A Novel Determination Method quantifying *Serratia marcescens* prodigiosin Concentrations Independent of Acidic and Alkali Conditions

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**ABSTRACT**

Prodigiosin is a bioactive secondary metabolite produced by *Serratia marcescens*. Due to the difference of the acidic and alkaline conditions, the absorption wavelength changed greatly using the absorbance value of 535 nm as the standard. It was difficult to accurately determine the prodigiosin concentration *in vitro* and *in vivo*. In this study, we proposed an accurate, reliable, and convenient method to determine the concentration of prodigiosin. Prodigiosin concentration was evaluated using the standard curve equation in accordance with the absorbance at a wavelength of 492 nm. For each standard curve equation, there remained steady linear correlations when prodigiosin was dissolved in methanol, ethanol, acetone, and butanol. Compared to the former method that used the absorbance level of 535 nm, the main advantage of this method is independent of pH value. The proposed method is conveniently used for general concentration measurements.

**Key words:** Prodigiosin, concentration, 492 nm, wavelength, *Serratia marcescens*, acidic and alkali conditions.

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**INTRODUCTION**

Prodiginines, a family of natural red pigments include the secondary metabolite prodigiosin produced by *Serratia marcescens* (Samrot et al, 2011). Prodigiosin is known for its antibiotic and antimicrobial activity against fungi, protozoa, and bacteria (Jin-Lan et al, 2011). In addition, prodigiosin is a promising immuno-suppressive and anti-proliferative drug (Anita, 2006), capable of preventing and treating acute graft-versus-host disease (Kim and Mook, 2007) treating rheumatic arthritis (Kim and Mook, 2004), diabetes mellitus (Kim and Mook, 2001) and cancer (Beatriz and Perez-Tomaz, 2003). The research of the biosynthetic pathway, physiology and regulation of the prodigiosin had made some progress (Neil et al, 2006). The concentration measurement is absolutely necessary for condition optimization of fermentation (Samrot et al, 2011). For the isolation, purification, and application of prodigiosin, the measurement method’s accuracy and convenience must be carefully considered. The former measurement method was based on the absorbance value of 535 nm under the condition of pH 3 using a UV-visible spectrophotometer (Samrot et al, 2011). In practice, it was difficult to accurately control the acidic and alkaline conditions. Slight deviation could cause the absorption wavelength to change using the absorbance value of 535 nm as the standard.

In accordance with the prepared standard curve, the concentration of investigated prodigiosin is evaluated. This method was considered feasible for determining the concentration of prodigiosin. However, the disadvantages of the method include the following:

1. The absorbance values at OD_{535} is dependent upon the pH value of the prodigiosin solution. The pH fluctuation of the prodigiosin solution can easily lead to deviations in the determination of the concentration determination (Neil et al, 2006);

2. The addition of other compounds for pH adjustment contaminates the prodigiosin solution, which is not applicable as a clinical drug.

3. The pH adjustment increases the cost of operation to some extent;
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Figure 1. The colony morphology of strain in this study.

(4) the acidic compounds affect the stability of the prodigiosin chemical bonds, which may change the physiological function. To combat these disadvantages, it is vital to develop a new measurement method with an independent pH value. Based on this idea, the present study provides a simple method to measure the concentration of prodigiosin without the effect of a pH value.

MATERIALS AND METHODS

Bacterial strain, culture and fermentation

The bacterial species used for prodigiosin production was *S. marcescens*, isolated from a soil sample efficiently producing prodigiosin at 28 to 30°C under aerobic conditions. This bacteria was grown on a streak plate containing solid isolation medium composed with 5 g/L yeast extract, 5 g/L polypepton and 10 g wheat bran, pH 6.8 at 30°C for 36 h. Then, the colony was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of growth media comprised of 20 g/L soybean oil, 1 g/L KH₂PO₄, 2 g/L NaCl, 0.5 g/L MgSO₄·7H₂O, on a reciprocal shaker at 30°C for 48 h. Growth media was adjusted to pH 8.0 (Min-Jung et al, 2006).

The isolated strain was identified by morphological examination and 16S rDNA analysis. The isolated bacteria colony was red, round, viscous, smooth surface, and 2 to 3 mm of diameter in 7 cm petri dish after 72 h incubation at 28°C (Figure 1). Physiological properties indicated the strain had characteristics of positive catalase production, positive methylene-blue reduction, positive ammonia production and negative diastase production. The similarity of 16S rDNA nucleotide sequences was 99% as compared with those from *S. marcescens* 16S RNA (ID: ref|NR_102509.1).

