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# Bioproduction of Prodigiosin from Fishery Processing Waste Shrimp Heads and Evaluation of Its Potential Bioactivities

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**Abstract:** The aim of this work was to reuse a fish processing waste, shrimp head powder (SHP), for the production of prodigiosin (PG) via microbial technology and to assess its potential bioactivities. PG was produced in a 12 L-bioreactor system, and the highest PG productivity of 6310 mg $L^{-1}$ was achieved when *Serratia marcescens* CC17 was used for fermentation in a novel designed medium (6.75 L) containing 1.5% C/N source (SHP/casein = 9/1), 0.02% $K_2SO_4$, ans 0.025% $Ca_3(PO_4)_2$, with initial pH 7.0, and fermentation was performed at 28 °C for 8 h. The purified PG showed moderate antioxidants, efficient anti-NO (anti-nitric oxide), and acetylcholinesterase (AChE) inhibitory activities. In a docking study, PG showed better binding energy scores (−12.3 kcal/mol) and more interactions (6 linkages) with several prominent amino acids in the biding sites on AChE that were superior to those of Berberine chloride (−10.8 kcal/mol and one linkage). Notably, this is the first investigation using shrimp heads for the mass bioproduction of PG with high productivity, and $Ca_3(PO_4)_2$ salt was also newly found to significantly enhance PG production by *S. marcescens*. This study also provided available data on the anti-NO and anti-AChE effects of PG, especially from the docking simulation PG towards AChE that was described for the first time in this study. The above results suggest that SHP is a good material for the cost-effective bioproduction of PG, which is a potential candidate for anti-NO and anti-Alzheimer drugs.

**Keywords:** fish industry waste; shrimp exoskeleton; marine chitinous wastes; microbial technology; prodigiosin; antioxidants; anti-Alzheimer activity; docking study

## 1. Introduction

The fish and shrimp processing industry has been producing large amounts of by-products, with accessible fish waste reported to be 27.85 million tons per year on a global scale [1]. In current marine science, aspects of recycling the marine discards to cost effectively prepare valuable products is an emerging research topic [2–5]. Among the fishery discards, chitinous marine waste, such as squid pens, crab shells, shrimp shells, and shrimp

heads, are obtained as fish industry waste that have received extensive research interest for the extraction and production of various valuable bioactive compounds via microbial fermentation in recent years [2]. Of these chitinous marine discards, shrimp exoskeleton is one of the most abundant chitinous wastes [6]. Shrimp exoskeleton is widely used for chitin/chitosan production via chemical processing [7] and to obtain several bioactive compounds, including pigments such as astaxanthin, astaxanthin and β-carotene esters [8], and essential amino acids [9]. Shrimp exoskeleton has been investigated for the production of numerous valuable active products via microbial and enzymatic fermentations, such as chitin, protein recovery [10,11], some enzymes such as proteases, chitosanases and exochitinases [11–13], chitosan oligosaccharides [12], *N*-acetyl-D-glucosamine [13], antidiabetic compounds [6], and dye adsorbent agents [11]. This low-cost material was also used as fish meal [14]. In this study, we approached the reuse of shrimp heads in the large-scale production of prodigiosin (a microbial pigment compound) via microbial fermentation and investigated its potential bioactivities.

Recently, microbial pigments have been widely investigated for their production and potential applications [15–17]. Of these, prodigiosin (PG), a red pigment belonging to the prodiginine family of compounds, has been extensively studied and assessed in numerous reorts [17]. This metabolite is mainly produced by *Serratia marcescens* and has been recognized for showing various bioactivities with many applications in medicine, food colorants, cosmetics, candles, textiles, and solar cells as well as in biocontrol in agriculture [17–24]. Due to various benefits of PG, the number of studies on PG production has increased dramatically in recent years [25]. However, in most previous studies, PG was biosynthesized using a commercial nutrient broth, such as yeast extract, casein, tryptone soy, yeast malt, tryptone yeast, glycerol-tryptone, Luria/Bertani broth, or peptone-glycerol [17,26–31]. For the low-cost production of PG, some nontraditional culture media such as cassava, sesame oil, peanut oil, coconut oil, copra seed, sesame seed, peanut seed, corn steep, crude glycerol, mannitol/cassava, and mannitol/corn steep have been used in the fermentation process [32–36]. Recently, it has also been claimed that various processing wastes may be used as potential C/N soures for PG production with a high yield via microbial fermentation [15].

Regarding environmental issues and the cost-effectiveness of the production process, we have investigated the reuse of chitinous marine discards for the biosynthesis of several bioactive compounds [6,11–13,37–41] and of PG [25,42,43]. Our previous study indicated that marine chitins play important roles in enhancing PG yield and that α-chitin was more effective than β-chitin [44]. Of the chitinous marine discards, squid pen, crab shell, and shrimp shell have been utilized for PG production by microbial conversion [25,42,43]. However, shrimp head, one of the most abundant chitinous wastes containing α-chitin, has not been investigated for the bioproduction of PG. In this work, we aim to demonstrate the bioprocessing of shrimp heads into PG with a cost-effective production process. The study addresses several major issues, including the optimization of culture conditions for PG biosynthesis in small scale (in a flask) production (1), in the scale-up of PG production to a 12 L-bioreactor system (2), and purification and evaluation of the potential bioactivities of PG (3). A docking study was also applied to clarify the mechanism of enzyme inhibitor targeting anti-Alzheimer drugs (4).

## 2. Results and Discussion

### *2.1. Establishment of Production Process of PG in Small Erlenmeyer Flask Scale*

#### 2.1.1. Biosynthesis of PG by Different Bacterial Strains of *Serratia marcescens*

Our earlier studies have indicated that different PG producing *S. marcescens* strains may produce PG with different yields using the same C/N source and fermentation conditions [25,42,43]. Thus, to screen for a suitable *S. marcescens* strain to effectively convert SHP into PG, a total for strains of *S. marcescens* (TUK011, TNU01, TNU02, and CC17) were tested. As shown in Table 1, the culture broth fermented by *S. marcescens* CC17

gave the highest PG productivity (3.862 mg/mL). Thus, this bacterial strain was used as a PG–producing strain in further investigations.

**Table 1.** The conversion of SHP into PG by various bacterial strains of *S. marcescens*.

| No. | PG Producing-Strain | PG Yield Detected in Fermented Medium (mg $L^{-1}$) |
|---|---|---|
| 01 | *S. marcescens* TKU011 | 2.43 ± 0.078 |
| 02 | *S. marcescens* TNU01 | 2.5746 ± 0.142 |
| 03 | *S. marcescens* TNU02 | 2.711 ± 0.136 |
| 04 | *S. marcescens* CC17 | 3.862 ± 0.145 |
| | Control | PG not detected |

*S. marcescens* CC17 is a rhizobacteriumoriginating from Vietnamese soil in our earlier report [45]. The CC17 strain was also investigated for PG production in our several previous studies. However, this bacterium showed a moderate PG–producing ability in the previous report, with a PG yield of 3.562, 2.73, and 2.1 mg/mL in media containing 1.6% (casein/de-SSP = 3/7), 1.6% (casein/de-CSP = 3/7), and 1.5% SPP [43], respectively, as the C/N sources. In this study, *S. marcescens* CC17 produced PG with a significant yield of 3.862 mg/mL. Thus, it may be suggested that SHP is a valuable C/N source for the conversion to PG by *S. marcescens* CC17.

