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Research Article

# Enhanced production of prodigiosin by *Serratia marcescens* FZSF02 in the form of pigment pellets

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ABSTRACT

*Background:* Prodigiosin has been demonstrated to be an important candidate in investigating anticancer drugs and in many other applications in recent years. However, industrial production of prodigiosin has not been achieved. In this study, we found a prodigiosin-producing strain, *Serratia marcescens* FZSF02, and its fermentation strategies were studied to achieve the maximum yield of prodigiosin.

*Results:* When the culture medium consisted of 16.97 g/L of peanut powder, 16.02 g/L of beef extract, and 11.29 mL/L of olive oil, prodigiosin reached a yield of 13.622 ± 236 mg/L after culturing at 26 °C for 72 h. Furthermore, when 10 mL/L olive oil was added to the fermentation broth at the 24th hour of fermentation, the maximum prodigiosin production of 15,420.9 mg/L was obtained, which was 9.3-fold higher than the initial level before medium optimization. More than 60% of the prodigiosin produced with this optimized fermentation strategy was in the form of pigment pellets. To the best of our knowledge, this is the first report on this phenomenon of pigment pellet formation, which made it much easier to extract prodigiosin at low cost. Prodigiosin was then purified and identified by absorption spectroscopy, HPLC, and LCMS. Purified prodigiosin obtained in this study showed anticancer activity in separate experiments on several human cell cultures: A549, K562, HL60, HepG2, and HCT116.

*Conclusions:* This is a promising strain for producing prodigiosin. The prodigiosin has potential in anticancer medicine studies.

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## 1. Introduction

Prodigiosin is a kind of pigment produced by bacterial strains such as *Serratia marcescens* and *Vibrio* spp. [1,2] and is one of the prodiginine derivatives containing a common 4-methoxy, 2-2 bipyrrole ring system [3]. Because of its color and bioactivity, prodigiosin has potential use in textiles [4,5], as food colorants [6], and as antimicrobials [7]. In particular, this pigment has shown low cytotoxicity in noncancerous cells and high apoptotic activity in many cancer cell lines both in vivo and in vitro [8,9,10], and new anticancer drugs may be discovered with in-depth research of prodigiosin.

There have been some reports focused on the factors affecting prodigiosin production and how to improve production with a low-cost medium. Temperature, culture time, and quorum sensing are the main factors impacting prodigiosin production [11,12,13]. For the medium, oil seed broth [14] and oil addition [15] were found to significantly improve prodigiosin production. Other nutrients such as brown sugar [16], squid pens [17], ethanol [18], and ram horn peptone [19] were also reported to improve prodigiosin production. In the process of prodigiosin extraction, large amounts of chloroform, ethyl acetate, and other organic solvents must be used to extract prodigiosin from liquid fermentation broth [20], but these organic solvents are always toxic and cause contamination. If the produced prodigiosin is collected in the pellet form, then it can be extracted with much less amount of organic solvents; thus far, to the best of our knowledge, there is no report on this concept. Therefore, fermentation strategies that decrease the amount of organic solvent used while ensuring high fermentation production should also be considered.

In this study, peanut and olive oil were chosen for prodigiosin production from *Serratia marcescens* FZSF02. Box-Behnken design (BBD) and olive oil addition during fermentation achieved high production of prodigiosin. Moreover, in our study, the prodigiosin was produced mainly in the form of pigment pellets, which could be extracted simply with a small amount of methanol. To verify the

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biological activity of the obtained molecule, its anticancer activity on several human cells was tested.

## 2. Materials and methods

### 2.1. Strains and medium

The strain FZSF02 was isolated from soil in Fuzhou, China, and was identified as *Serratia marcescens* by 16S rDNA sequencing (NCBI KU145144). This strain was isolated and preserved in a medium (pH 7.0) consisting of beef extract (5 g/L), soya peptone (10 g/L), and NaCl (1 g/L). The solid medium consisted of 20 g/L of agar in addition to the composition of the liquid medium.

### 2.2. Preparation of seed culture and culture conditions of Serratia marcescens FZSF02

For seed culture preparation, a loop of *Serratia marcescens* FZSF02 was inoculated into 50 mL of screening medium in a 250-mL Erlenmeyer flask, followed by incubation and shaking at 37 °C and 180 rpm for 12 h. All experiments for medium optimization were carried out at an inoculation dose of 2% and at 26 °C for 72 h.