Isolation and determination of prodigiosin in concentration

The crude prodigiosin was purified (Min-Jung et al, 2006). The processes included the following: the cells of the *S. marcescens* were harvested with a centrifugation of 8000 xg at 4°C for 10 min. After the supernatant was discarded, the red cells were re-suspended using 99.5% methanol solvent at room temperature for 10 h. The suspension was centrifuged for a prodigiosin isolation from pellets at 10000 xg at 4°C for 10 min. The supernatant containing prodigiosin was transferred to a 500 ml Bunsen beaker. The concentrated solution of prodigiosin was created using the vacuum evaporation method. Then, the prodigiosin was separated with a silica gel column chromatography (Kieselgel 60, 2.5×30 cm). A mixture of n-hexane: ethyl acetate (2:1 [v/v]) was used for the eluted.
The $R_f$ of the prodigiosin by TLC was nearly 0.13. The concentrated prodigiosin was separated with a mixture of chloroform: methanol 95:5 (v/v) by TLC. Then, the single red pigment from prodigiosin was collected and dissolved in methanol. Finally, the collected prodigiosin was purified using the HPLC technique. Prodigiosin concentration was estimated by the absorbance at 535 nm of wavelength using a double beam UV-visible spectrophotometer in acidic methanol adjusted to pH 3.

**Standard curve construction**

In the former study, prodigiosin concentration was estimated by the absorbance at 535 nm of wavelength using a double beam UV-visible spectrophotometer in acidic methanol adjusted to pH 3. The standard curve was constructed based on the relationship of prodigiosin concentration and absorption. In the present study, the conditions were adjusted to meet the requirements of the study. The standard curves were prepared using the relation between the absorption value at 492 nm and the prodigiosin content. The prodigiosin concentration was determined using the prepared standard under the same wavelength. Between the two methods, the greatest difference existed in the absorption value from different wavelengths.

**RESULTS AND DISCUSSIONS**

**Effects of pH value on absorbance value**

The pH values were increased from pH 0 to pH 11 with 3 mg/L prodigiosin. The full wavelength scanning was conducted with wavelengths between 400 nm and 570 nm. Figure 2 shows the pH values that affected the maximum wavelengths and the peak values under the same prodigiosin concentration. There was a significant absorbance peak at 535 nm. Another absorbance peak (at 470 nm) was gradually conspicuous with the increase of the pH value. Between the wavelengths of 470 and 535 nm, there were the same absorbance values at a wavelength of 492 nm, which indicates the absorbance values were invariant at 492 nm. It is interesting to note that the results showed that the absorbance value at 492 nm depended on the prodigiosin concentration independently of the pH values.

**Effects of pH values on the prodigiosin color**

Figure 3 shows that the color of the prodigiosin changed with the pH values under 3 mg/L prodigiosin. The color gradually changed from deep red to light red with the increase of pH 0 to 7.2. At the beginning of pH 8.1, the yellow gradually deepened with the increase of pH value. There was a close connection between the absorbance spectrum (Figure 2) and the color (Figure 3). With the increase of absorbance value at 535 nm, the color gradually changed from red to yellow. Meanwhile, with the increase of absorbance value at 470 nm, the yellow gradually deepened. The solution of the pH 11 showed a deep yellow color. The absorbance peak remained at 535 nm. However, the absorbance value significantly decreased. According to the curve from Figure 2 and solution color from Figure 3, the solution color significantly changed under different acidic and alkali
Figure 3. The effect of pH value on the color under the same concentration for prodigiosin

Comparisons with two standard curve equations

As the control, the standard curve was prepared using the prodigiosin concentration and absorbance value at 535 nm of the wavelength with a UV-visible spectrophotometer in 95% acidic methanol-HCl adjusted to pH 3. Figure 4 shows that the standard curve was prepared at 535 nm wavelength in acidic methanol (pH 3), which represented a good linear relationship between the prodigiosin concentration and the absorbance value. Linear regression equation was $y = 0.313x + 0.015$ ($R^2 = 0.999$).