#### 2.1.2. Effect of Free Protein and Salt Compositions Added into Culture Medium on PG Production by *S. marcescens* CC17

Casein was investigated as a suitable free protein source [25,42,44]. Thus, this protein was mixed with SHP at various proportions and was used as C/N source for the cultivation of the *S. marcescens* CC17 strain. As shown in Figure 1a, the ratios SHP/casein of 8/2 and 9/1 were suitable substratesthat were able to produce a high PG yield. Regarding a cost-effective PG production, the SHP/casein mix in 9/1 proportions was the best choice for further investigation. In several previous studies, casein at different concentrations was also added to a medium containing marine chitins to enhance PG production by *S. marcescens* strains, such as α-chitin/casein = 5/3 [44], demineralized crab shell/casein = 7/3 [42], and demineralized shrimp shell/casein = 7/3 [25], resulting in PG yields of 3.23, 3.50, and 4.18 mg/mL, respectively. In the current work, this commercial free protein was added to (3.911 mg/mL) to that previous of work (3.23–4.18 mg/mL). The medium in a minor amount (SHP/casein = 9/1), and PG was produced at a similar level.

In *S. marcescens* fermentation to biosynthesize PG, supplementary phosphate and sulfate salts are required to enhance PG yield [21,44]. To reach a higher PG productivity in biosynthesis, the culture broth was supplemented with many kinds of phosphate and sulfate salts and was then fermented by CC17 strain. The results indicate that $Ca_3(PO_4)_2$ was a most suitable phosphate salt (Figure 1b), and its optimal added concentration was at 0.025% (Figure 1c). In the next experiments (Figure 1d), $K_2SO_4$ demonstrated potential as a sulfate salt for *S. marcescens* CC17 by producing the highest PG level, and its optimal added concentration was 0.02%. Similarly, $K_2SO_4$ was a potential sulfate salt source for PG enhancement at the same added concentration of 0.02% as in prior work [25]. In various reported studies, $K_2HPO_4$ was found to be a suitable phosphate salt source playing a significant role in maximizing the PG yield [21,25,42–44]. However, $Ca_3(PO_4)_2$ was newly investigated for its significant effects on PG productivity when biosynthesized by *S. marcescens* in this current report. Based on the reviewed literature, the phosphate salts and their addition levels play an important role in Prodigiosin production by *S. marcescens* [21,43,44]. However, the mechanism of this effect remains unclear.

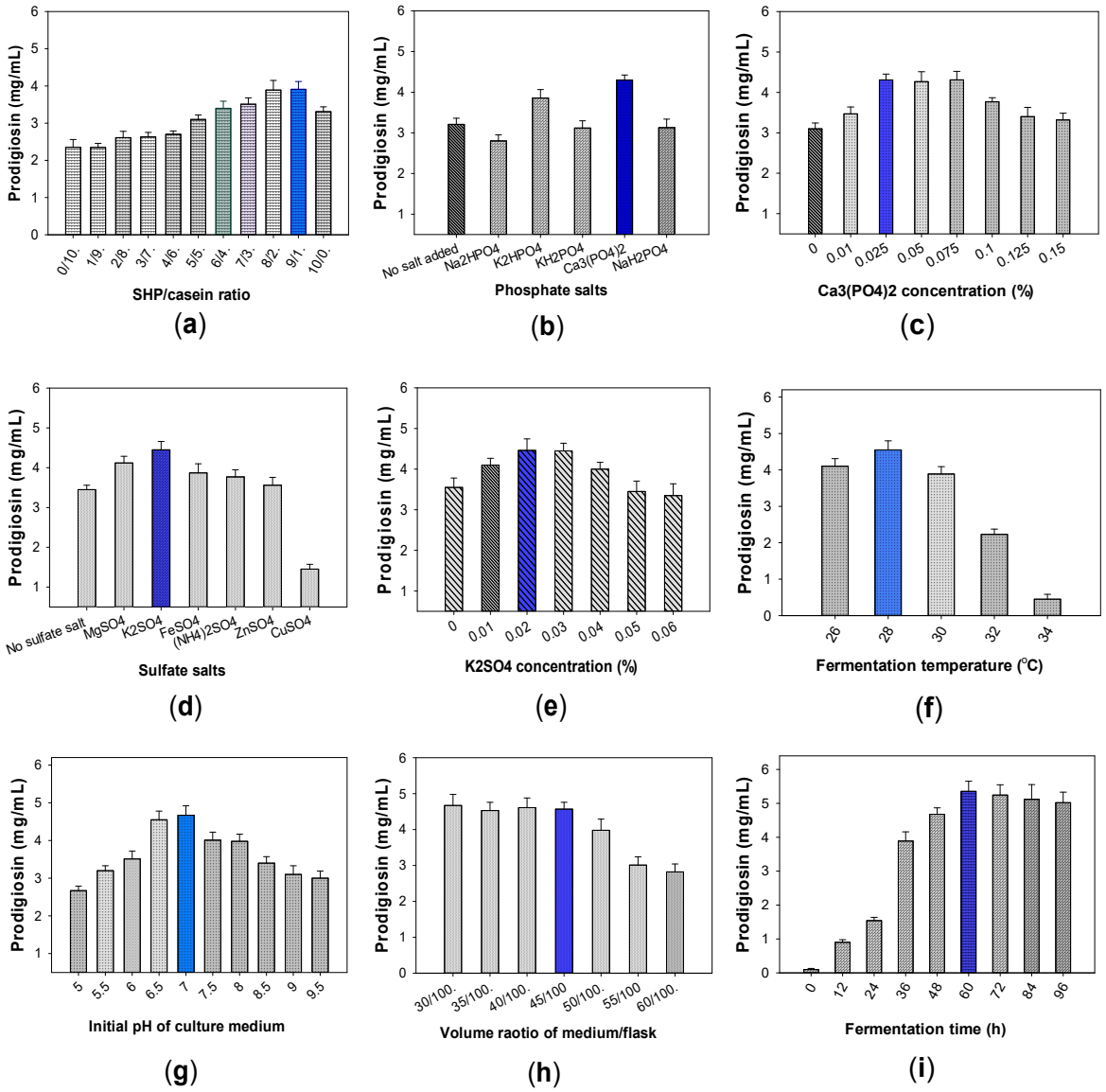



**Figure 1.** The effect of the SHP/casein ratio (**a**), sources of phosphate salt (**b**), $Ca_3(PO_4)_2$ concentration (**c**), sources of sulfate salt (**d**), $K_2SO_4$ concentration (**e**), temperature of cultivation (**f**), the starting pH of culture broth (**g**), volume ratio of culture broth/flask (**h**), and the time duration of cultivation (**i**). Standard errors (SE) are shown by the error bars in all the figures. The blue bars indicate the parameters chosen for further experiments.

#### 2.1.3. The Effect of Fermentation Parameters on PG Productivity by CC17 Strain

To achieve more effective PG production via fermentation, some fermentation parameters such as fermentation temperature (26, 28, 30, 32. and 34 °C), the pH of culture broth (pH in the range of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 9.5), the volume ratio of the liquid medium/flask (30/100, 35/100, 40/100, 45/100, 50/100, 55/100, and 60/100 mL/mL), and the fermentation time (0–96 h) were investigated for their effects on PG productivity (Figure 1f–i). Overall, PG synthesized by *S. marcescens* CC17 reached the highest yield (5.355 mg/mL) in the novel designed culture broth containing 1.5% C/N source (SHP/casein = 9/1), 0.02% $K_2SO_4$, and 0.025% $Ca_3(PO_4)_2$ at and initial pH 7.0 and with culture volume of 45 mL medium in a 100 mL flask. The fermentation was conducted at 28 °C for 60 h. Notably, the PG yield significantly increased by approximately 1.4-fold from 3.862 to 5.355 mg/mL after the optimization of the culture conditions.