### 2.3. Effect of medium composition on prodigiosin production

To select the optimal nitrogen source, 10 g/L soya peptone, beef extract, tryptone, yeast extract, fish meal, corn steep liquor, soybean powder, and peanut powder were chosen separately as the sole nutrition source to ferment FZSF02. The effects of carbon source on the production of prodigiosin were evaluated by adding 5 g/L (w/v) of a given carbon source to the medium with the optimal nitrogen source. Different kinds of oil were separately added to the medium at a volume of 10 mL/L to study their effect on prodigiosin production. Oil addition for the second time was carried out at the 24th hour to test the effect of oil addition in batches on the prodigiosin production.

The data of of prodigiosin production were presented as mean values (±SD) of three repeats.

Box-Behnken design (BBD) was used to calculate the combined influence of nutrition factors. Depending on the results of single-factor experiments, peanut powder, beef extract, and olive oil were selected as the three factors. Three levels of each factor and a 17-experiment design were prepared. For each experiment, 1 mL of seed broth was inoculated into a 250-mL evaporator flask containing 50 mL of culture medium, and then, these flasks were incubated at 26 °C for 72 h. The prodigiosin production in each flask was then assayed.

Design Expert 8.0.6 was used to design the experiments and analyze the results.

### 2.4. Isolation, purification, and identification of prodigiosin

In this study, pigment pellets formed during the fermentation process. Pigment pellets of every 50 mL of fermentation broth in one flask were first collected with a medical gauze; then, these pellets were diluted with 50 mL of acidified methanol (4 mL of 1 mol/L HCl/96 mL of methanol) [21] after ultrasonication for 30 min. After standing for 1 h, the supernatant was concentrated by rotary evaporation, and the crude pigment was obtained. The residual fermentation broth with pellets removed was mixed with an equal volume of ethyl acetate and then sufficiently shaken; the ethyl acetate phase was obtained with a separating funnel. The crude pigment was obtained after rotary evaporation.

Crude prodigiosin was further purified with a silica gel column. The loaded column was eluted first with petroleum ether for five volumes and then with petroleum ether:ethyl acetate (1:1) until the orange portion flowed out. Purified prodigiosin was obtained when the orange eluate was collected and concentrated by rotary evaporation at 40 °C.

Purified prodigiosin was diluted with acidified methanol for the following characterization. Absorption spectra were obtained by scanning the wavelengths from 200 to 800 nm with a UV-visible spectrometer (BECKMAN DU-800). High-performance liquid chromatography (HPLC) was performed to identify the purified prodigiosin and the standard prodigiosin (Cat#529685, CALBIOCHEM, Germany). HPLC was performed on an Agilent 1220 Infinity LC with a 4.6 × 250 mm, 5 μm LC column ZORBAX (ZORBAX SB-C18, Agilent). The mobile phase was acidified methanol:water:acetonitrile (73:20:7), and the detection wavelength was 535 nm [20]. Liquid chromatography–mass spectrometry (LCMS) was performed to measure the molecular weight of the pigment [16].

### 2.5. Quantitation of prodigiosin

Prodigiosin quantitation was carried out by a reported method [22]. In practice, the fermentation broth with pigment pellet removed was properly diluted with acidified methanol, and the absorbance at 535 nm was then measured. The pigment solution extracted with acidified methanol from the pellets was also properly diluted, and the absorbance was measured by the same method. The absorbance values were then converted to concentration using purified prodigiosin as the standard. The prodigiosin concentration per milliliter fermentation broth was the sum of the prodigiosin amounts measured in the pellet part and the pellet-free fermentation broth part.

### 2.6. Cell lines and method for anticancer property test

The human lung carcinoma cell line A549 (ATCC® CCL185™) was cultured in DMEM + 10% FBS + 1% P/S; the human bone marrow chronic myelogenous leukemia cell line K562 (ATCC® CCL-243™), human acute promyelocytic leukemia cell line HL60 (ATCC® CCL-240™), human hepatocellular carcinoma cell line HepG2 (ATCC® HB-8065™), and human colorectal carcinoma cell line HCT116 (ATCC® HB-247™) were cultured in RPMI1640 medium + 10% FBS + 1% P/S. The prodigiosin doses used for the anticancer property test were set as a series: 100 μM, 50 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, 0.1 μM, 0.05 μM, 0.01 μM, and 0 μM. Cell viability was determined by the CCK method with a Transdetect Cell Counting Kit (Transgen, Beijing, China).

