Utilization of Crab Waste for Cost-Effective Bioproduction of Prodigiosin

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Abstract: This study aimed to establish the culture process for the cost-effective production of prodigiosin (PG) from demineralized crab shell powder (de-CSP), a fishery processing byproduct created via fermentation. Among the tested PG-producing strains, Serratia marcescens TNU02 was demonstrated to be the most active strain. Various ratios of protein/de-CSP were used as the sources of C/N for PG biosynthesis. The PG yield was significantly enhanced when the casein/de-CSP ratio was controlled in the range of 3/7 to 4/6. TNU02 produced PG with a high yield (5100 mg/L) in a 15 L bioreactor system containing 4.5 L of a newly-designed liquid medium containing 1.6% C/N source (protein/de-CSP ratio of 3/7), 0.02% (NH_4)_2SO_4, 0.1% K_2HPO_4, and an initial pH of 6.15, at 27 °C for 8 h in dark conditions. The red pigment was purified from the culture broth and then quantified as being PG by specific Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and UV spectra analysis. The purified PG demonstrated moderate antioxidant and effective inhibition against four cancerous cell lines. Notably, this study was the first to report on using crab wastes for PG bioproduction with high-level productivity (5100 mg/L) in a large scale (4.5 L per pilot) in a short period of fermentation time (8 h). The salt compositions, including (NH_4)_2SO_4 and K_2HPO_4, were also a novel finding for the enhancement of PG yield by S. marcescens in this report.

Keywords: Serratia marcescens; prodigiosin; bioreactor system; crab shells; bioprocessing; antioxidants; anticancers

1. Introduction

Crab shells, a marine chitin waste that can be abundantly obtained from fishery processing byproducts, have been used for the production of various bioactive products, including chitin [1–3], enzymes [4], coagulants [5] antioxidants [6], and anti-cancer components [7]. Crab shells are also used in Portland cement matrices [8]. Recently, this material has been used for the bioproduction of anti-diabetic agents [9]. In this study, we investigated the use of crab shells for the production of the active medical compound prodigiosin.
Prodigiosin (PG), a microbial red pigment belonging to the family of prodiginines (Figure 1), holds numerous valuable bioactivities and has the potential of being an antiparasitic, antibacterial, anti-inflammatory, analgicidal, immunosuppressant, antioxidant, and insecticidal agent. PG has also been investigated for its use in textiles, cosmetics, and candles [10], as well as recently for its anti-Alzheimer activities [11]. In addition, PG was newly reclaimed as a novel and useful material for solar cells [12].

![Figure 1](image-url) The chemical structures of the pigments belonging to the family of prodiginines. Prodigiosin (1), Undecylprodigiosin (2), Metacylprodigiosin (3), Cycloprodigiosin (4), and Streptorubin B (5).

This pigment compound is produced by various bacterial strains, including *Serratia rubidaea*, *Alteromonas rubra*, *Janthinobacterium lividum* BR01, *Rugamonas rubra*, *Streptomyces longisporus ruber* 100-19, *S. coelicolor*, *S. spectabilis* BCC 4785, *S. fusant* NRCF69, *V. gazogenes*, *V. psychoerythrus*, *P. magnesiorubra*, *P. putida* KT2440, *S. rubireticuli*, *P. rubra*, and *Actinomyces* [10]. Of these, *S. marcescens* is the major producer of PG [11].

Recently, the study of PG has been renewed and increased due to its beneficial effects, especially its high anticancer activity [11,13] and lack of toxicity to normal cells [11]. To enhance the anticancer effect, PG was combined with other agents [14], formed nanoparticle sizes [15], and synthesized its derivatives [16]. Thus, the large-scale production of PG for further clinical studies has been considerable. Numerous studies have focused on PG production [10]; however, most of the C/N sources used for fermentation have been commercial nutrient mediums such as broths of peptone glycerol [18], tryptone yeast, tryptone soy, glycerol, yeast malt [19], nutrient broth (NB) [20], *Luria-Bertani* (LB) broth, 3-[N-morpholino]-ethanesulfonic acid [21], and yeast extract [22]. For the low-cost production of PG via fermentation, several C/N sources have been investigated for fermentation, such as crude glycerol, coconut oil, sesame oil, peanut oil, peanut seed, sesame seed, copra seed, corn steep, and cassava, as well as mixtures of sunflower oil/Luria–Bertani broth, mannitol/corn steep, olive oil/beef extract, and mannitol/cassava [23–28].

For the green production of PG, under the considerations of environmental problems and low-cost production, we established the utilization of marine chitin-containing wastes for the production of PG [11,29–31]. These previous studies focused on reusing squid pens as a C/N source for the production of PG via fermentation by *S. marcescens* TKU011 [29–31] and *S. marcescens* TNU01 [11]. Recently,
we found that the addition of chitin to the medium has a significant effect on enhancing PG yield in the culture broth and that α-chitin (extracted from crab shells) has a better effect compared to β-chitin (extracted from squid pens) [32]. This evidence indicated that materials containing α-chitin may be a good source for PG production. Crab shell contains α-chitin as a major component, and as such it was chosen as the target material for the cost-effective production of PG in this investigation.