### *2.2. Scale-Up of PG Production to a 12 L-Bioreactor System*

Several ways to ferment have been reported for PG biosynthesis, such as immobilized cultures, batch cultivation, fed-batch cultivation, continuous cultivation, and cultivation using various systems of bioreactors [25]. Of these, a bioreactor system was found to be a strong tool for the mass bioproduction of PG (large scale production) with high-level productivity and performance in a short cultivation duration [42]. To approach the goal of biosynthesis of PG at a mass scale, the novel designed medium obtained in the previous experiments (in Section 2.1 of this study) was used for fermentation in the 12 L-bioreactor. The results (Figure 2) show that PG was induced by *S. marcescens* CC17 with the PG yield increasing with the fermentation time and reaching the maximal PG yield (6310 mg $L^{-1}$) after 8 h of cultivation. Compared to fermentation in the small flask scale (Figure 1i), PG was produced at a higher yield at the larger scale. Notably, PG was induced with the highest yield during a much shorter cultivation time. In recent years, the studies on PG biosynthesis have dramatically increased [17]. However, in most prior studies, PG was produced from a commercial nutrient medium at a small scale (Erlenmeyer flasks). In contrast, we established bioprocessing to produce PG from low-cost shrimp discard feedstock at a large scale, by using a 12 L-bioreactor.

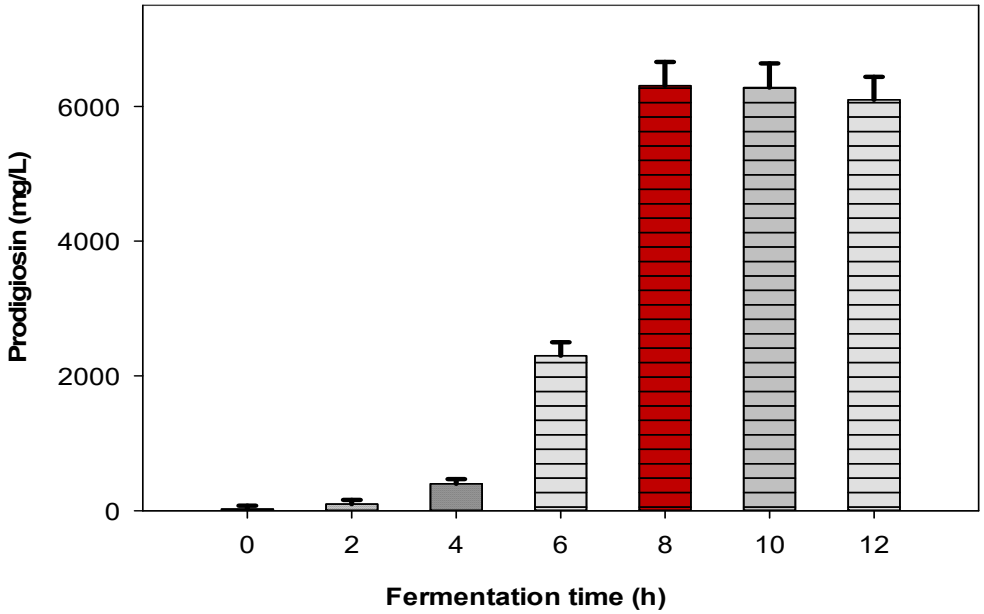



**Figure 2.** Scale-up of PG production to a 12 L-bioreactor system. Standard errors (SE) are shown as error bars. The red bar indicates that PG reached maximal yield at 8 h of fermentation.

To date, beyond some our previous reports [25,42,43], several studies have also achieved mass PG production using various bioreactors systems. This pigment compound was biosynthesized from a mixture of commercial nutrients in large scale of 1.5 L, 5 L, 7 L, and 100 L-bioreactor systems with true working culture medium volumes of 0.935 L, 2.7 L, 6.5 L, and 50 L, respectively. The optimal recorded fermentation times are in the range 20–65 h with moderate PG yields of 521.64–872 mg $L^{-1}$ [46–49]. In the current work, *S. marcescens* CC17 mass produced PG in a 12 L-bioreactor system with a true culture medium volume of 6.75 L with the notably high PG yield of 6310 mg $L^{-1}$ and with the much shorter fermentation time of 8 h than those of previous reports [46–49].

### *2.3. The Purification of PG in Culture Broth*

In this study, PG was purified via two simple steps (Figure 3). First, the culture broth fermented by *S. marcescens* CC17 in 12 L-bioreactor (Figure 3a) was mixed with ethyl acetate for layer separation (Figure 3b), and the ethyl acetate layer rich in red pigment was dried to powder (using rotary evaporator and hot air oven dry) and was loaded on to a silica open

column to isolate red pigment compound by elution using methanol and chloroform at the mixture ratios from 0/10 to 2/8 (*v*/*v*) (Figure 3c). The purified pigment was confirmed as PG via HPLC, UV/vis, and mass spectral analyses (Figures 4 and 5).