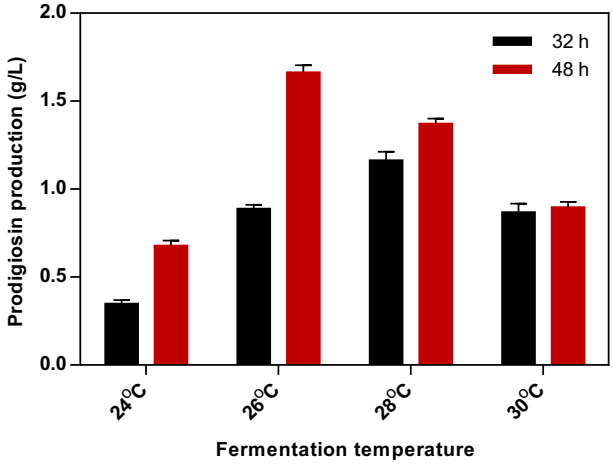


**Fig. 1.** Effect of fermentation temperature on prodigiosin production by *Serratia marcescens* FZSF02.

**Table 1**
Effect of carbon source on prodigiosin production.

| Carbon source (5 g/L) | Prodigiosin production (mg/L) |
|---|---|
| Control | 1735.53 ± 54.87 |
| Lactose | 1601.1 ± 585.82 |
| Dextrin | 2040.7 ± 102.62 |
| Sucrose | 1743.77 ± 225.92 |
| Glycerol | 1674.87 ± 504.73 |
| Maltitol | 2116.72 ± 125.5 |
| Starch | 1939.23 ± 296.39 |
| Glucose | 2396.43 ± 48.95 |
| Citrate | 0 |

## 3. Results and discussion

### *3.1. Effects of fermentation conditions and nutrients on prodigiosin production*

The optimal temperature for prodigiosin production was 26 °C for *Serratia marcescens* FZSF02 (Fig. 1). By employing the screening medium, different types of carbon sources were used to examine their effect on prodigiosin production. Prodigiosin production increased by 38.1%, from 1735.53 ± 54.87 mg/L to 2396.43 ± 48.95 mg/L, with 5 g/L of glucose; maltitol, dextrin, and starch could also promote prodigiosin production to different extents (Table 1). The optimal carbon source type may be different for different strains of *Serratia marcescens*. For *Serratia marcescens* B6 [23], glycerol was determined as the best carbon source, and glucose was the worst among the tested carbon sources, which was different for FZSF02. With 5 g/L of glucose as the carbon source in the medium, the effect of the nitrogen source was studied (Table 2). Prodigiosin production reached 1774.49 ± 84.94 mg/L, 1699.6 ± 347.66 mg/L, and 3762.07 ± 260.82 mg/L with soya peptone, beef extract, and peanut powder as the sole nitrogen sources, respectively. This bacterium could also produce prodigiosin with tryptone and yeast extract as the nitrogen source, but the production was less than 400 mg/L. This strain could not produce prodigiosin with fish meal, soybean powder, or corn steep liquor. Pigment pellets were found on the surface of the fermentation broth; the prodigiosin in the pellets accounted for 19.2%, 11.3%, and 20.2% of the total prodigiosin with soya peptone, beef extract, and peanut powder as the sole nitrogen source, respectively. When beef extract (10 g/L) and peanut powder (10 g/L) were combined as the nitrogen source, prodigiosin production reached 5062.16 ± 325.79 mg/L, which was 2.98 times higher than that of beef extract alone and 1.35 times higher than that of peanut powder alone. Furthermore, the prodigiosin content in the pigment pellets also increased significantly to 65.33% of the total prodigiosin production. In some reports [24,25], yeast extract was the optimal nitrogen source, and beef extract inhibited prodigiosin production, which was different from the results for *Serratia marcescens* FZSF02. Some reports demonstrated that seeds rich in oil could stimulate *Serratia marcescens* strains to produce prodigiosin [12,26,27]. In our study, the combination of beef and peanut powder strongly promoted prodigiosin production. Moreover, more than 60% of the produced prodigiosin was in the pigment pellets; this is the first report to date on this phenomenon (Fig. 2). The pellets were easily collected from the fermentation broth, and prodigiosin could be simply extracted with a small amount of methanol, which is very valuable for the industrial production of prodigiosin with low cost and high efficiency. Oil was another important factor that could improve prodigiosin production [15]. In our study, six kinds of oil increased prodigiosin production to different degrees (Table 3). Olive oil was found to be the optimal oil for prodigiosin production; a high amount of prodigiosin,

**Table 2**
Effect of nitrogen source on prodigiosin production.

| Nitrogen source (10 g/L) | Prodigiosin in broth (mg/L) | Prodigiosin from pellet (mg/L) | Total prodigiosin (mg/L) |
|---|---|---|---|
| Soya peptone | 1433.19 ± 158.63 | 341.3 ± 173.7 | 1774.49 ± 84.94 |
| Beef extract | 1508.08 ± 337.52 | 191.52 ± 20.76 | 1699.6 ± 347.66 |
| Peanut powder | 3002.89 ± 25.02 | 759.18 ± 246.77 | 3762.07 ± 260.82 |
| Soya peptone and peanut powder | 1262.3 ± 21.2 | 326.55 ± 31.04 | 1588.85 ± 52.24 |
| Beef extract and peanut powder | 1755.22 ± 387.61 | 3306.94 ± 293.02 | 5062.16 ± 325.79 |
| Tryptone | 353.28 ± 17.01 | 0 | 353.28 ± 17.01 |
| Yeast extract | 380.24 ± 12.97 | 0 | 380.24 ± 12.97 |
| Fish meal | 0 | 0 | 0 |
| Soybean powder | 0 | 0 | 0 |
| Corn steep liquor | 0 | 0 | 0 |

