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The Influence of Environmental Conditions on the Production of Pigment by *Serratia marcescens*

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Abstract *Serratia marcescens* biovar A2/A6, isolated from an Indonesian freshwater source, was identified based on extensive morphological, biochemical and genetic characterization. Formation of pigment was found to be strongly influenced by environmental conditions. Placket-Burman design was used to analyze the effect of carbon and nitrogen sources. Based on results of physiological and biochemical studies, the optimum conditions for growth and pigment formation were incubation 30°C in a neutral to slightly alkaline medium containing lactic acid and beef extract.

Keywords: pigment production, physico-chemical factors, *Serratia marcescens*

INTRODUCTION

Serratia marcescens, a Gram-negative bacterium characterized by production of a nondiffusible red pigment, prodigiosin [1-3], is an opportunistic pathogen, with the nonchromogenic biotypes posing a public health threat [3]. Chromogenic biotypes from the natural environment have only rarely implicated in infections. Interestingly, the water insoluble red pigment produced by *Serratia marcescens* has been reported to have antibiotic activity [4-7]. *Serratia marcescens* also produces a water-soluble, reddish-violet pigment with superoxidase dismutase mimetic activity [8,9].

The production of pigment by a non-clinical strain is meaningful and scientifically important as it was found in this investigation that there might be a relationship, between pigment production to the absence of plasmid that was also reported by Gargallo-Viola [3]. Thus, pigment production by non-clinical strains of *Serratia marcescens* was of interest fur further studies to determine a relationship, if any, of loss of pigment production to pathogenicity. *Serratia marcescens* also produces pigment fractions of various characteristics [1,12], influenced by both genetic and environmental factors. L-proline serves as a sole source of carbon and nitrogen for growth and prodigiosin production [10]. Production of the water-soluble pigment is enhanced by addition of polymyxin B [9], gramicidin, and valinomycin to the growth medium [11]. No doubt, pigment production by *Serratia marcescens*, will continue to be intrigued to both microbiologist, clinicians and bioprocess engineer. L-lactic acid, found in this study to serve as carbon source for growth and pigment production, has not been reported previously as stimulant of pigment production.

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MATERIALS AND METHODS

Microorganism

The bacterial strain employed in this study was isolated from an Indonesian freshwater source and identified using biochemical, morphological, and genetic features. Biochemical and morphological characteristics were determined following standard bacteriological methods.

Growth Media

Luria Bertani agar medium (Difco) and overnight incubation at 30°C were used to maintain the strain. The medium employed to investigate temperature and pH relationship to pigment production contained beef extract (Lab-Lamco Powder, Oxoid) 3 g/L, peptone (Difco) 5 g/L and NaCl 5 g/L. Experiments were carried out using 250 mL flasks containing 100 mL medium adjusted to pH 7.0 and incubated with shaking (150 rpm), at 30°C. Growth temperatures used were 25, 30, 37°C. The growth rate was defined as the slope of the regression line during exponential phase of growth.

Experimental Design

The Placket-Burman design [13] was used to analyze the effect of carbon and nitrogen sources, employing succinic acid, citric acid, L-lactic acid, and L-glutamic acid as C-source and yeast extract, beef extract, peptone, and $(NH_4)_2SO_4$ as N-source at concentrations of 3.0 g/L

Table 1. Placket-Burman matrix for the study of carbon and nitrogen sources with 12 experiments

Experiments	Variables							
	C1	C2	C3	C4	N1	N2	N3	N4
1	+	−	+	−	−	−	+	+
2	+	+	−	+	−	−	−	+
3	−	+	+	−	+	−	−	−
4	+	−	+	+	−	+	−	−
5	+	+	−	+	+	−	+	−
6	+	+	+	−	+	+	−	+
7	−	+	+	+	−	+	+	−
8	−	−	+	+	+	−	+	+
9	−	−	−	+	+	+	−	+
10	+	−	−	−	+	+	+	−
11	−	+	−	−	−	+	+	+
12	−	−	−	−	−	−	−	−

+: high level of particular variable (3.0 g/L)
−: in the absence of C or N-source
The growth temperature and initial pH were 30°C and 7.0, respectively.
C1: succcinic acid, C2: citric acid, C3: L-lactic acid, C4: L-glutamic acid, N1: yeast extract, N2: beef extract, N3: peptone, N4: ammomium sulfate.

or in the absence of N/C-source. Details of the experimental design are given in Table 1.

Determination of Pigment and Cell Concentration

The cell concentration was monitored spectrophotometrically by measuring Optical Density (OD) at 600 nm and the dry weight of the cells. The dry weight cell concentration (g/L) was found to be $0.4 \times OD_{600nm}$.