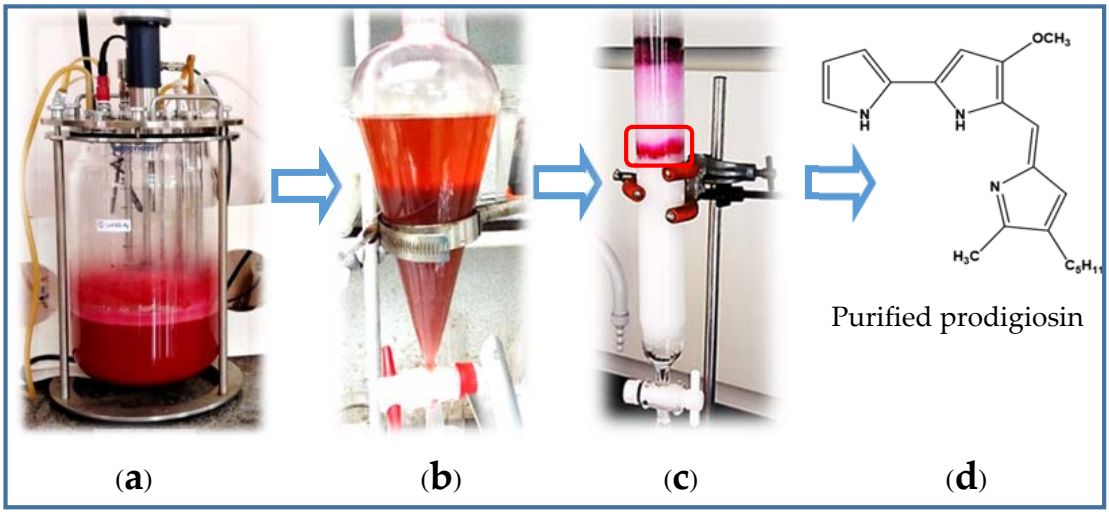
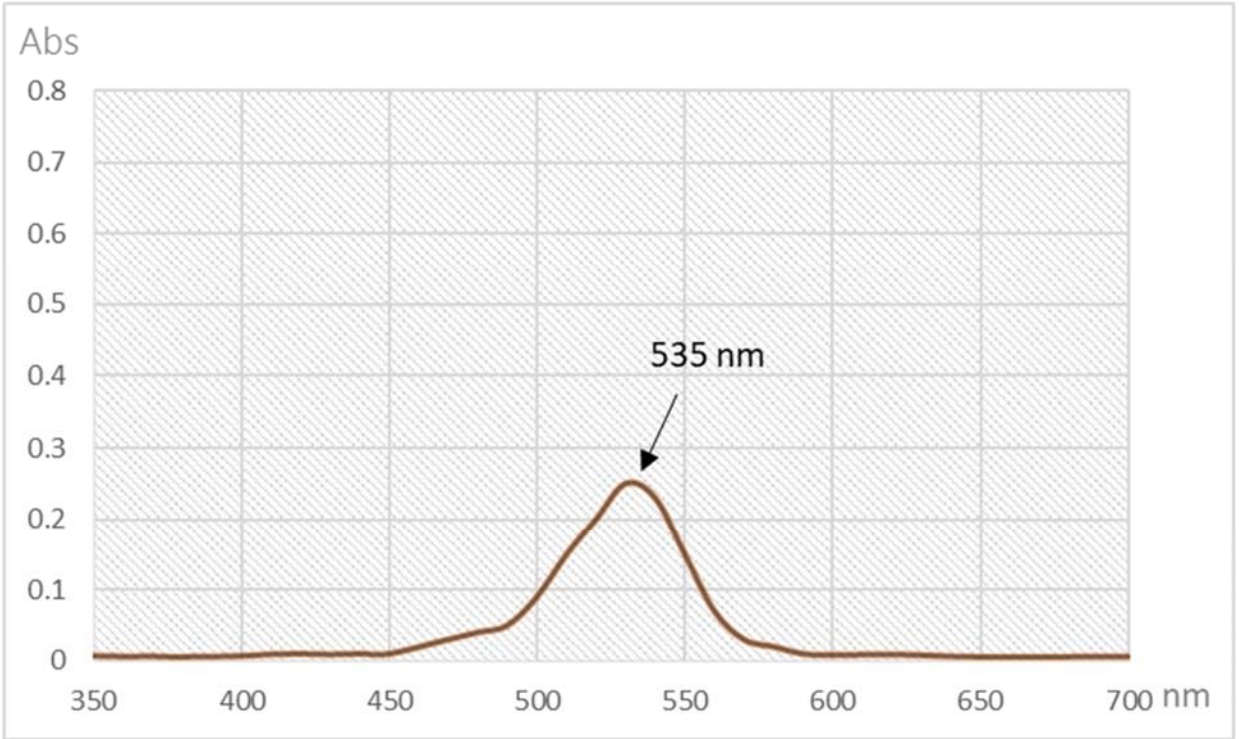
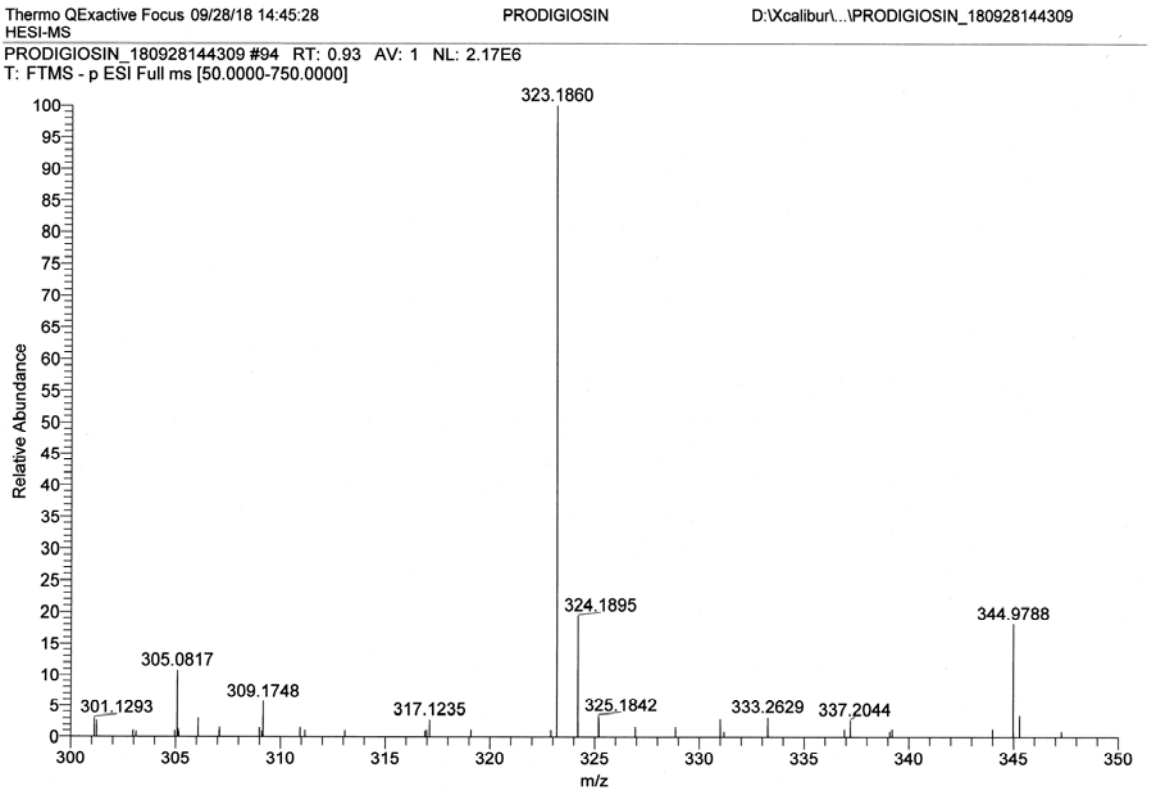



**Figure 3.** The purification of PG. The red pigment compound in the culture broth after 8 h of fermentation in a 12 L-bioreactor system (**a**) was first extracted by ethyl acetate (**b**) and was then isolated to pure compound in a silica gel separation column (**c**). The chemical structure of PG (**d**).



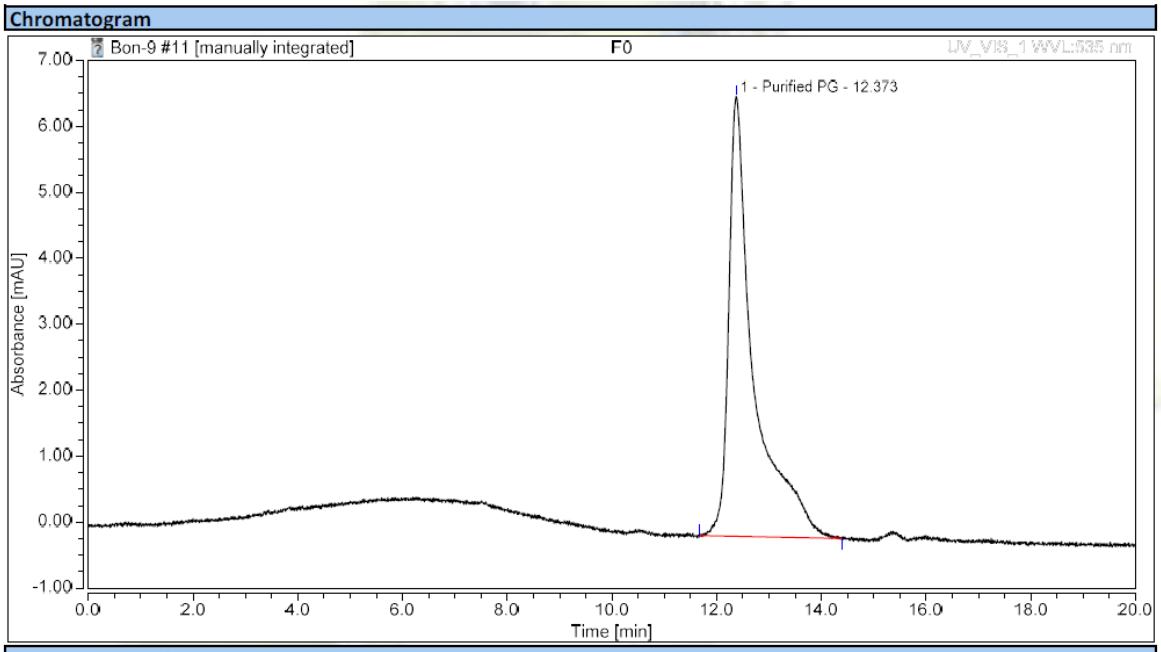

(**a**)

**Figure 4.** *Cont.*




(**b**)

**Figure 4.** The UV/vis spectrum (**a**), and the mass (M = 323.186) (**b**) of the red pigment compound biosynthesized in this study.