In this study, crab shell was used as the major C/N source with the supplementation of free protein for fermentation by various strains of *S. marcescens*. This study focused on investigating the most suitable added free protein, the effect of the salt composition, and the optimal conditions for fermentation to produce PG in a 100 mL flash. Production of the PG was then scaled up by using a 15 L bioreactor system for fermentation. Finally, the PG was extracted, isolated from the culture broth and its biological activities were evaluated, including its antioxidant and anticancer effects.

2. Results and Discussion

2.1. The Effect of Different Added Free Proteins and Different Strains of *S. marcescens* on PG Production

For the investigation of the effect of different protein sources added to a medium containing demineralized crab shell powder (de-CSP) on PG production, five types of protein, including casein, beef extract, nutrient broth, peptone, and yeast extract, were supplemented for fermentation to produce PG. As presented in Figure 2a, casein was the most suitable free protein for PG production, with the highest yield of 3.01 mg/mL. To investigate the optimal added concentration of casein, this protein source was combined with de-CSP at various ratios (Casein/de-CSP) of 1/9, 2/8, 3/7, 4/6, 5/5, and 6/4 then used as the C/N source for fermentation. This sub-experimental result is illustrated in Figure 2b. The casein/de-CSP ratios of 3/7 and 4/6 reached the highest PG yield of ≥3.5 mg/mL. With regard to the utilization of crab waste for the cost-effective production of PG, a casein/de-CSP ratio of 3/7 was conducted for further investigation.

![Figure 2](image-url)

**Figure 2.** The effect of sources of protein added (a) and casein/de-CSP ratio (b) on PG production. The C/N was a mixture of 1% crab shell and 0.6% free protein (a), or the combination of casein and de-CSP at different ratios and used at 1.6% as the C/N source (b) in a basal salt solution of 0.1% CaSO₄, 0.05% K₂HPO₄ and a pH of 6.15 for fermentation by *S. marcescens* TKU011 under the fermentation conditions of 25 °C, a duration of 2 days, a shaking speed of 150 rpm in the dark, and a culture medium/flask volume ratio of 3/7 (v/v) (30 mL of liquid culture medium in a 100 mL flask). The column with red color indicated that it is the factor chosen for further investigation.

For comparison of the PG production ability of different bacterial strains, four strains of *S. marcescens*, including TKU011, CC17, TNU01, and TNU02 were fermented in the same conditions. The data are summarized as shown in Table 1. The results indicated that three strains, including TKU011,
TNU01 and TNU01 were promising PG-producing strains that could produce an equal PG yield of 3.52–3.61 mg/mL. *S. marcescens* TNU02 was chosen for further investigation.

**Table 1.** PG production by various strains of *Serratia marcescens*.

| No. | Bacterial Strains     | Prodigiosin (mg/mL) |
|-----|-----------------------|---------------------|
| 1   | *S. marcescens* TKU011| 3.52 ± 0.134        |
| 2   | *S. marcescens* TNU01 | 3.59 ± 0.153        |
| 3   | *S. marcescens* TNU02 | 3.61 ± 0.163        |
| 4   | *S. marcescens* CC17  | 2.73 ± 0.102        |

| No bacterial strain | - |

The liquid medium contained 0.1% CaSO$_4$, 0.05% K$_2$HPO$_4$, 1.6% C/N source (casein/de-CSP = 3/7) and had a pH 6.15. These designed mediums were fermented by four strains of *S. marcescens* at 25°C at a shaking speed of 150 rpm in the dark for 2 days. (•) No PG production.

2.2. The Effect of Salt Composition and Some Parameters of Fermentation on PG Production

Our previous studies revealed that the salt composition, including sulfate and phosphate salts, demonstrate a significant effect on PG production using *S. marcescens* [11,30–32]. Thus, six kinds of sulfate salts (MgSO$_4$·7H$_2$O, CaSO$_4$, ZnSO$_4$·7H$_2$O, MnSO$_4$·7H$_2$O, FeSO$_4$·7H$_2$O, and (NH$_4$)$_2$SO$_4$) and five kinds of phosphate salts (K$_2$HPO$_4$, KH$_2$PO$_4$, Ca$_3$(PO$_4$)$_2$, NaH$_2$PO$_4$, and Na$_2$HPO$_4$) were conducted to evaluate their effect on PG production via fermentation (Figure 3).