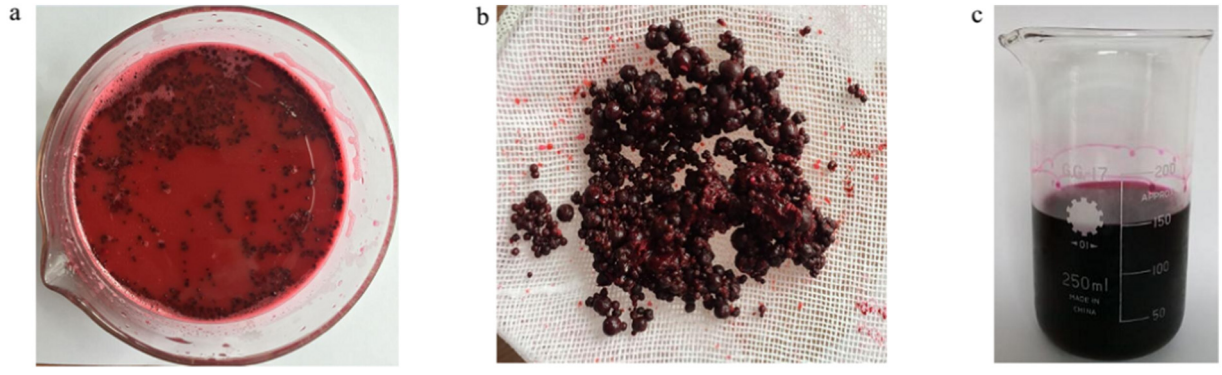


**Fig. 2.** Pigment pellets containing prodigiosin produced during the fermentation course. (a) Pellets on the surface of the fermentation broth; (b) Pellets collected with medical gauze; (c) Pigment pellets dissolved with acidified methanol.

**Table 3**
Effect of oil application on prodigiosin production.

| Oil (10 mL/L) | Prodigiosin production (mg/L) |
|---|---|
| Soybean oil | 9283.16 ± 907.46 |
| Canola oil | 7142.8 ± 45 |
| Olive oil | 11,366.6 ± 433.52 |
| Maize oil | 9119.9 ± 274.55 |
| Peanut oil | 9539.29 ± 539.83 |
| Tea oil | 10,348.1 ± 1490.52 |

11,366.6 ± 433.52 mg/L, was obtained when 10 mL/L olive oil was added to the medium, which was more than twofold higher than that produced without olive oil.

### 3.2. Optimization of culture medium by response surface analysis

With the results of the single-factor experiments (Fig. 3), peanut powder, beef extract, and olive oil were chosen for response surface analysis with the Box-Behnken design (BBD) (Table 4). ANOVA results showed that this model fit $R = -15.55729 + 1.39789X_1 + 0.73592X_2 + 1.92056X_3 - 7.31136X_1X_2 - 0.014667X_1X_3 - 6.4696X_2X_3 - 0.032844X_1^2 - 0.016725X_2^2 - 0.069289X_3^2$. R represents prodigiosin production, and $X_1$, $X_2$, and $X_3$ are the amounts of peanut powder, beef extract, and olive oil, respectively. The model p-value < 0.0001 and F-value of 320.24 imply that the model is significant. The 3D surfaces of this design are shown in Fig. 4. The maximum prodigiosin production should be 13,045.2 mg/L with the predicted optimal combination of 16.97 g/L of peanut powder, 16.02 g/L of beef extract, and 11.29 mL/L of olive oil. To confirm this model, a verification experiment was carried out with the optimal combination. The prodigiosin production reached 13.622 ± 236 mg/L, which fit the predicted value. The pigment pellets accounted for 62.3% of the total prodigiosin.

