain WPRA3 to closely related bacteria based on neighbour-joining tree analysis of 16S rRNA gene sequence data. 16S rRNA sequences of representative strains of the genus *Serratia* were obtained from GenBank. A sequence of *Klebsiella pneumonia* was used as the out-group and bootstrap values (expressed as percentage of 1,000 replications) are shown at branch nodes. Sequence accession numbers are shown in parentheses. Scale bar, 0.01 substitutions per nucleotide position.

Antimicrobial activity

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The minimum inhibitory concentration (MIC) was determined using the 96 well plate according to the microbroth dilution method (Mbosso *et al*., 2010) in Mueller Hinton broth (MHB; Difco) supplemented with glucose (10%) and phenol red (0.5%) added as indicator, changed from red to yellow colour due to the formation of acidic metabolites associated with microbial growth. In a first step, 50 µl MHB was distributed from the first to the twelfth wells. Dry extracts were initially dissolved in 100 µl Dimethylsulfoxide (DMSO, an organosulfur compound) and subsequently in MHB, to final concentration of 1,600 µg/ml. 50 µl of this solution was added to the first well of each microtiter line. Dilution was done by transferring 50 µl of the solution/mixture from the first to eleventh well. The twelfth well served as growth control since no sample was added. Subsequently, 50 µl of microbial suspension (10^5 colony forming units), was added to each well. The microtiter plates were incubated at 37°C for 24 h. The minimum bactericidal concentration (MBC) was determined by subculture of the well showing no apparent growth in a MHA. The least concentration showing no visible growth on agar subculture was taken as MBC value.

Statistical analysis

All the assays were carried out in triplicate. Experimental results were expressed as means±standard deviation (SD) of three parallel measurements. The differences between the samples were analyzed using one-way analysis of variance (ANOVA) and were considered significant at $P<0.05$. Statistical analysis was carried out using SPSS version 15 (IBM Inc).

Results

Identification of bacterium

The isolate was a Gram-negative, facultative anaerobic, red pigmented and short rod-shaped bacterium. It was positive for catalase, the citrate utilization test, β-galactosidase, lysine decarboxylase and ornithine decarboxylase. Oxidase, indole and the Voges Proskauer test were negative. The isolate could grow on MacConkey agar, a selective medium for the cultivation of enteric bacteria. The organism was found to be positive for gelatinase, and was negative for urease. Acid was produced from the utilization of D-glucose, D-mannitol, inositol, D-sorbitol, D-sucrose, D-melibiose, amygdalin, and L-arabinose while L-rhamnose was not a utilizable substrate for the isolate. Comparative 16S rRNA gene sequence analysis showed that the strain WPRA3 should be positioned within the genus *Serratia*, being most closely related to *Serratia nematodiphila* strain DZ0503SBS1 and *Serratia marcescens* subsp. *Sakuensis* JCM 11315^T (97% similarity). The 16S rRNA gene sequence similarities of the novel strain with type strains of other established *Serratia* species were ≤96%: WPRA3 shared 96% similarity with *Serratia marcescens* subsp. marcescens, 95% with *Serratia ureilytica*, *Serratia ficaria* strain DSM 4569, *Serratia entomophila* strain DSM 12358, *Serratia odorifera* strain PADG 1073, *Serratia rubidaea* strain JCM

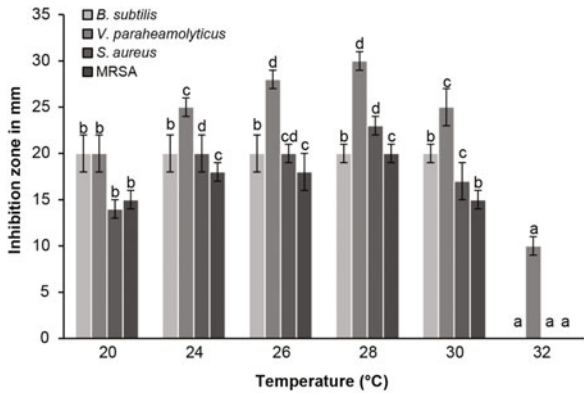

Fig. 2. Effect of temperature on the production of antibacterial compound by isolate WPRA3.

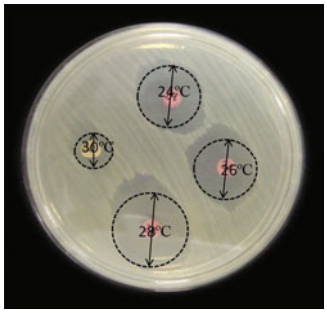

Fig. 3. Temperature effects on antibacterial activity of isolate WPRA3 against *S. aureus*. The zones of inhibition were measured as shown in the Figure.