Among the various tested salts, (NH$_4$)$_2$SO$_4$ and KH$_2$PO$_4$ were screened as the most suitable salt compositions (Figure 3a,c) at the optimal added concentrations of 0.02% and 0.1%, respectively (Figure 3b,d). K$_2$HPO$_4$ and MgSO$_4$ have been found to be the most suitable sources of phosphate and sulfate salts, respectively, in many previous reports [11]. Recently, K$_2$HPO$_4$ and CaSO$_4$ have been found to be the best salt compositions for PG production [32]. However, in this study, KH$_2$PO$_4$ and (NH$_4$)$_2$SO$_4$ were newly found to play a significant role in PG yield enhancement in the fermentation of *S. marcescens*.

For the maximum PG production by *S. marcescens* TNU02 fermentation, some fermentation parameters, such as temperature, pH of the culture medium, culture volume and cultivation time, were investigated. The results illustrated in Figure 3e–k show that *S. marcescens* TNU02 induced the highest PG yield of 4.51 mg/mL in the designed liquid medium containing a 1.6% C/N source (a casein/de-CSP ratio of 3/7), 0.02% (NH$_4$)$_2$SO$_4$, 0.1% K$_2$HPO$_4$, an initial pH of 6.15, and a percentage of culture medium in the flask of 30% (30 mL of liquid culture medium in a 100 mL flask), at 27°C for 2 days in dark conditions. After optimization of the culture conditions, some nutrients and parameters changed and the PG yield dramatically increased by approximately 1.5-fold, from 3.01 mg/mL to 4.51 mg/mL (Table 2).
The percentage of culture medium.

Using a 15 L bioreactor system. The production of PG in a 100 mL flask was also performed at the

2.3. Scaled up Biosynthesis, Extraction and Qualification of PG Produced by S. marcescens TNU02

To achieve the purpose of PG production in mass, we used the optimal culture conditions obtained from the above experiments in a minor scale (a 100 mL flask) to scale up the production of PG by using a 15 L bioreactor system. The production of PG in a 100 mL flask was also performed at the
same time for comparison. The PG productivity was detected during fermentation from 2 to 12 h (fermentation in the bioreactor), and for 8 to 48 h (fermentation in the 100 mL flask). As shown in Figure 4, the PG yield reached the maximum (5100 mg/L) at 8 h of fermentation in the 15 L bioreactor system, while the PG yield of fermentation in the 100 mL flask reached the maximum productivity of 4514 mg/L at 36 h of fermentation. Thus, the PG production in the 15 L bioreactor system resulted in reaching a higher PG yield in a much shorter period of fermentation time compared to the PG production in the 100 mL flask. A review of the recent literatures found that numerous studies have reported on PG production. However, in most previous studies, PG production was conducted in a minor scale (in flasks) using commercial nutrients as C source for fermentation [11]. Different to previous research, our study successfully engaged in cost-effective PG production at a large scale (a 15 L bioreactor system) using low-cost material (crab waste) as the C/N source for fermentation. PG was also produced by S. marcescens TKU011 in a 10 L bioreactor system with a lower maximal yield (3450 mg/L) at a longer period of fermentation time (12 h) in our previous report [11].

![Figure 4](image_url)

Figure 4. Production of PG by S. marcescens TNU02 in a 15 L bioreactor system and in a 100 mL flask. 450 mL of S. marcescens TNU02 was previously fermented in a 1000 mL flask for 2 days and then injected into a 15 L bioreactor system containing 4.05 L of a culture medium containing 1.6% C/N source (a casein/de-CSP ratio of 3/7), salt compositions of 0.1% KH2PO4 and 0.02% (NH4)2SO4, and an initial pH of 6.15. Fermentation was also conducted in a 100 mL flask for comparison. Sampling and determination of the PG concentration was performed every 2 h until 12 h of fermentation had passed in the bioreactor system. Fermentation in the 100 mL flask was also performed at the same time for comparison, and the sampling and determination of the PG concentration was performed every 8 h, up to 48 h during the cultivation.

The PG was isolated and purified from the culture broth in a 15 L bioreactor via several steps according to the method previously reported by Wang et al., 2012 [30], including layer separation by using ethyl acetate (Figure 5c) and fractionation by a silica column (Figure 5d), and was finally purified via TLC isolation (Figure 5e). The production, isolation, and purification process of the PG is illustrated in Figure 5. The red purified compound was confirmed as PG using a number of rapid techniques, including its UV absorption and MALDI-TOF MS spectrum. The results shown in Figures 6 and 7 reveal that this red pigment possessed maximal UV absorption at 535 nm and a molecular weight of
323.045 g/mol. These two items represented the specific UV absorption and molecular weight of the PG [30]; thus, this red pigment was confirmed to be PG.

Figure 5. The process of PG production and purification in a 15 L bioreactor system. The liquid medium at the start of fermentation (A) turned red after being fermented by S. marcescens TNU02 over 8 h (B). The red pigment PG was purified via the separation layer by ethyl acetate (C) and then further separated in a column containing silica gel (D) and was finally isolated by TLC separation (E).