**Table 4**
Three-factor experimental design based on Box-Behnken.

| Experiment | Peanut powder (g/L) | Beef extract (g/L) | Olive oil (mL/L) | Prodigiosin production ($10^3$ mg/L) |
|---|---|---|---|---|
| 1 | 17.5 | 15 | 12.5 | 12.9 |
| 2 | 17.5 | 15 | 12.5 | 12.9 |
| 3 | 17.5 | 15 | 12.5 | 12.6 |
| 4 | 17.5 | 5 | 20 | 6.4 |
| 5 | 17.5 | 15 | 12.5 | 13 |
| 6 | 10 | 5 | 12.5 | 8.9 |
| 7 | 17.5 | 25 | 5 | 9.3 |
| 8 | 25 | 15 | 20 | 4.6 |
| 9 | 10 | 25 | 12.5 | 10.4 |
| 10 | 10 | 15 | 5 | 8.1 |
| 11 | 17.5 | 5 | 5 | 7.8 |
| 12 | 10 | 15 | 20 | 7.1 |
| 13 | 17.5 | 25 | 20 | 5.9 |
| 14 | 25 | 25 | 12.5 | 8.8 |
| 15 | 25 | 5 | 12.5 | 9.5 |
| 16 | 17.5 | 15 | 12.5 | 13.2 |
| 17 | 25 | 15 | 5 | 8.9 |

### 3.3. Effect of the addition of olive oil on prodigiosin production

When the initial addition of olive oil was 20 mL/L, the prodigiosin production was much lower than 10 mL/L (Fig. 3). We supposed that although this strain can use olive oil to synthesize prodigiosin, high concentrations of olive oil may suppress the growth of the strain. Therefore, we studied whether olive oil addition during the fermentation course can enhance prodigiosin production. With the optimized medium, the addition of olive oil significantly promoted

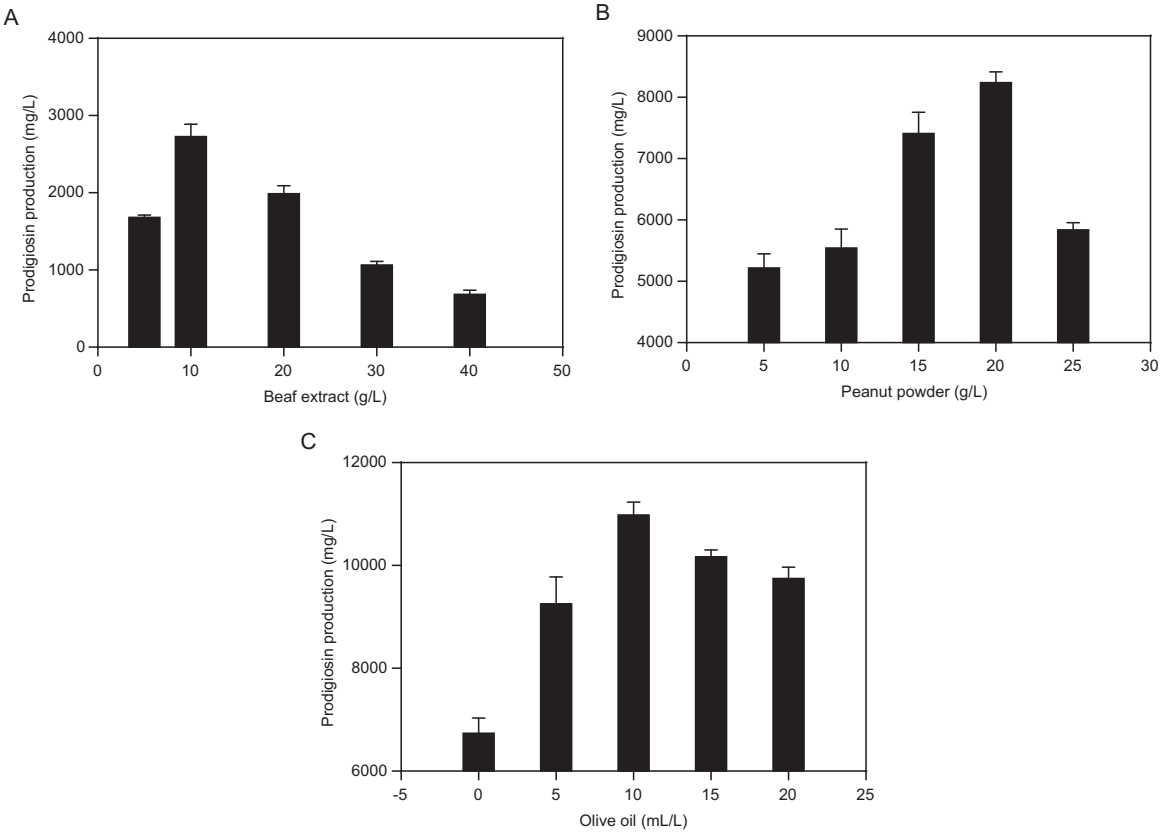


**Fig. 3.** Single-factor experiments for prodigiosin fermentation. (a) Effect of beef extract concentration on prodigiosin production; (b) Effect of peanut powder concentration on prodigiosin production; (c) Effect of olive oil addition on prodigiosin production.