The cell-bound pigment was extracted using a modified method of William *et al.* [16]. The cell-bound and extracellular pigment was measured spectrophotometrically at 540 nm, and the total pigment was calculated by adding the cell-bound and extracellular values. A 5 mL broth was centrifuged at 10,000 rpm for 5 min. The extracellular pigment was determined directly from cell-free supernatant at pH 5 by adding hydrochloric acid, while the cell-bound pigment was extracted by adding 5 mL ethanol-chloroform (2:18). A typical spectrophotometric scan of the pigment is presented in Fig. 1.

Statistical Analysis

The effect of investigated variables on cell growth and pigment production were calculated employing the following equation:

$$E_{i,j} = \frac{\sum_{i,j}^{n_{i,j}(+)} R_{i,j}(+)}{n_{i,j}(+)} - \frac{\sum_{i,j}^{n_{i,j}(-)} R_{i,j}(-)}{n_{i,j}(-)}$$

Variance was calculated employing the following equation:

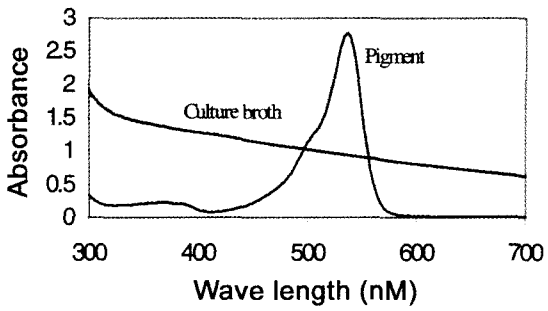

Fig. 1. The UV/VIS spectophotometric scan of culture broth (CB) and pigment (P).

$$V_{i,j} = \frac{\sum_{i,j}^{n_{(i,j)^-}} \{R_{(i,j)^-} - \bar{R}_{(i,j)^-}\}^2 + \sum_{i,j}^{n_{(i,j)^+}} \{R_{(i,j)^-} - \bar{R}_{(i,j)}\}^2}{(n_{(i,j)^-} - 1) + (n_{(i,j)^-} - 1)}$$

$$SE_{i,j} = \sqrt{V_{i,j}}$$

Significance of each variable was determined using the t-test

$$t = \frac{E_{i,j}}{SE_{i,j}}$$

Genetic Characterization of Microorganism

Confirmation of identification as *Serratia marcescens* was achieved using 16S rRNA analysis,i.e., by amplifying the 16S rDNA gene by the polymerase chain reaction (PCR) employing two universal primers, 27f and 1525r [14]. The PCR product was analyzed by horizontal gel electrophoresis. The 16S rDNA was isolated and extracted, using an Ultrafree-MC filter unit (0.45 μm; Millipore Co, Bedford, USA), following manufacturer's instructions. Ligation of the PCR-amplified 16S rDNA into the pGEM-T vector was done following standard procedures.

The cloned 16S rDNA was sequenced using a PRISM™ DyeDeoxy Terminator cycle sequencing kit (Applied Biosystem, California, USA) and an Applied Biosystems atomated DNA sequencer (ABI 377).

RESULTS AND DISCUSSION

Biochemical characteristics of the isolate are presented in Table 2, showing a good match with the reference strain of *Serratia marcescens* ATCC 13880, except for growth and acid production on D-xylose and hydrolysis of gelatin. Benzoate was not utilized and red pigment was produced, indicating the strain belongs to biogroup A2/A6. Fig. 2 shows the bacterium is rod-shaped with peritrichous flagella, and 0.5 μm in diameter and 0.5-0.9 μm in length. Plasmid DNA could not be detected.

Table 2. Biochemical characteristics of the isolate examined in this study and comparative data for *Serratia marcescens*

Characteristic tested	Freshwater isolate	*S. marcescens*
Growth on and acid production from		
L-Arabinose	-	-
D-Melibiose	-	-
D-Xylose	+	-
L-Rhamnose	-	-
D-Sorbitol	+	+
D-Arabitol	-	-
Adonitol	+	+
Raffinose	-	-
D-Lactose	-	-
Glycerol	+	+
Benzoate	-	v
Sucrose	+	+
Salicin	+	+
Indole production	-	-
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
Argine dehydrolase	-	-
Methyl red test	-	-
Voges-Proskauer test	+	+
Gelatin, hydrolyzed	-	+
DNAse	+	+
Catalase	+	+
Oxidase	-	-

v: varied.

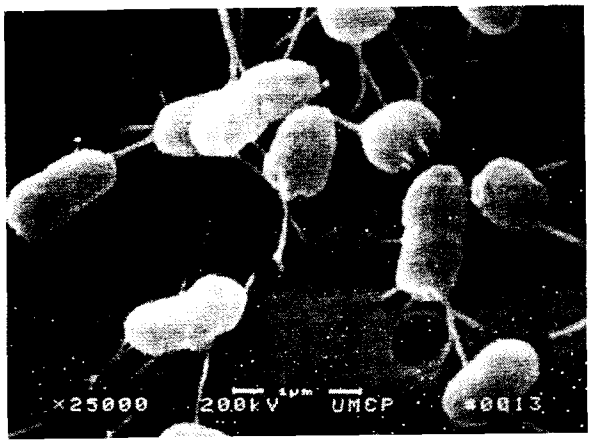

Fig. 2. Scanning electron microscope photograph of the freshwater isolate of *Serratia marcescens*.