Figure 6. MALDI-TOF MS spectrum of the purified PG produced by S. marcescens TNU02 analyzed using the method described in our previous report [32].

Figure 7. The UV absorption spectrum of S. marcescens TNU02 PG produced in a 15 L bioreactor system.
2.4. Antioxidant and Anticancers Activities of Purified PG

PG has been investigated for its vast array of medical effects [10]. To confirm that the PG produced by our novel medium with the fermentation of *S. marcescens* TNU02 in a bioreactor system and purified by the reported protocol [30] was active molecule, some biological activities, including the antioxidant and anticancer activities of the PG, were tested.

Antioxidant compounds may protect some major components of cells, such as proteins, DNA, and lipids from the damage of free radicals [33]. In this study, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays were used for the evaluation of the antioxidant activity of the PG. As shown in Figure 8a, the PG demonstrated both DPPH and ABTS radical scavenging capacities. Of these, the ABTS radical scavenging activity of the PG was higher than the DPPH radical scavenging capacity of the PG, with a max activity of 98.3% (at a tested PG concentration of 4 mg/mL) and 96% (at a tested PG concentration of 8 mg/mL), respectively. To provide more clarity, the IC$_{50}$ values (the concentration of an antioxidant compound that may reduce 50% of the radical scavenging [11]) of the ABTS and DPPH radical scavenging activity of the PG were also expressed as 1.25 mg/mL and 2.64 mg/mL, respectively. The α-tocopherol, a standard antioxidant compound was tested for the comparison activity and showing its ABTS and DPPH radical scavenging activity of 13.3 μg/mL and 23.1 μg/mL, respectively.

![Figure 8. Antioxidant (a) and anticancer effects (b) of PG produced by *S. marcescens* TNU02 PG in a 15 L bioreactor system.](image)

The potential antioxidant capacity of PG has been reported in some studies, such as Muthukumar et al. [34], Arivizhivendhan et al. [35], and Nguyen et al. [11], who found the max activity of the DPPH radical scavenging capacity of PG to be 86%, 99%, and 98%, respectively. However, few data on the ABTS radical scavenging of PG have been reported so far [35]. Notably, almost all of the studies did not report the IC$_{50}$ values on antioxidant activity of PG [11]. Thus, the results of the antioxidant activities reported in this study could support and enrich the available data on the antioxidant effects of PG.

PG has been reported to be effective in inhibiting various cancerous cell lines without being toxic to normal cells; thus, it has been suggested as a potential molecular target in anticancer drugs [36]. To evaluate the inhibitory activity of the PG produced in this study, some cancerous cell lines, including A549, Hep G2, MCF-7, and WiDr, were investigated and tested. As illustrated in Figure 8b, the PG demonstrated a high inhibitory effect with max inhibition values of 92.1%, 93.1%, 94%, and 92% against all the tested cell lines (MCF-7, A549, Hep G2, and WiDr), respectively. To clarify the result, the IC$_{50}$ values were also calculated and showed that the PG possessed potent anticancer activity against MCF-7, A549, Hep G2, and WiDr with the low IC$_{50}$ values of 0.102 μg/mL, 0.182 μg/mL,
S. marcescens was performed under the above conditions using TKU011, TKU02, TNU01, TNU02, and CC17 under the same conditions for PG production. Five types of protein, including casein, beef extract, nutrient broth, peptone, and 0.6% yeast extract were supplemented in a culture medium containing 1% de-CSP, 0.1% CaSO₄, 0.05% K₂HPO₄, and a pH of 6.15. This mixture medium was fermented by Serratia marcescens TKU011 at 25 °C under a shaking speed of 150 rpm in the dark for 2 days. The PG produced in the culture broth was the optimal casein added concentration of (NH₄)₂SO₄, which expressed its best effect on PG production. The fermentation process was performed under the above conditions using S. marcescens TKU011. The optimal casein/de-CSP ratio of 3/7 was used for fermentation by different strains of S. marcescens, including S. marcescens TKU011, S. marcescens TKU01, S. marcescens TNU02, and S. marcescens CC17 under the same conditions of fermentation for PG production. S. marcescens TNU02 was used for the following experiments.

The effect of different free protein combinations with CSP and different strains of S. marcescens on PG production: Five types of protein, including casein, beef extract, nutrient broth, peptone, and 0.6% yeast extract were supplemented in a culture medium containing 1% de-CSP, 0.1% CaSO₄, 0.05% K₂HPO₄, and a pH of 6.15. This mixture medium was fermented by Serratia marcescens TKU011 at 25 °C under a shaking speed of 150 rpm in the dark for 2 days. The PG produced in the culture broth was the determined its concentration. Casein was found as the most suitable protein source to be added. Thus, casein was combined with de-CSP at various ratios of 1/9, 2/8, 3/7, 4/6, 5/5, and 6/4 and used as the C/N source for fermentation at 1.6% for investigation on PG production. The fermentation process was performed under the above conditions using S. marcescens TKU011. The optimal casein/de-CSP ratio of 3/7 was used for fermentation by different strains of S. marcescens, including S. marcescens TKU011, S. marcescens TKU01, S. marcescens TNU02, and S. marcescens CC17 under the same conditions of fermentation for PG production. S. marcescens TNU02 was used for the following experiments.