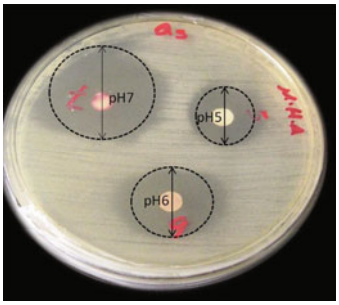

Fig. 5. Initial pH effects on antibacterial activity of isolate WPRA3 against *B. subtilis*. The zones of inhibition were measured as shown in the Figure.

1240 and *Serratia plymuthica* strain K-7, and 94% similarity with *Serratia grimesii* strain DSM 30063, *Serratia proteamaculans* strain 4364, *Serratia fonticola* strain DSM 4576, *Serratia proteamaculans* strain DSM 4543, and *Serratia liquefaciens* strain CIP 103238. The phylogenetic tree (Fig. 1) showed that strain WPRA3 was most closely related to *Serratia marcescens* subsp. Sakuensis JCM 11315.

Optimization of media and culture condition

Effect of different range of temperature: There was a gradual increase in antibacterial compounds production with an increase in temperature from 24–28°C. No antibacterial activity against *B. subtilis*, MRSA and *S. aureus* was observed at 32°C. Optimum temperature for the production of antibacterial compounds was observed to be 28°C (Fig. 2). Temperature effects on antibacterial activity of isolate WPRA3 against *S. aureus* is shown in Fig. 3.

Effect of different range of initial pH: There was a gradual increase in antibacterial compounds production with an increase in pH from 4–7. No antibacterial activity against MRSA, *S. aureus* and *V. parahaemolyticus* was detectable at pH 4. Isolate WPRA3 did not show any antibacterial activity against MRSA at pH 10 and pH 11 (Fig. 4). Optimum pH for production of antibacterial compounds by strain WPRA3 was obtained at pH 7. Initial pH effects on antibacterial activity of isolate WPRA3 against *B. subtilis* is shown in Fig. 5.

Effect of different levels of aeration: Increase in the levels of aeration leads to a gradual increase in activity against *S. aureus* and MRSA. On the other hand, it cuased a decrease in activity against *B. subtilis*. Optimum aeration for antibacterial compounds production by strain WPRA3 was obtained at 200 rev/min. The effect of aeration on the antibacterial compounds production is shown in Fig. 6.

Effect of NaCl concentration: Addition of different concentrations of NaCl into the media enhanced the production of antimicrobial compounds, as concentration increased from 0% to 2%, while further increases inhibited the production of antimicrobial compounds. Optimum production of antibacterial compounds by the strain was obtained at 2% NaCl concentration in the minimal medium (Fig. 7).

Effect of different concentrations of glucose: Optimum glucose concentration for the production of antibacterial compounds was recorded to be 0.75% (Fig. 8). Addition of different concentrations of glucose in minimal medium resulted in a reduction in the production of antimicrobial compounds, as concentration increased from 0.75%.

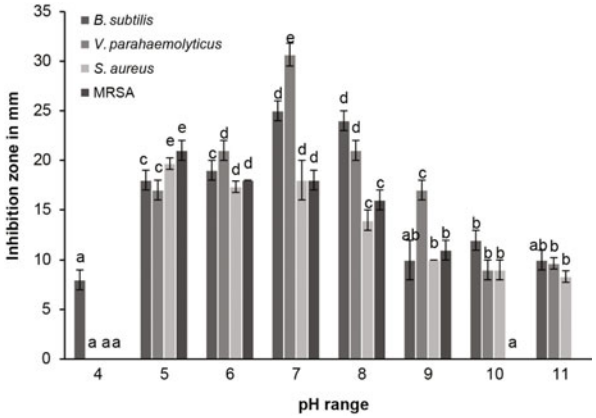

Fig. 4. Effect of pH change on the production of antibacterial compounds by isolate WPRA3.

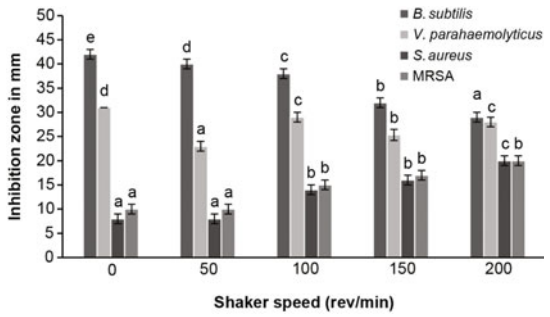

Fig. 6. Effect of aeration level on the production of antibacterial compounds by isolate WPRA3.