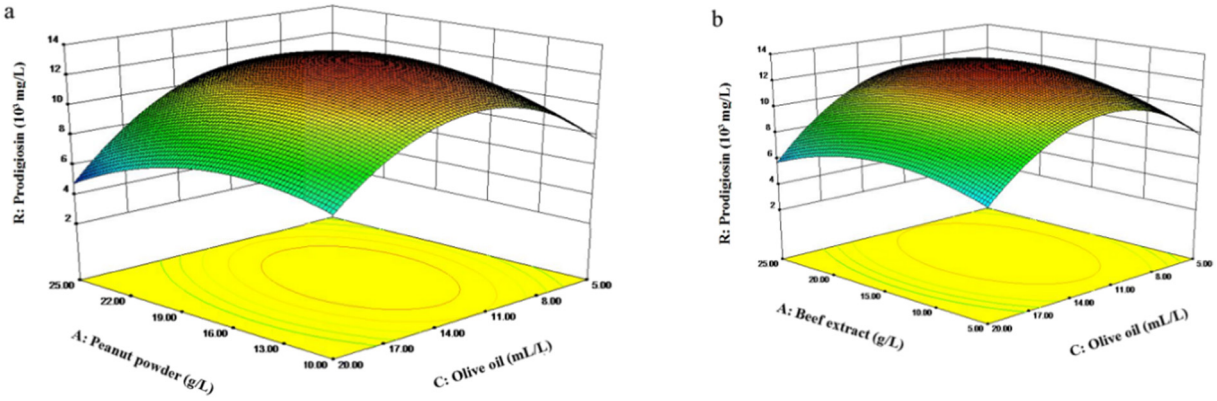


**Fig. 4.** 3D surface of the Box-Behnken design (BBD). (a) 3D surface showing the combined effect of peanut powder concentration and olive oil addition level on prodigiosin production; (b) 3D surface showing the combined effect of beef extract concentration and olive oil addition level on prodigiosin production.

**Table 5**
Effect of olive oil addition at 24 h on prodigiosin production.

| Olive oil (mL/L) | Prodigiosin production (mg/L) |
|---|---|
| 0 | 12,824.22 |
| 5 | 14,397.34 |
| 10 | 15,420.90 |
| 15 | 14,102.21 |

prodigiosin production. When the olive oil addition was 10 mL/L at 24 h, the prodigiosin production was 15,420.9 mg/L (Table 5). After the combined optimization strategy, the final prodigiosin production was higher than that in some reported strains such as *Serratia marcescens* UTM1 (8000 mg/L) [16], *Serratia marcescens* B6 (583 mg/L) [23], *Serratia marcescens* TKU011 (2480 mg/L) [6], *Serratia marcescens* N10612 (1307 mg/L) [24], and *Serratia marcescens* MO-1 (277.74 mg/L) [19].

### *3.4. Identification of prodigiosin*

Prodigiosin purified by silica gel column chromatography was identified by absorption spectroscopy and compared with standard prodigiosin and LCMS. Prodigiosin had a significant absorption peak at 535 nm (Fig. 5), which was in accordance with previous reports [11,28]. When detected with HPLC, the largest peak of the purified pigment (Fig. 6b) had the same retention time as that of the standard prodigiosin (Fig. 6a). There were also peaks at 535 nm by HPLC, which indicated that there might be other prodigiosin-like pigments produced in the fermentation course. Some studies have also demonstrated that prodigiosin-producing strains can produce other prodigiosin-like pigments [29]. The LC-MS data showed a main peak at the molecular weight of 323.9 (Fig. 7), which was in accordance with the molecular weight of prodigiosin [29]. Therefore, from the characteristics above, we confirmed that the product was prodigiosin.