The effect of phosphate salts on PG production: Six kinds of sulfate salts (MgSO₄·7H₂O, CaSO₄, ZnSO₄·7H₂O, MnSO₄·7H₂O, FeSO₄·7H₂O, and (NH₄)₂SO₄) were tested for their effect on PG yield production. The liquid medium contained 0.1% sulfate salt and 0.05% K₂HPO₄ and had a pH of 6.15 and a 1.6% C/N source (casein/de-CSP = 3/7). These designed mediums were fermented at 25 °C under a shaking speed of 150 rpm in the dark for 2 days. (NH₄)₂SO₄ expressed the best effect on PG production. This salt was added into the medium at various concentrations of 0.01, 0.02, 0.035, 0.05, and 0.1%. The fermentation was performed with no changes to the other factors so as to investigate the optimal added concentration of (NH₄)₂SO₄.

The effect of phosphate salts on PG production: Five kinds of phosphate salts (K₂HPO₄, KH₂PO₄, Ca₃(PO₄)₂, NaH₂PO₄, Na₂HPO₄) were investigated to evaluate their effect on PG production. The liquid medium contained 0.02% (NH₄)₂SO₄ and 0.05% phosphate salt and had a pH 6.15 and a 1.6% C/N source (casein/de-CSP = 3/7). These designed mediums were fermented at 25 °C under a shaking speed of 150 rpm in the dark for 2 days. KH₂PO₄ expressed its best effect on PG production. This salt was added into the medium at various concentrations of 0.025, 0.05, 0.1, 0.15, and 0.2%. The fermentation
was performed with no changes to the other factors so as to investigate the optimal added concentration of KH$_2$PO$_4$.

Effect of some fermentation parameters on PG production: A 1.6% C/N source (casein/de-CSP = 3/7) in a liquid medium containing the optimal salt composition of 0.02% (NH$_4$)$_2$SO$_4$ and 0.1% K$_2$HPO$_4$ was used to investigate the effect of the following parameters: culture temperature (23, 25, 27, 30, and 33 °C); initial pH of the culture medium (5.15, 5.65, 6.15, 6.65, 7.15, 7.65, 8.15, 8.65, 9.15, and 9.65 pH); percentage of culture medium volume compared to that of its flask (20, 30, 40, 50, and 60%); cultivation time (0, 1, 2, 3, 4, and 5 days).

Scaled up production of PG using a BioFlo/CelliGen 115 15 L bioreactor system (Eppendorf North America, Connecticut, US): The optimal culture conditions obtained from the above experiments were used for the investigation of PG production in mass using a 15 L bioreactor system, in which 450 mL of S. marcescens TNU02 was first fermented in a 1000 mL flask for 2 days and then injected into 4.05 L of a liquid culture medium (in the 15 L bioreactor system) containing a 1.6% C/N source (casein/de-CSP ratio of 3/7), 0.1% KH$_2$PO$_4$ and 0.02% (NH$_4$)$_2$SO$_4$, with an initial pH of 6.15. The sampling and determination of the PG concentration was performed every 2 h until 12 h of fermentation had passed.

3.2.2. Extraction, Purification and Qualification of PG

Qualification of the PG was performed as per the assay earlier presented by Wang et al., 2012 [30]. A mixture containing 4 mL methanol, 0.5 mL fermented medium broth, and 0.5 mL 2% AlK(SO$_4$)$_2$·12H$_2$O was centrifuged at 1400 × g for 5 min. The supernatant was collected and mixed with 0.5 N HCl in methanol at a ratio of 1/9 and then used for measuring the optical density at 535 nm. The purified PG provided from our previous study [11] was used to establish the standard for converting the optical density at 535 nm into the content of the PG.

The PG was purified from the fermented culture broth (FCB) using the protocol recently described in a previous study [32]. The supernatant harvested from the FCB by centrifugation at 10,000 × g for 15 min was mixed with ethyl acetate (EA) at a ratio of 1/1. This mixture was kept in a funnel for around 3 h, with shaking occurring every half hour. The EA layer containing red pigment PG was collected and dried to a powder by evaporation of the EA at 55 °C in an oven air drier. The crude PG powder was then separated via a silica open column Geduran® Si 60, size: 0.040–0.063 mm; Merck KGaA, Darmstadt, Germany) and the PG was eluted by using the gradient of solvents system of methanol in chloroform changed from the ratio of 0/10 to 2/8 (v/v). Finally, the PG was purified via the separation of PG on a TLC (thin layer chromatography) plate. After separation of PG on the TLC plate, the lane with red pigments on the TLC plate was cut into various small pieces, and the PG was then dissolved using methanol. The PG was dried to a powder by evaporation of the methanol in an oven air drier at 55 °C. This isolated PG was then used for the detection of MALDI-TOF MS, UV, and medical effects.