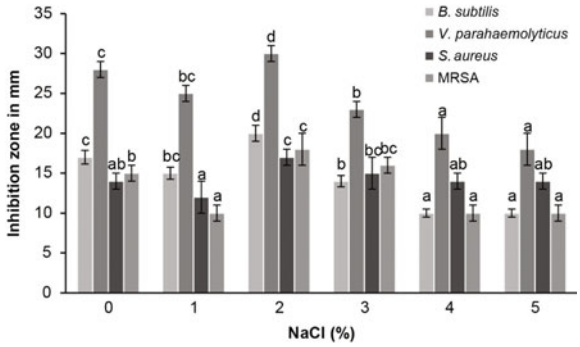

Fig. 7. NaCl concentration effects on antibacterial activity of the isolate WPRA3.

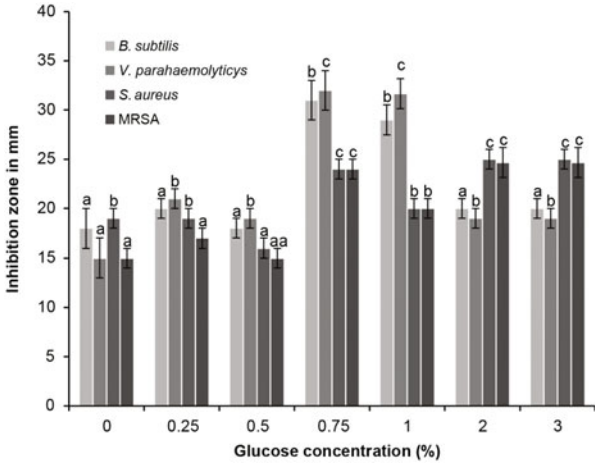

Fig. 8. Effect of glucose concentration on the production of antibacterial compounds by isolate WPRA3.

Effect of different concentrations of yeast extract: Maximum production of antibacterial compounds was observed at 0.5% yeast extract concentration. The concentration of yeast extract had a significant influence on the production of antibacterial compounds. Without an added nitrogen source, no effective antibacterial activity was detectable against *V. parahaemolyticus*, MRSA and *S. aureus*. A similar observation was recorded against MRSA when a low concentration of yeast extract (0.25%) was added. There was a gradual decrease in the production of effective antibacterial compounds against test microorganisms as the concentration of yeast extract increased from 1 to 3% (Fig. 9).

Effect of incubation period: There was a gradual increase in antibacterial compounds production with time. However, no antibacterial activity against *S. aureus* and MRSA was observed after 24 h. None was also observed for MRSA after 48 h of incubation period. Maximum production of antibacterial compounds by the isolate was obtained after 72 h (Fig. 10).

Partial purification of bioactive metabolite

UV absorption: Crude extracts namely RCE and OCE were dissolved in MeOH and scanned at wavelengths ranging from 350–600 nm. Figure 11 indicates maximum UV absorptions for RCE at 533 nm, 543 nm and a shoulder at 500

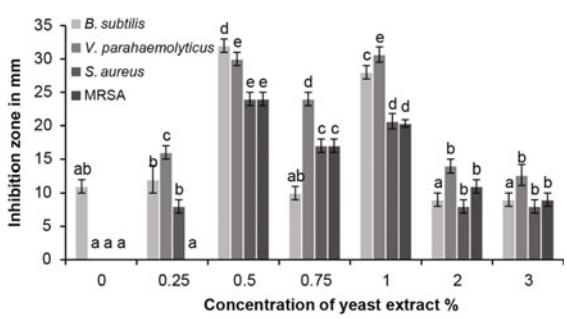

Fig. 9. Effect of yeast extract concentration on the production of antibacterial compounds by isolate WPRA3.

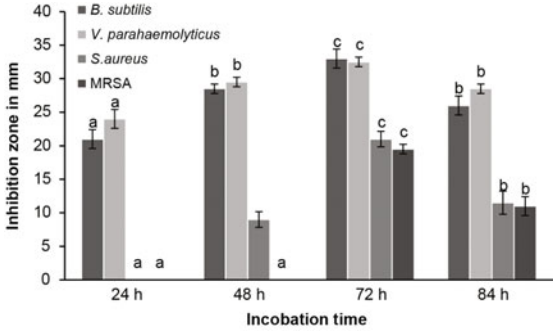

Fig. 10. Effect of incubation time on the production of antibacterial compounds by isolate WPRA3.

nm and maximum UV absorption for OCE at 540 nm.