**Integration Results**

| No. | Peak Name | Retention Time min | Area mAU*min | Height mAU | Relative Area % | Relative Height % | Amount |
|---|---|---|---|---|---|---|---|
| 1 | Purified PG | 12.373 | 3.684 | 6.674 | 100.00 | 100.00 | n.a. |
| **Total:** | | | **3.684** | **6.674** | **100.00** | **100.00** | |

**Figure 5.** The high-performance liquid chromatography (HPLC) fingerprint of purified prodigiosin (PG) biosynthesized by *S. marcescens* CC17 in this study.

As presented in Figure 4, this pigment metabolite possessed a max absorption wavelength at 535 nm and a molecular weight of 323.186 g/mol, and these recorded spectra are in agreement with those of PG in previous reports [42–44,50]. In addition, in its HPLC fingerprint, this purified PG appeared as a major single peak at the retention time (RT) of 12.373. This RT of purified PG was approximately similar to that of PG in the previous report with RT of 12.283–12.40 [25]. Therefore, the purified red pigment was identified as PG. The HPLC spectrum also indicated that the PG compound was isolated with high grade purity, and therefore, it was used to test biological activities in the following experiments.

### *2.4. Detection of Biological Activities of Purified PG*

PG has a vast array of biological activities with numerous applications in medicine, food colorants, cosmetics, candles, textiles, solar cells, and biocontrol in agriculture [17–24]. To confirm that the PG biosynthesized and purified in this work is active, we tested its antioxidant and anti-NO activities. The potential enzyme inhibitory effects related to the anti-Alzheimer efficacy of PG were also evaluated. The experimental data are illustrated in Figure 6.

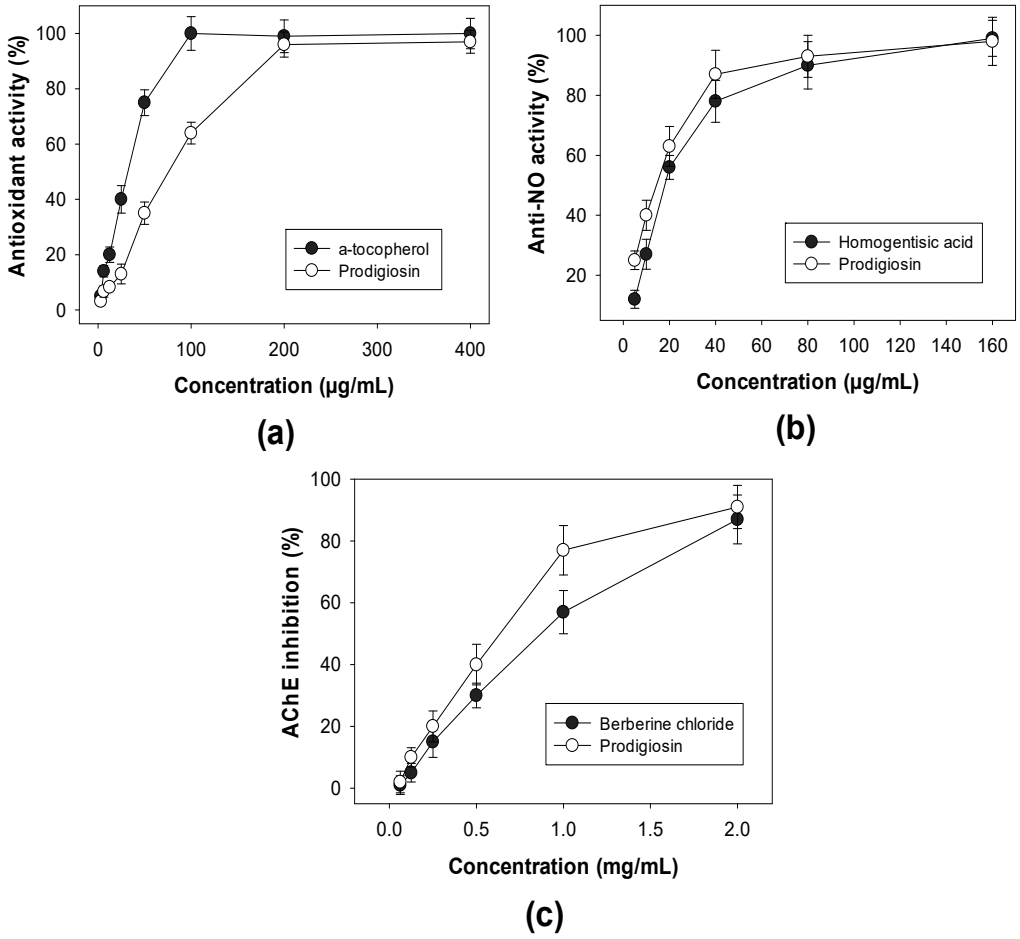



**Figure 6.** Bioactivities of prodigiosin (PG) including antioxidant activity (**a**), anti-NO activity (**b**), and acetylcholinesterase (AChE) inhibitory activity (**c**). Standard errors (SE) are shown as error bars in all the figures.

Antioxidant agents have been recognized to protect several important types of molecules (proteins, deoxyribonucleic acids, and lipids) from being damaged by free radicals, and thus, antioxidants may play important roles in reducing and preventing various diseases [51]. To evaluate the anti-oxidant effects of PG, the DPPH radical scavenging activity was assayed. As seen in Figure 6a, PG showed potent max DPPH radical scavenging activ-

ity of 97% at 200 μg/mL, which is comparable to α-tocopherol, a commercial antioxidant compound (99%) at 100 μg/mL. The antioxidant activities of PG and α-tocopherol were further converted to $IC_{50}$ values. While α-tocopherol displays an excellent antioxidant effect with a low $IC_{50}$ of 32.5 μg/mL, PG processes a higher $IC_{50}$ of 77.4 μg/mL. Therefore, compared to α-tocopherol, PG was found to show moderate antioxidant activity. The antioxidant effect of PG has been previously reported to have effective max inhibition in the range of 86–99% [25,42,43,52]. Though showing potent max antioxidant activity, PG was also determined to be a moderate antioxidant compound due its high $IC_{50}$ values of 79.1 μg/mL [43], 235 μg/mL [25], and 2640 μg/mL [43].