The 16S rDNA sequence data confirm the freshwater isolate to be *Serratia marcescens* based on homology study using nucleotide BLAST from NCBI (National Center for Biotechnology Information). The identity is 97%.

Growth at temperatures of 25-37°C did not affect significantly the growth rate of *Serratia marcescens*, which varied from 0.14 h^{-1} to 0.18 h^{-1}. The optimum temperature of growth was 30°C and the strain did not grow at pH 4.0, but did grow at pH 5.0 to 11.0. Pigment production, as a function of temperature, is shown in Fig. 3(b). Pigment was produced during late exponential phase at 12 h (Fig. 3(a)). At temperature of 30° C, pigment production was greater than that of 25°C. Pigment was produced at pH values of 5.0 to 9.0. At the lower pH, pigment was produced at 18 h, while at pH 7.0-9.0, its production occurred at 12 h (see Fig. 3(b)).

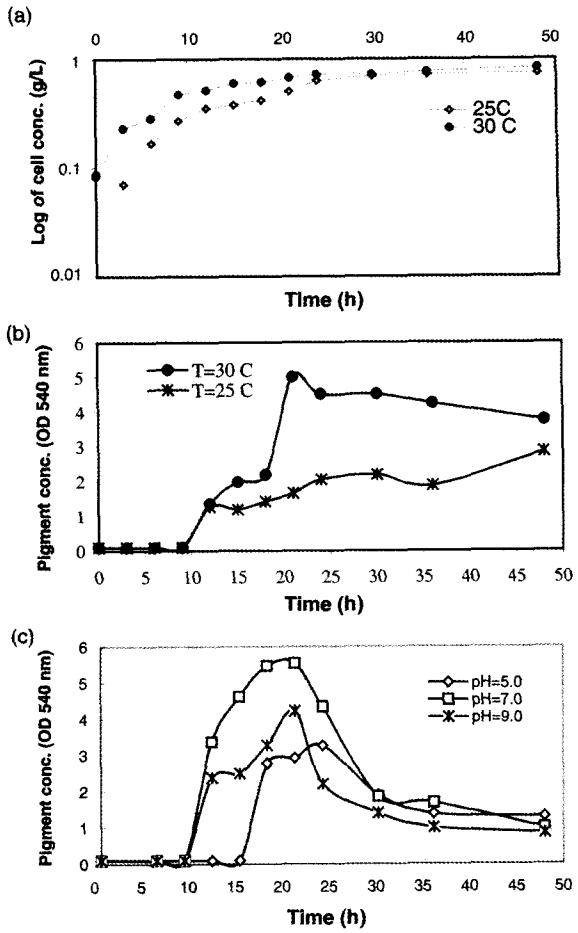

Fig. 3. Growth of bacteria as function of temperature (a) and pigment production as a function of growth temperature (b) and pH (c).

Growth in a medium containing beef extract (3.0 g/L), peptone (5.0 g/L), NaCl (5.0 g/L), with and without glucose addition (5.0 g/L) revealed the bacterium did not produce pigment in media containing glucose. Succinic acid, L-glutamic acid, and ammonium sulfate did not support growth of *Serratia marcescens*, whereas citric acid, L-lactic acid, yeast extract, beef extract and peptone were utilized as carbon and nitrogen sources for growth. From the results of the statistical analyses, it was found that L-lactic acid, yeast extract and beef extract are suitable substrates for growth (see Table 3).

The combination of citric acid and ammonium sulfate was not found to be suitable as carbon and nitrogen

Table 3. The significant level of variable tested on the growth of bacterium and its pigment production

Variable	The growth of bacteria (g/L)		Pigment production (OD_{540nm})	
	Response	$t_{calculated}$	Response	$t_{calculated}$
Succinic acid (C1)	-0.269	0.914	0.061	1.241
Citric acid (C2)	0.161	0.545	-0.069	1.417
Lactic acid (C3)	0.640	2.245*	0.147	3.212*
L-Glutamic acid (C4)	-0.092	0.309	0.031	0.633
Yeast extract (N1)	1.633	7.805***	0.035	0.708
Beef extract (N2)	1.146	4.448**	0.189	4.418**
Peptone (N3)	0.263	0.891	0.055	1.115
Ammonium sulfate (N4)	-0.356	1.212	-0.149	3.264*

$t_{(\alpha=0.05)} = 2.228$; $t_{(\alpha=0.1)} = 1.812$.
*, **, *** indicate the degree of significance, where *** shows the most significant effect.

sources for pigment production, whereas succinic acid, lactic acid, L-glutamic acid, yeast extract, beef extract and peptone supported pigment production. However, lactic acid and beef extract were the most effective substrates for pigment production (Table 3).