3.2.3. Biological Activity Assays

The purified PG obtained in this study was tested for its antioxidant and anticancer activities. The antioxidant activity was evaluated via DPPH and ABTS radical scavenging capacity assays. Of these, the DPPH radical scavenging capacity was performed following the method recently and described by Nguyen et al., 2020 [39], and the ABTS radical scavenging capacity assay was performed according to the assay presented by Arivizhivendhan et al., 2018 [35]. The anticancer activity was investigated following the protocol mentioned in detail in a previous report [40]. α-Tocopherol and Mitomycin were used as standard compounds for testing antioxidant and anticancer activities, respectively.

4. Conclusions

Demineralized crab shell powder (de-CSP) was reused for the cost-effective production of bioactive PG. In this study, PG was notably produced in a large scale (4.5 L per pilot bioreactor) with a high-level PG yield (5100 mg/L) in a short period of fermentation time (8 h). The PG was purified and then quantified by specific MALDI-TOF MS and UV spectra analysis. The PG produced
by S. marcescens TNU02 and isolated in this study demonstrated moderate antioxidant and effective anticancer activities. The results suggested that de-CSP could be a valuable material for mass and cost-effective PG production.

**Author Contributions:** Conceptualization, V.B.N. and S.-L.W.; methodology V.B.N.; software, V.B.N.; validation, S.-L.W., and V.B.N.; formal analysis, V.B.N., D.N.N., S.-L.W., A.D.N., T.Q.T., T.P.P., C.T.D., and T.P.H.T.; investigation, V.B.N., D.N.N., V.A.N.; resources, V.B.N. and S.-L.W.; data curation, S.-L.W., V.B.N.; writing—original draft preparation, V.B.N.; writing—review and editing, V.B.N. and S.-L.W.; visualization, V.B.N., A.D.N., S.-L.W.; supervision, S.-L.W., A.D.N., and V.B.N.; project administration, V.B.N. and S.-L.W. All authors have read and agreed to the published version of the manuscript.