Anti-nitric oxide activity was used as an indication of pro-inflammatory property related to inflammatory disorders [53]. In the current work, we used LPS-stimulated-RAW264.7 cells for the detection of anti-NO activity of PG. The experimental data in Figure 6c show that PG displays effective anti-nitric oxide activity. This purified pigment displays great max anti-NO effect of 99% and a low $IC_{50}$ value of 16.22 μg/mL, which is comparable to that of homogentisic acid, a commercial anti-NO agent (98% and 14.6 μg/mL). To date, the medical effects of PG have been increasingly reported upon, its anti-cancer properties, especially [44]. However, only little data on anti-NO effects have been reported beyond the previous study [25]. Thus, the data on the bioactivity obtained in this study support improved assessments of the anti-NO effects of PG.

The inhibitors of acetylcholinesterase (AChE) have been proven as the main class of drugs for Alzheimer's disease treatment; this disease is the main cause of dementia [54]. In this report, we evaluate the potential anti-Alzheimer's activity of PG via testing its in vitro inhibition against acetylcholinesterase and via a docking study. As shown in Figure 6c, PG was found to be an effective AChE inhibitor with a max inhibition of 91% and a low $IC_{50}$ of 0.64 mg/mL. To clarify the potential AChE inhibition by PG, berberine chloride (BC), a commercial AChE inhibitor, was tested for comparison, and it showed lesser activity (87% and 0.871 mg/mL) than PG. Up to now, little data on AChE inhibition by PG have been reported. Only one previous study reported the AChE inhibitory activity of PG, showing a moderate max AChE inhibition (62%) and a high $IC_{50}$ (1.12 mg/mL) [43]. In contrast, the PG compound biosynthesized by *S. marcescens* CC17 and isolated in two steps (Figure 3) with high purity (Figure 5) was found to be a potent AChE inhibitor with a greater max AChE inhibition (91%) and a lower $IC_{50}$ value (0.64 mg/mL). The interaction and affinity of PG towards AChE enzyme has not been described, and a numerical simulation study was performed to elucidate these important points, as discussed below.

### *2.5. Docking Study*

To understand the potential AChE inhibitory activity of PG, molecular docking studies were also performed to elucidate the interactions and affinity between these two inhibitor compounds (PG and BC) and the target enzyme AChE. Before docking performance, the ligands and protein enzyme were prepared using MOE-2015.10 software, and the molecular structures are shown in Figure 7.

As shown in Figure 7 based on the output of MOE-2015.10, PG exists in the two forms of cation-PG (Figure 7a) and neutral-PG (Figure 7b) in 78% and 22% proportions, while BC only exists in its neutral form (neutral-BC, Figure 7c) at virtual pH 8.0. Thus, these ligands (cation-PG, neutral-PG, and neutral-BC) were investigated for their binding energies and interactions with the target protein AChE (Figure 7d). In enzyme docking studies, inhibitor compounds may interact and bind to the target enzyme proteins at numerous binding sites (BSs). Thus, normally, only the one BS possessing the lowest biding energy is chosen for description in detail [55]. The docking results are summarized in Table 2 and are illustrated in Figure 8.

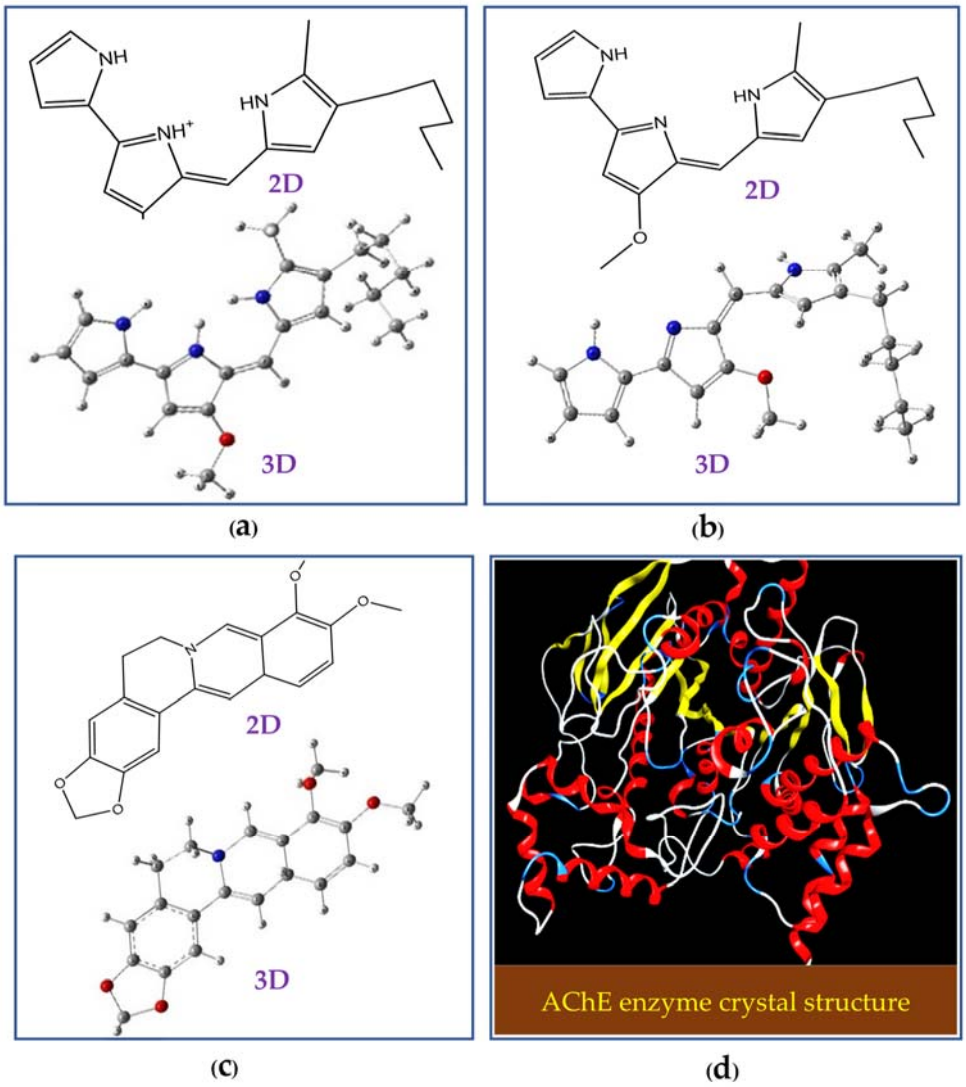



**Figure 7.** The 2D and 3D structures of cation-PG (**a**), neutral-PG (**b**), neutral-BC (**c**), and the AChE enzyme crystal structure (**d**).