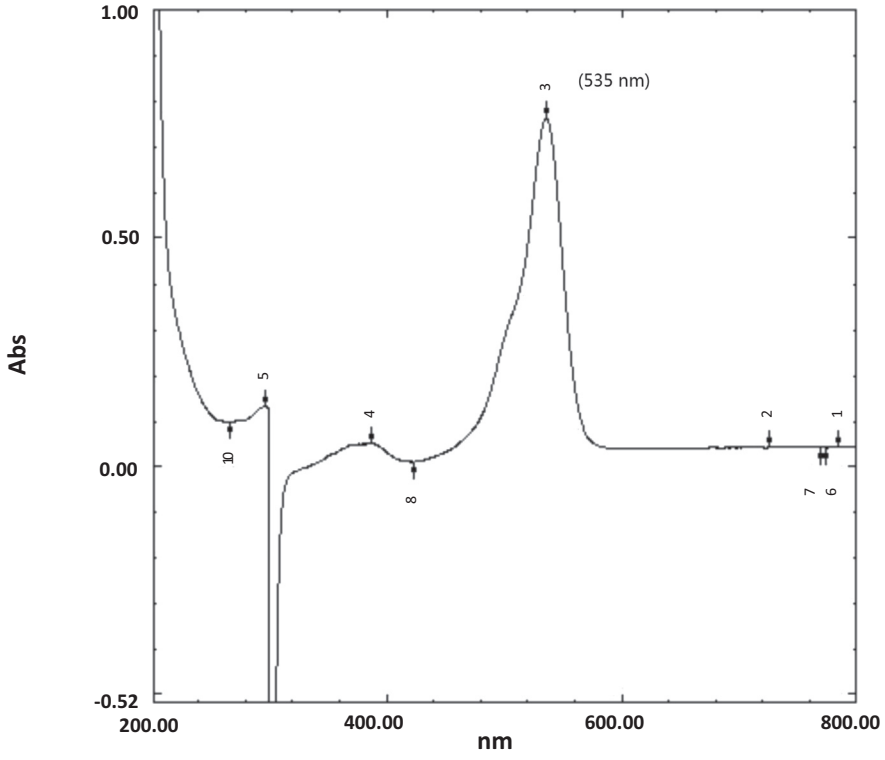


**Fig. 5.** Absorption spectra of the purified prodigiosin.

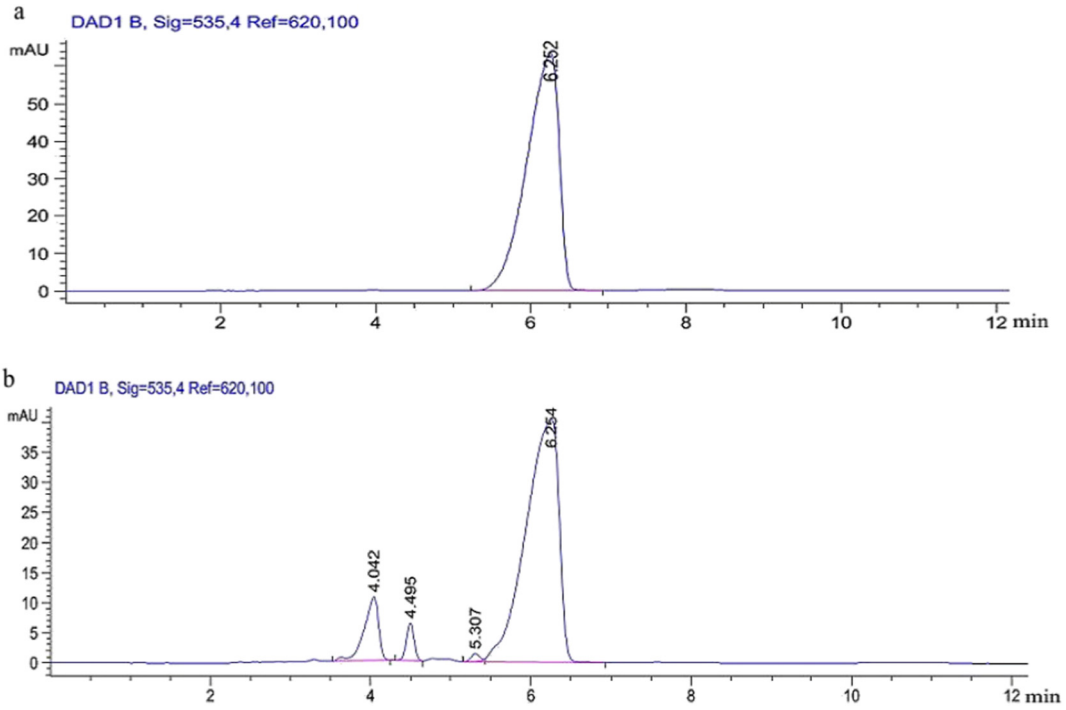


**Fig. 6.** Detection of purified prodigiosin with HPLC. (a) Detection of standard prodigiosin samples; (b) Detection of purified prodigiosin samples.