Thin Layer Chromatography (TLC): Several major bands with different R_f value were obtained for two crude extracts namely RCE and OCE on TLC plates (Fig. 12). Two major bands with R_f values 0.75 and 0.81 were detected for RCE, while OCE showed five major bands with R_f values 0.4, 0.23, 0.14, 0.1, and 0.07 on TLC plates with dichloromethane-methanol (DCM-MeOH) (6:4) solvent system.

Flash Column Chromatography (FCC): Six and eight combined fractions were obtained from FCC of OCE and RCE respectively. Out of the eight combined fractions obtained from RCE, four fractions (R4, R5, R6, and R7) were active against MRSA and five (R3, R4, R5, R6, and R7) against *V. parahaemolyticus*. Three fractions (R1, R2, and R8) however, did not show activity against any of the test pathogenic bacteria. Out of the six fractions obtained from OCE, four fractions (O2, O3, O4, and O5) showed antibacterial activity against MRSA and three (O2, O3, and O5) were active against *V. parahaemolyticus*. Two fractions (O1 and O6) however, did not inhibit the growth of pathogenic bacteria.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The crude extract RCE and fraction FR4-RCE did not show any activity against *P. aeroginosa*, while they showed strong antibacterial activity against *B. subtilis* and *V. parahaemolyticus*. The crude extract OCE and fraction FO5-OCE showed strong antibacterial activity against all five test microorganisms in this assay. The RCE and FR4-RCE did not show

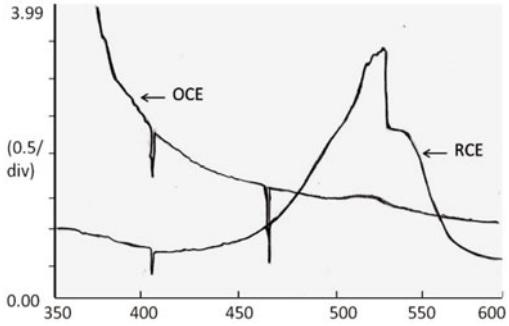

Fig. 11. UV spectrums of ethyl acetate crude extracts (OCE & RCE) of isolate WPRA3.

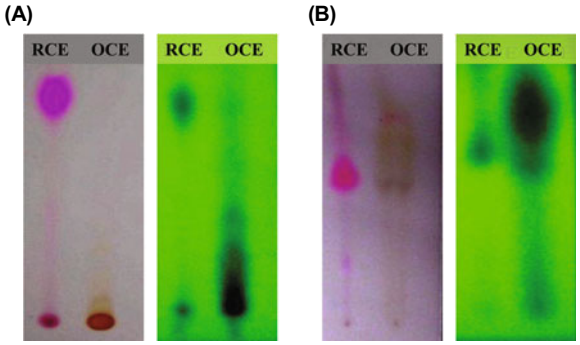

Fig. 12. TLC analysis of crude extracts (RCE and OCE) of isolate WPRA3 with different solvent systems. (A) MeOH/DCM (4:6), (B) MeOH/DCM (8:2).

any bactericidal activity against *P. aeroginosa*, however they exhibited bactericidal activity against others test microorganisms in this assay at concentration ranging from 25–400 µg/ml. The OCE and FO5-OCE showed bactericidal activity against all five test microorganisms in this assay ranging from 50–400 µg/ml. Table 1 show the MIC and MBC values of crude extracts and fractions.

Discussion

Identification of bacterium

Serratia sp. is an opportunistic Gram-negative, facultative anaerobic, motile, catalase positive, and oxidase negative bacterium. It can grow at 20–37°C (Giri *et al.*, 2004). *Serratia* is well-known as a producer of prodigiosin (Song *et al.*, 2006). The absorption spectra of pigments from isolate WPRA3 was nearly identical to that of prodigiosin isolated from *S. rubidaea*, *S. marcescens*, *S. nemathodiphila* DZ0503-SBS1 (Zhang *et al.*, 2009), and *S. plymuthica* (Spröer *et al.*, 1999). A distinctive feature of this isolate was its ability to produce a large amount of red and orange pigment under certain conditions. In comparison of isolate WPRA3 with other pigment producing *Serratia* sp., the closest species was *S. nemathodiphila*, which could be distinguished by Voges Proskaure test, arginine dihydrolase ability, raffinose, L-phenylalanine, threonine, and L-ornithine utilization (Zhang *et al.*, 2009). On the basis of physiological, morphological and fatty acid analysis (data not shown), isolate WPRA3 proposed to be assigned to a novel species, *Serratia* sp. WPRA3 (JX020764).