**Table 2.** The results of the docking simulation between cation-PG, neutral-PG, and neutral-BC with the target protein (AchE).

| Compound Form (Inhibitor) | Symbol (Inhibitor-Enzyme) | DS (kcal/mol) | RMSD (Å) | Number of Interactions (Bonds) | Amino Acids Interacting with the Ligand (Inhibitor Compound) |
|---|---|---|---|---|---|
| Cation-PG | CPG–AchE | −12.3 | 1.35 | 6 bonds (1 H-donor, 3 ionic, and 2 pi-H) | Asp 326 (3.2 Å), Asp 326 (3.89 Å), Asp 393 (2.75 Å), Asp 393 (3.87 Å), Lys 325 (4.13 Å), Asp 393 (3.62 Å) |
| Neutral-PG | NPG–AchE | −11.1 | 1.75 | 3 bonds (1 H-pi, and 2 pi-H) | Trp 84 (3.94 Å), Trp 84 (4.51 Å), Gly 118 (3.85 Å) |
| Neutral-BC | NBC–AchE | −10.8 | 1.93 | 1 bond (1 H-pi) | Trp 84 (4.62 Å) |

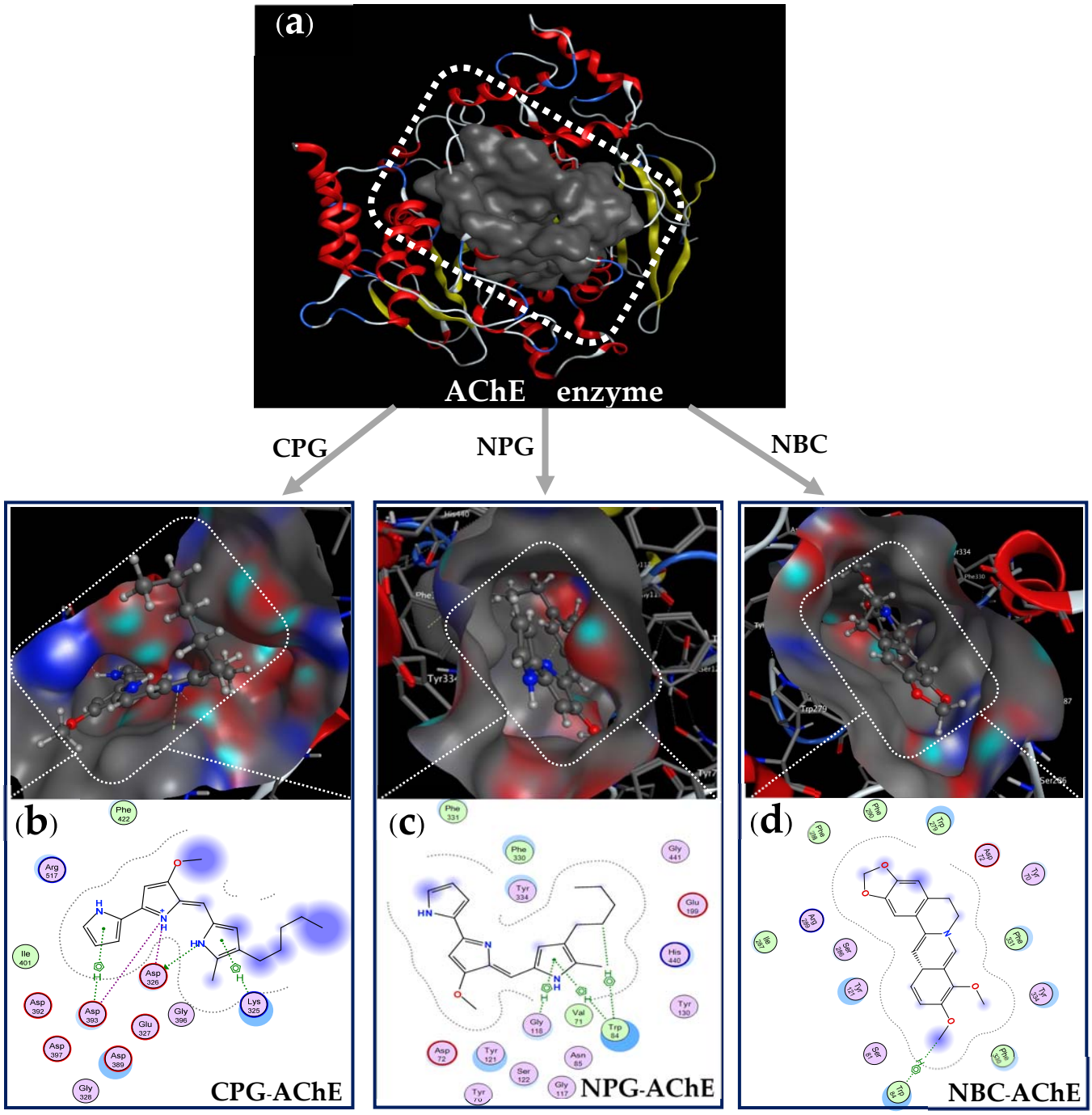



**Figure 8.** The 3D structures of binding site on AChE enzyme (**a**). The interactions between the ligands (cation-PG, neutral-PG, and neutral-BC) and the AChE enzyme, labeled as CPG-AChE (**b**), NPG-AChE (**c**), and NBC-AChE (**d**).

In the docking simulations, the binding energy was considered to be the major indicator for comparing the inhibitory activities of the compounds towards the target enzyme. The lower the associated docking score (DS) that an inhibitor possesses, the greater inhibition it displays. When a ligand interacts and binds to an enzyme with a DS lower than −3.20 kcal/mol, this indicates that it has good binding capacity towards the enzyme [56,57]. Moreover, Root Mean Square Deviation (RMSD) is also considered important in the in silico approach. When this exceeds 3.0 Å, the predicted inhibition is not significant. The results of docking are widely accepted when the RMSD stays below 2 Å [57,58]. In this study, all the three tested ligands interact and bind to the AChE enzyme with the recorded

RMSD values in the range 1.35–1.93 Å. This shows that all the ligands successfully interact with the binding site of the enzyme, so the docking threshold success is accepted [57,58]. Based on the docking score, all of these ligands (cation-PG, neutral-PG, and neutral-BC) demonstrate good inhibition effect due to their very low DS values of −12.3, −11.1, and −10.8, respectively. In the comparison based on the DS value, the inhibition effect of these ligands has the rank order Cation-PG > Neutral-PG > Neutral-BC. In particular, both forms of PG appear to be possibly more effective enzyme inhibitors than the commercial compound. These virtual data are in agreement with the experimental results (in vitro activity test, Figure 6c).

To better understand the interactions at the biding sites of the ligands towards the target enzyme, the simultaneous interactions are also presented in Figure 8. The biding site (BS) on the AchE (Figure 8a) contains up to 31 amino acids: Gln 69–Tyr 70–Val 71–Asp 72–Gln 74–Ser 81–Trp 84–Asn 85–Pro 86–Gly 117–Gly 118–Gly 119–Tyr 121– Ser 122–Tyr 130–Glu 199–Ser 200–Trp 279–Leu 282–Ser 286–Ile 287–Phe 288–Arg 289–Phe 290–Phe 330–Phe 331–Tyr 334–Gly 335–His 440–Gly 441–Ile 444, based on the output from MOE-2015.10.

As shown in Figure 8b, the ligand Cation-PG was found to be the most effective in binding to the BS on AChE via interacting with some prominent amino acids, including Asp 326, Asp 393, and Lys 325, resulting in six linkages being created. Of these, Asp 326 was found creating an H-donor and an ionic bond with the N2 and the N3 of the cation-PG with the recorded distances of 3.2 Å and 3.89 Å, respectively. The amino acid Asp 393 created two ionic and one pi-H bond with the N3, N3, and 5-ring of the cation-PG with recorded distances of 2.75, 3.87, and 3.62 Å, respectively. In addition, the 5-ring of the cation-PG was found forming one linkage pi-H with Lys 325 with distance of 4.13 Å.

The ligand neutral-PG was ranked for lower binding efficacy to the target enzyme than Cation-PG. The docking study presented in Figure 8c indicates that this ligand interacts with two prominent amino acids (Trp 84 and Gly 118) and that three bonds were formed. The 5-ring of the neutral-PG connected with Trp 84 and Gly 118 to form two pi-H bonds with the distances of 4.51 and 3.85 Å, respectively, while C10 of this ligand interacted with the 6-ring of Trp 84 to create a H-pi linkage with distance of 3.94 Å.