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**References**

1. Setoguchi, T.; Kato, T.; Yamamoto, K.; Katokawa, J. Facile production of chitin from crab shells using ionic liquid and citric acid. *Int. J. Biol. Macromol.* 2012, 50, 861–864. [CrossRef]
2. Hamdi, M.; Hammami, A.; Hajji, S.; Jridi, M.; Nasri, M.; Nasri, R. Chitin extraction from blue crab (*Portunus segnis*) and shrimp (*Penaeus kerathurus*) shells using digestive alkaline proteases from *P. segnis visceras*. *Int. J. Biol. Macromol.* 2017, 101, 455–463. [CrossRef] [PubMed]
3. Oh, K.T.; Kim, Y.J.; Nguyen, V.N.; Jung, W.J.; Park, R.D. Demineralization of crab shell waste by *Pseudomonas aeruginosa F722*. *Process Biochem.* 2007, 42, 1069–1074. [CrossRef]
4. Wang, C.H.; Doan, C.T.; Nguyen, V.B.; Nguyen, A.D.; Wang, S.L. Reclamation of fishery processing waste: A mini-review. *Molecules* 2019, 24, 2234. [CrossRef] [PubMed]
5. Jun, J.Y.; Jung, M.J.; Jeong, I.H.; Kim, G.W.; Sim, J.M.; Nam, S.Y.; Kim, B.M. Effects of crab shell extract as a coagulant on the textural and sensorial properties of tofu (soybean curd). *Food Sci. Nutr.* 2019, 7, 547–553. [CrossRef]
6. Soundarapandian, P.; Shyamalendu, R.; Varadharajan, D. Antioxidant activity in hard and soft shell crabs of *Charybdis lucifera* (Fabricius, 1798). *J. Aquac. Res. Dev.* 2014, 5, 7. [CrossRef]
7. Leila, R.; Zahra, R.; Pegah, M.; Mozafar, K. Antiproliferatory effects of crab shell extract on breast cancer cell line (MCF7). *J. Breast Cancer* 2014, 17, 219–225.
8. Roldão, P.A.J.; Adão, L.B.M.; José, E.C.S.; Sergio, D.A.; José, M.R.L. Use of crab shell (*Ucides cordatus*) in Portland cement matrices. *J. Agric. Sci.* 2020, 12, 200–208.
9. Nguyen, V.B.; Wang, S.L. Reclamation of marine chitinous materials for the production of α-glucosidase inhibitors via microbial conversion. *Mar. Drugs* 2017, 15, 350. [CrossRef] [PubMed]
10. Wang, S.L.; Nguyen, V.B.; Doan, C.T.; Tran, T.N.; Nguyen, M.T.; Nguyen, A.D. Production and potential applications of bioconversion of chitin and protein-containing fishery byproducts into prodigiosin: A review. *Molecules* 2020, 25, 2744. [CrossRef]
11. Nguyen, V.B.; Nguyen, D.N.; Wang, S.L. Microbial reclamation of chitin and protein-containing marine by-products for the production of prodigiosin and the evaluation of its bioactivities. *Polymers* 2020, 12, 1328. [CrossRef] [PubMed]
12. Patricia, H.V.; Irene, M.A.; Melissa, R.D.; José Manuel, R.D.; Donato, L.M.; Francisco Guadalupe, A.A.; Juan Francisco, V.C. Photoelectric evaluation of dye-sensitized solar cells based on prodigiosin pigment derived from *Serratia marcescens* 11E. *Dyes Pigments* 2020, 177, 108278–108287.
13. Cheng, M.F.; Lin, C.S.; Chen, Y.H.; Sung, P.J.; Lin, S.R.; Tong, Y.W.; Weng, C.F. Inhibitory growth of oral squamous cell carcinoma cancer via bacterial prodigiosin. *Mar. Drugs* 2017, 15, 224. [CrossRef] [PubMed]
14. Anwar, M.M.; Shalaby, M.; Embaby, A.M.; Saeed, H.; Agwa, M.; Hussein, A. Prodigiosin/PU-H71 as a novel potential combined therapy for triple negative breast cancer (TNBC): Preclinical insights. *Sci. Rep.* 2020, 10, 14706. [CrossRef]
15. El-Batal, A.; El-Hendawy, H.; Faraag, A. In silico and in vitro cytotoxic effect of prodigiosin-conjugated silver nanoparticles on liver cancer cells (HepG2). *Biol. Technol.* 2017, 98, 225–243. [CrossRef]
16. Nisha; Kumar, K.; Kumar, V. Prodigiosin alkaloids: Recent advancements in total synthesis and their biological potential. *RSC Adv.* 2015, 5, 10899–10920. [CrossRef]
17. Montaner, B.; Navarro, S.; Piqué, M.; Vilaseca, M.; Martinell, M.; Giralt, E.; Gil, J.; Perez-Tomas, P. Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. *Br. J. Pharmacol.* 2000, 131, 585–593. [CrossRef]

18. Wang, X.; Tao, J.; Wei, D.; Shen, Y.; Tong, W. Development of an adsorption procedure for the direct separation and purification of prodigiosin from culture broth. *Biotechnol. Appl. Biochem.* 2004, 40, 277–280.

19. Gulani, C.; Bhattacharya, S.; Das, A. Assessment of process parameters influencing the enhanced production of prodigiosin from *Serratia marcescens* and evaluation of its antimicrobial, antioxidant and dyeing potential. *Malays. J. Microbiol.* 2012, 8, 116–122.

20. Kandra, P.; Challa, M.M.; Kalangi, P.J.H. Efficient use of shrimp waste: Present and future trends. *Appl. Microbiol. Biotechnol.* 2012, 93, 17–29. [CrossRef]

21. Wei, Y.H.; Yu, W.J.; Chen, W.C. Enhanced undecylprodigiosin production from *Serratia marcescens* SS-1 by medium formulation and amino-acid supplementation. *J. Biosci. Bioeng.* 2005, 100, 466–471. [CrossRef] [PubMed]

22. Wei, Y.H.; Chen, W.C. Enhanced production of prodigiosin-like pigment from *Serratia marcescens* UCP 1549 using renewable-resources as a low-cost substrate. *Molecules* 2010, 15, 6931–6940. [CrossRef]

23. De Casullo Araujo, H.W.; Fukushima, K.; Campos Takaki, G.M. Prodigiosin production by *Serratia marcescens* TKU011 in media containing squid pen. *Electron. J. Biotechnol.* 2019, 20, 58–64. [CrossRef]

24. Lin, C.; Jia, X.; Fang, Y.; Chen, L.; Zhang, H.; Lin, R.; Chen, J. Enhanced production of prodigiosin by *Serratia marcescens* FZSF02 in the form of pigment pellets. *Electron. J. Biotechnol.* 2019, 40, 58–64. [CrossRef]

25. Chang, C.C.; Chen, W.C.; Ho, S.F.; Wu, H.S.; Wei, Y.H. Development of natural anti-tumor drugs by microorganisms. *J. Biosci. Bioeng.* 2011, 111, 501–511. [CrossRef]