Optimization of media and culture condition

Maximum antibacterial compounds production by isolate WPRA3 was obtained at 2% salt, 0.75% glucose, and 0.5% yeast extract concentration. The addition of some small-molecular-weight organic compounds to the culture media of microorganisms can also trigger the uncovering of metabolic pathways. As a result, it is suggested that glucose and yeast extract influence the production of antibacterial compounds by isolate WPRA3. As a result, the optimum culture conditions for antibacterial compounds production by isolate WPRA3 was observed at pH 7, incubation at 28°C for 72 h with aeration (200 rev/min). Aeration had significant influence on pigment production. The amount of red pigment reduced by increasing the aeration; whereas the yellow-orange pigment increased significantly with increase in aeration (data not shown) the results suggested that there was a relationship between the colour of crude extracts, OCE and RCE, and the effect of antibacterial compounds against test organisms. Therefore different culture conditions such as pH, temperature and aeration have significant impact on the types of secondary metabolites produced by strain WPRA3.

Partial purification of bioactive metabolite

Based on the UV absorption of pigment WPRA3, the red colour extract (RCE) was proposed as prodigiosin (Samort *et al.*, 2011), while the orange colour extract (OCE) may be considered as being norprodigiosin (2-methyl-3-pentyl-6-hydroxyprodigiosene) (Song *et al.*, 2006). Different fractions of prodigiosin were separated by column chromatography. In this study, eight fractions were obtained after subjecting the RCE crude extract to flash column chromatography (FCC). Initial elution with dichloromethane resulted in the separation of a black-purple fraction that remained at the top of the column, then a light purple fraction that was followed by pink, red (FR4-RCE), purple, red-pink, and finally an orange and a yellow fractions that was eluted by methanol. From FCC of OCE, six fractions were obtained. Initial elution with dichloromethane resulted in separation of a dark brown fraction that remained at the top of the column, then a yellow fraction that was followed by orange, yellow, orange (FO5-OCE) and finally a yellow fraction that was eluted by methanol. However, Weiss (1949) noted three red fractions upon column chromatography with magnesium oxide and acetone petroleum ether mixture. Maximum of four fractions was reported for prodigiosin by Williams *et al.* (1956). Lynch *et al.* (1968) also found that further separation procedures yielded a total of six fractions which were easily

Table 1. Minimum inhibitory concentration (µg/ml) and minimum bactericidal concentration (µg/ml) of crude extracts (RCE & OCE) and fractions (FR4 & FO5). Experimental results were expressed as means ± standard deviation (SD) of three parallel measurements.

Test organisms	Crude extracts and fractions							
	MIC				MBC			
	RCE	FR4	OCE	FO5	RCE	FR4	OCE	FO5
MRSA	200±0	10.4±3.5	41.6±14.4	12.5±0	400±0	83.3±28.8	175±50	100±0
B. subtilis	16.6±7.2	1.06±0.4	50±0	33.3±14.4	400±0	100±0	200±0	100±0
V. parahaemolyticus	12.5±0	0.4±0	50±0	1±0.4	175±50	33.3±14.4	200±0	41.6±14.4
P. aeroginosa	800±0	800±0	175±50	50±0	-	-	400±0	175±50
S. aureus	83.3±28.8	33.3±14.4	25±0	5.3±1.6	175±50	100±0	175±50	50±0

separated by column chromatography. It is suggested that the culture condition influenced the pigment formation of isolate WPRA3, that produce red pigment at pH 7 without aeration. However, in contrast to Song *et al*. (2006), an orange pigment of isolate WPRA3 was obtained in acidic condition (with aeration) and, not in alkaline condition.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Strain WPRA3 showed a broad antibacterial spectrum against Gram-negative, Gram-positive bacteria and fungi when tested with ethyl acetate crude extracts, RCE and OCE, and semi-purified fractions with different R_f values, FR4-RCE (R_f; 0.5) and FO5-OCE (R_f; 0.75). The red pigment, Prodigiosin, and its analogues have been reported to have significant biological potential due to their antifungal, antibacterial, antimalarial, immunosuppressive and anticancer activities (Han *et al*., 1998; D'Alessio *et al*., 2000; Montaner *et al*., 2000; Montaner and Pérez-Tomás, 2003). The MICs of the crude extracts and fractions against test photogens demonstrated their potential to be candidates for the generation of new antimicrobial agents.

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