Figure 5 shows the standard curve was prepared at a wavelength of 492 nm under the same conditions. The linear regression equation was $y = 0.092x + 0.006$ ($R^2 = 0.999$). Compared to the standard curve equations for
wavelengths between 492 and 535 nm, the standard curve of 492 nm was efficient for measuring the prodigiosin concentration. Note that the absorbance value at 492 nm was lower than that at 535 nm. Therefore, the concentration determination at 492 nm of wavelength for prodigiosin was accurate and feasible.

The accuracy and reliability of standard curve equations

The prodigiosin solutions adjusted to different pH were used to determine the accuracy and evaluate the prodigiosin concentration according to their standard curve equations. The rates of the evaluated and the true are shown in Table 1. The results showed that in order to investigate the accuracy of prepared standard curves from two determination methods, the evaluated values under pH 3 for a 535 nm wavelength were close to the true value. However, the values larger than pH 5 declined rapidly. The rate of the evaluated and the true was only 0.02, which meant that the absorbance at 535 nm was significantly affected by the pH values. Meanwhile, the absorbance values from the OD 492 nm wavelength fluctuated to a lesser degree. The evaluated values were very close to the true values (2 mg/L). Thus, the measurement technique

Table 1. Differences of two prepared standard curves with true concentration under different pH value.

<table>
<thead>
<tr>
<th>pH</th>
<th>Absorbance (mg/L)</th>
<th>Evaluated value</th>
<th>Rate of the evaluated and true</th>
<th>Absorbance (mg/L)</th>
<th>Evaluated value</th>
<th>Rate of the evaluated and true</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.652</td>
<td>2.04</td>
<td>1.02</td>
<td>0.192</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0.651</td>
<td>2.03</td>
<td>1.02</td>
<td>0.191</td>
<td>1.99</td>
<td>0.99</td>
</tr>
<tr>
<td>3</td>
<td>0.648</td>
<td>2.02</td>
<td>1.01</td>
<td>0.192</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.518</td>
<td>1.61</td>
<td>0.80</td>
<td>0.192</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
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<td>1.31</td>
<td>0.65</td>
<td>0.193</td>
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<td>1.01</td>
</tr>
<tr>
<td>9</td>
<td>0.122</td>
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<td>0.17</td>
<td>0.192</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
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<td>0.04</td>
<td>0.02</td>
<td>0.192</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 5. The standard curve of prodigiosin based on absorbance at 492 nm of wavelength.
was stable with the independence of pH value.

**Alternative organic solvents**

In order to investigate the effects of the organic solvents on the measurement method, purified prodigiosin was dissolved separately in ethanol, acetone, and butanol. Each standard curve was prepared by setting the relation between the prodigiosin concentration and corresponding absorbance value at a wavelength of 492 nm. The standard curve equation \( y=0.090x+0.006 \) \( R^2=0.999 \) was established by using the absorbance at 492 nm of wavelength in ethanol. With the same measurement conditions earlier mentioned, the standard curves for prodigiosin dissolved in acetone and butanol respectively were \( y=0.101x-0.001 \) \( R^2=0.999 \) and \( y=0.086x \) \( R^2=0.999 \).

The proposed method is applicable for general measurement as well as, modern automated production lines of prodigiosin. Prodigiosin is a promising drug because of its beneficial medical applications, including but not limited to: immuno-suppressive, anti-cancer (Kawauchi, 1997), antifungal, antibacterial, anti-protozoal and anti-malarial (Jeong, 2005). The accurate determination of the prodigiosin concentration is extremely important. More specifically, a rapid and reproducible on-line automated determination technique is required. The proposed method in this study assesses the prodigiosin concentration in real-time based on the defined standard curve equation. The absorbance value is obtained at a wavelength of 492 nm. This method can eliminate interference at different pH values, which used to be an obstacle for the previous measurement method at a wavelength of 535 nm.

The absorption value using A492 was smaller than using A535 at the same concentration. The concentration determination was calculated according to the prepared standard curve. The change of concentration surely led to the change of absorption value. In theory, with a diluted sample, the corresponding concentration was also calculated. In the real application, a UV machine of higher precision would exert more advantage of the provided method.

**Conclusions**

To avoid the pH value effect upon the determination of the prodigiosin concentration, the present study provides a simple method to measure the concentration of prodigiosin independent of pH. The standard curve equation was prepared based on the relationship between the absorbance value at 492 nm and prodigiosin concentration. Based on the standard curve equations, the concentration of the investigated prodigiosin was evaluated according to the absorbance value at a wavelength of 492 nm.

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**REFERENCES**