The biochemical, morphological and genetic data accumulated in this study led to the taxonomic conclusion that the red pigment-producing bacterium isolated from a freshwater source in Indonesia is *Serratia marcescens*. Based on lack of utilization of benzoate and the production of a red pigment, the bacterium is concluded to belong to the biogroup A2/A6. Gargallo-Viola [3] reported that 99 *Serratia marcescens* strains isolated from clinical and environmental sources could be grouped into nonchromogenic biotypes, most of which were from clinical samples (97.3%) and chromogenic biotypes, with an absolute correlation between ability to produce prodigiosin and absence of plasmids. No plasmids were detected in the freshwater chromogenic isolate.

The pigment showed maximum absorbance at 540 nm in acidic condition, indicating the pigment comprised prodigiosin, confirmed by the pigment having antimicrobial activity (data not given). Lynch *et al.* [12] reported that pigment produced by *Serratia marcescens* contained six fractions with different infrared absorption spectra. The pigment is a complex mixture of yellow-orange [15], red [3], and reddish-violet [8,9]. The red color prodigiosin, has a maximum absorbance of 535 nm in acidic-ethanol [16]. In addition, Feng *et al.* [17] reported maximum absorbance at 537 nm, close to the value reported here. The water-soluble reddish-violet pigment has a maximum absorbance of 540 nm [8], while the yellow pigment has a maximum absorbance at 380 nm [15]. Further studies of the pigment of the freshwater isolate are in progress.

Optimal growth was obtained at 30°C and pH 7.0–9.0. Pigment was produced as a secondary metabolite, a finding in agreement with the results of William *et al.* [16] who found that pigment is synthesized maximally during stationary phase by *Serratia marcescens* strain Nima. This freshwater isolate produced pigment at 25 and 30°, but not at 37°C, as observed by William *et al.* [16] and Rosenberg *et al.* [18] in their analyses.

Carbon and nitrogen sources are important in growth and pigment production phase by *Serratia marcescens*. In this study, it was found that glucose inhibited the formation of pigment, a phenomenon observed for other microorganisms, *i.e.*, in antibiotic production. Martin and Demain [19] found that polysaccharide or oligosaccharide was better than glucose as a carbon source for antibiotic production. Specifically, they reported that glucose inhibited production of prodigiosin while galactose did not. Furthermore, inhibition by glucose of prodigiosin production was due to a lowering of pH [20]. Also in a medium containing glucose, bacteria may produce the glucose-6-phosphate dehydrogenase alloenzyme (G6PD), which inhibits pigment production [2].

L-lactic acid was found to serve best for growth and pigment production, of the several nutrient sources included in this study, a new finding. Previous studies showed that cyclodextrin stimulated production of prodigiosin by *Serratia marcescens* ATCC 21639 [21]. Also, dulcitol and lactose are good carbon sources for production of prodigiosin by Nima strain [20]. The role of lactic acid as stimulant of pigment production was not clear. Further studies of the use of lactic acid as a carbon source for pigment production are in progress.

From results of this study, it is concluded that beef extract was the best of the nitrogen sources of those tested in this study for production of pigment. It might be due to the amino acid content of beef extract. Ammonium inhibited production of the pigment, as observed by Rokem and Weitzman [22] who found that, in chemostat culture of *Serratia marcescens* ATCC 21639, cells growing in $(NH_4)_2SO_4$ containing medium did not produce prodigiosin. These data suggest that ammonium is a poor nitrogen atom donor in the Schift-base conversion of orange to red pigment, because it has a weak inhibitory effect on pigment synthesis, notably in the production of water-soluble red pigments by *Monascus* sp. [23]. Pigment production by *Serratia marcescens*, no doubt, will continue to be intriguin to both microbiologist, clinicians and bioprocess engineer.

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NOMENCLATURE

$E_{i,j}$: The effect of i th on jth -variable (i = 1 to 8 & j= 1 to 4)
$n_{i,j}$ (+) : The number of experiments at high concentrations of ith–variable which is equal to 6
$n_{i,j}$ (-) : The number of experiments at low concentrations of ith–variable which is equal to 6
$R_{i,j}$ (+) : Average final concentration of jth at high concentration of ith-variable
$R_{i,j}$ (-) : Average final concentration of jth at low concentration of ith-variable
$SE_{i,j}$: Standard error of ith on jth-variable (i = 1 to 8 & j= 1 to 4)
t : t-value
$V_{i,j}$: The variance of ith on jth-variable (i = 1 to 8 & j= 1 to 4)

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