26. Wei, Y.H.; Chen, W.C. Enhanced production of prodigiosin-like pigment from *Serratia marcescens* SS-1 by medium improvement and oil-supplementation strategies. *J. Biosci. Bioeng.* 2005, 99, 616–622. [CrossRef]

27. Giri, A.V.; Anandkumar, N.; Muthukumaran, G.; Pennathur, G. A novel medium for the enhanced cell growth and production of prodigiosin from *Serratia marcescens* isolated from soil. *BMC Microbiol.* 2004, 4, 11. [CrossRef]

28. Elkenawy, N.M.; Yassin, A.S.; Elhifnawy, H.N.; Amin, M.A. Optimization of prodigiosin production by *Serratia marcescens* using crude glycerol and enhancing production using gamma radiation. *Biotechnol. Rep.* 2017, 14, 47–53. [CrossRef]

29. Wang, S.L.; Chen, S.Y.; Yen, Y.H.; Liang, T.W. Utilization of chitinous materials in pigment adsorption. *Food Chem.* 2012, 135, 1134–1140. [CrossRef] [PubMed]

30. Wang, S.L.; Wang, C.Y.; Yen, Y.H.; Liang, T.W.; Chen, S.Y.; Chen, C.H. Enhanced production of insecticidal prodigiosin from *Serratia marcescens* TKU011 in media containing squid pen. *Process. Biochem.* 2012, 47, 1684–1690. [CrossRef]

31. Liang, T.W.; Chen, S.Y.; Chen, Y.C.; Chen, Y.C.; Yen, Y.H.; Wang, S.L. Enhancement of prodigiosin production by *Serratia marcescens* TKU011 and its insecticidal activity relative to food colourants. *J. Food Sci.* 2013, 78, 1743–1751. [CrossRef] [PubMed]

32. Nguyen, V.B.; Nguyen, T.H.; Nguyen, M.T.; Tran, T.T.; Doan, C.T.; Tran, T.N.; Nguyen, A.D.; Kuo, Y.H.; Wang, S.L. Novel efficient bioprocessing of marine chitins into active anticancer prodigiosin. *Mar. Drugs* 2020, 18, 15. [CrossRef] [PubMed]

33. Lobo, V.; Patil, A.; Phatak, A.; Chandra, N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev.* 2010, 4, 118–126. [CrossRef] [PubMed]

34. Muthukumar, A.; Pradeep, P.; Thigale, I.; Mohanasrinivasan, V.; Jemimah, N.S.; Devi, C.S. Exploring the bioactive potential of *Serratia marcescens* VITAPI (Acc: 1933637) isolated from soil. *Front. Biol.* 2016, 11, 476–480. [CrossRef]

35. Arivizhivendhan, K.V.; Mahesh, M.; Boopathy, R.; Swarnalatha, S.; Regina Mary, R.; Sekaran, G. Antioxidant and antimicrobial activity of bioactive prodigiosin produces from *Serratia marcescens* using agricultural waste as a substrate. *J. Food Sci. Technol.* 2018, 55, 2661. [CrossRef] [PubMed]

36. Yip, C.; Yarkoni, O.; Ajjoka, J.; Wan, K.; Nathan, S. Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin. *Appl. Microbiol. Biotechnol.* 2019, 103, 1667–1680. [CrossRef] [PubMed]

37. Nguyen, V.B.; Wang, S.L.; Nguyen, T.H.; Nguyen, T.H.; Trinh, T.H.T.; Nong, T.T.; Nguyen, T.U.; Nguyen, V.N.; Nguyen, A.D. Reclamation of rhizobacteria newly isolated from black pepper plant roots as potential biocontrol agents of root-knot nematodes. *Res. Chem. Intermed.* 2019, 45, 5293–5307. [CrossRef]
38. Wang, S.L.; Yeh, P.Y. Production of a surfactant- and solvent-stable alkaliphilic protease by bioconversion of shrimp shell wastes fermented by Bacillus subtilis TKU007. Process Biochem. 2006, 41, 1545–1552. [CrossRef]

39. Nguyen, V.B.; Ton, T.Q.; Nguyen, D.N.; Nguyen, T.T.; Ngue, T.N.; Nguyen, T.H.; Doan, C.T.; Tran, T.N.; Nguyen, M.T.; Ho, N.D.; et al. Reclamation of beneficial bioactivities of herbal antioxidant condensed tannin extracted from Euonymus laxiflorus. Res. Chem. Intermed. 2020, 46, 4751–4766. [CrossRef]

40. Kuo, Y.H.; Liang, T.W.; Liu, K.C.; Hsu, Y.W.; Hsu, H.C.; Wang, S.L. Isolation and identification of a novel antioxidant with antitumor activity from Serratia ureilytica using squid pen as fermentation substrate. Mar. Biotechnol. 2011, 13, 451–461. [CrossRef]

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