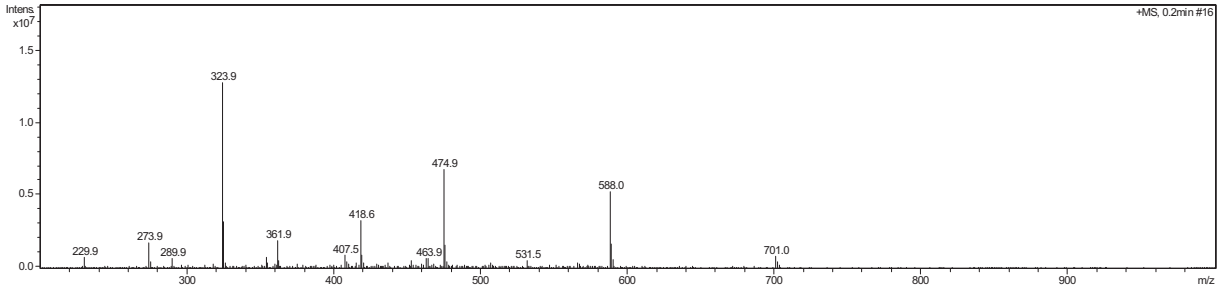


**Fig. 7.** Molecular weight of prodigiosin sample examined by LC-MS.

### *3.5. Anticancer activity of purified prodigiosin in this study*

The IC50 values ranged from 79.6 to 6160 nM for the five cancer cell lines (Table 6). Human acute promyelocytic leukemia cells HL60 and human bone marrow chronic myelogenous leukemia cells K562 were more sensitive to prodigiosin. Apoptosis of HL60 cell line induced by prodigiosin was also observed under a microscope (Fig. 8). Prodigiosin was reported to have anticancer activity against more than 60 cancer cell lines [30]. Anticancer mechanisms of prodigiosin seem to vary for different cancer cells according to several studies. It can restore p53 tumor suppressor activity in chemoresistant colorectal cancer stem cells [10]. In advanced breast cancers, prodigiosin exerted its anticancer role by inhibiting Wnt/β-catenin signaling and reducing cyclin D1 levels [31]. Our results also demonstrate that prodigiosin from *Serratia marcescens* FZSF02 has anticancer potential.

**Table 6**
Anticancer activity of prodigiosin against different cell lines.

| Cell line | Cell description | IC50 (nM) |
|---|---|---|
| A549 (ATCC® CCL185™) | Human Lung Carcinoma | 3740 |
| K562 (ATCC® CCL-243™) | Human Bone Marrow Chronic Myelogenous Leukemia | 283 |
| HL60 (ATCC® CCL-240™) | Human Acute Promyelocytic Leukemia | 79.6 |
| HepG2 (ATCC® HB-8065™) | Human Hepatocellular Carcinoma | 6160 |
| HCT116 (ATCC® HB-247™) | Human Colorectal Carcinoma | 1399 |

## 4. Conclusions

In this paper, the fermentation conditions and medium of a prodigiosin-producing strain, *Serratia marcescens* FZSF02, were optimized. After response surface optimization and olive oil addition during the fermentation course, prodigiosin production reached 15,420.9 ± 427 mg/L with the medium containing glucose, peanut powder, beef extract, and olive oil. Moreover, prodigiosin in pellet form accounted for more than 60% of the total prodigiosin in the fermentation broth. High production and pellet formation make the process suitable for industrial production of prodigiosin at low cost. The main product of this strain was prodigiosin, and there may be other prodigiosin-like pigments in the fermentation broth. The observed anticancer potential ensured the application value of the produced prodigiosin.

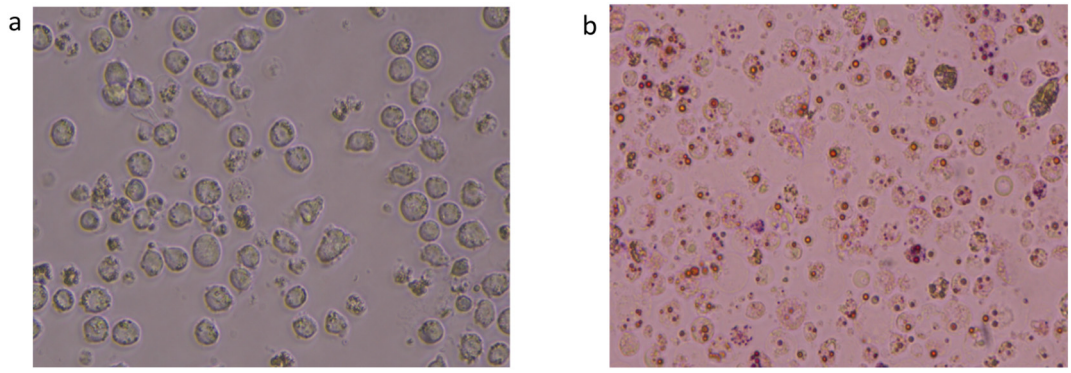


**Fig. 8.** Cell apoptosis of HL60 induced by prodigiosin. (a) HL60 cells with no prodigiosin added to culture for one day (400×). (b) HL60 cells with 50 μM of prodigiosin added to culture for one day (400×).

## Conflict of interest

None declared.

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