The interaction between the neutral-BC and the AChE enzyme is also described in Figure 8d. Different from the two above ligands, the neutral-BC was found binding to the BS of AChE via only one interaction of the H-pi bond (4.62 Å), which formed between the C26 of the ligand and the 6-ring of Trp 84. Maybe the comparatively weak interaction with only one bond of the neutral-BC toward the target enzyme caused the low docking score of the NBC–AchE complex relative to those of CPG–AchE and NPG–AchE. This docking study seems reasonable and also agrees well with the experimental results from testing AChE inhibitory activities (Figure 6c).

In recent years, the studies related to PG have been of great interest due to their various beneficial medical effects and other applications [17–25]. Additionally, in silico studies related to PG have been previously reported [59–63]. However, the interactions and biding of PG towards the enzyme target for anti-Alzheimer's activity was first reported in this current study. Based on the in vitro and in silico studies, PG is a potential candidate for Alzheimer's disease treatment, and further studies including the evaluation of its anti-Alzheimer's effects via various cells and animal models as well as in clinical studies should be pursued for the development of PG into an anti-Alzheimer's drug.

## 3. Materials and Methods

### *3.1. Materials*

Shrimp head powder (SHP) was acquired from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). The 4 *S. marcescens* strains TKU011 [21], TNU01, TNU02 [44], and CC17 [45] were sourced from earlier studies. Acetylcholinesterase was acquired from Sigma Aldrich (USA). Silicagel (Geduran® Si 60, size: 0.040–0.063 mm) was obtained from Merck Sigma Chemical Co. (St. Louis City, MO, USA). The 2, 2-diphenyl-1-picrylhydrazyl was purchased from

Sigma Chemical Co. (St. Louis City, MO, USA). The highest grade of solvents and of common chemical agents available were used in this study.

### *3.2. Methods*

#### 3.2.1. Experiments of Fermentation for PG Biosynthesis in Flask and 12 L-Bioreactor

##### Experiments of PG Biosynthesized by Different PG Producing Strains

A total of four strains of *S. marcescens*, namely TUK011, TNU01, TNU02, and CC17, were tested for the biosynthesis of PG via fermentation. The liquid culture broth containing 1.5% C/N source (SHP/casein = 7/3), 0.02% $K_2SO_4$, and 0.05% $K_2HPO_4$ and of a medium pH 7.0 was fermented by these *S. marcescens* strains. The fermentation was conducted in 100 mL-flasks for 2 days at 27.5 °C and 150 rpm. The most active PG-producing strain CC17 was selected based on the PG yield it produced in culture broth (this protocol denoted by *).

##### The Effect of Added Free Protein in Culture Medium on PG Yield

Casein was used as a free protein to be added into the culture medium. SHP was combined with casein at the ratios of 0/10, 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, 9/1, and 10/0 and was used at the concentration 1.5% for fermentation. The basal salt solution and culture conditions were as in above-described protocol (*).

##### The Effect of Phosphate Salts on PG Production

Some phosphate salts, namely $Ca_3(PO_4)_2$, $KH_2PO_4$, $K_2HPO_4$, $Na_2HPO_4$, and $NaH_2PO_4$ were added to the medium for the examination of their effects on PG biosynthesis. The liquid medium contained 1.5% C/N source (casein/SHP = 1/9), 0.05% phosphate salts, and 0.02% $K_2SO_4$ with an initial pH of 7. The culture conditions were as in the above-described protocol (*). $Ca_3(PO_4)_2$ was the most effective for PG yield, so it was chosen for the investigation of its optimal added concentration (0.01–0.15%), and the cultivation was also performed following the above protocol (*).

##### The Effects of Sulfate Salts on PG Production

Various sulfate salts, namely $(NH_4)_2SO_4$, $MgSO_4$, $K_2SO_4$, $CaSO_4$, $FeSO_4$, and $ZnSO_4$ were tested for fermentation to determine their effects on PG yield. The liquid medium contained 1.5% C/N source (casein/SHP = 1/9), 0.02% sulfate salts, and 0.025% $Ca_3(PO_4)_2$ and was determined to have an initial pH of 7. The culture conditions were as described in the above protocol (*). $K_2SO_4$ was the most effective for PG yield, so it was chosen for investigation of its optimal added concentration in the range of 0.01–0.06%, and the cultivation was performed as described in the above-mentioned protocol (*).

##### The Effects of Fermentation Parameters on PG Productivity Produced by CC17 Strain

To reach higher PG yield, some culture condition parameters were tuned. A medium containing 1.5% C/N source (casein/SHP = 1/9), 0.02% $K_2SO_4$, and 0.025% $Ca_3(PO_4)_2$ and that was determined to have an initial pH of 7 was fermented by *S. marcescens* CC17 in different cultivation conditions, including varied fermentation temperatures (26, 28, 30, 32. and 34 °C), initial pH of the culture medium (pH of 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 9.5), volume ratio of the liquid medium/flask (30/100, 35/100, 40/100, 45/100, 50/100, 55/100, and 60/100 mL/mL), and fermentation time (0–96 h). The subsequent experiments were then conducted based on the optimal parameters from these tuning experiments.

##### The Experiments to Scale-Up PG Biosynthesis to 12 L-Bioreactor System

The optimal cultivation conditions obtained from the fermentations in flask were used to design the study for the scale-up of PG production to a 12 L-bioreactor system. The CC17 strain seeds were pre-cultivated in several 1000 mL flasks at 28 °C for 1.5 days, and675 mL of this bacterium seed solution was then injected into the bioreactor system containing 6.075 L of liquid medium. This culture medium contained 1.5% C/N source

(SHP/casein = 9/1), 0.02% $K_2SO_4$, and 0.025% $Ca_3(PO_4)_2$ and was determined to have a medium pH of 7. The cultivation was performed at 28 °C in dark conditions for 12 h, and the PG concentration was determined every 2 h.

#### 3.2.2. Experiments of Qualification and Purification of PG

The qualification of PG was done according to protocols described in a prior study [64]. A mixture including 2.0 mL methanol and 0.25 mL of 2.0% $AlK(SO_4)_2{\cdot}12H_2O$, and 0.25 mL of cultured broth was centrifuged at 1400× *g* for 5 min. The collected supernatant (1 mL) was then placed into a glass flask with 9 mL acidic methanol adjusted by 0.5 HCl. This final mixture solution was used to detect the optical density ($OD_{535nm}$). Purified PG obtained from the earlier work [42] was used to create the calibration curve to convert the optical density into the concentration of PG. The process of PG purification was also performed according to the protocol presented in detail in our previous work [64]. The purified pigment compound was measured for its UV/vis and mass spectra, HPLC profile, and some of its biological activities.

#### 3.2.3. High-Performance Liquid Chromatography (HPLC) Analysis of PG

Methanol was used to dissolve the sample. An amount of 3 µL of PG (1 mg/mL) was injected into the HPLC system. C18 column was used to separate the compound by using 70% MeOH in water adjusted to pH 3.0 by using 10 mM ammonium acetate as mobile phase. The column was set for a flowrate of 0.8 mL/min and was kept at 30 °C for 20 min. The PG was detected at 535 nm.

#### 3.2.4. Biological Activity Assays

Some biological effects such as antioxidant capacity, anti-nitric oxide activity, and acetylcholinesterase inhibition were tested. Of these, the DPPH radical scavenging activity assay was used to evaluate the antioxidant effect of PG, and this assay was conducted according to the method in the previous study [65]. The anti-nitric oxide and acetylcholinesterase inhibitory activities were assayed following Nguyen et al. 2018 [66] and Tan et al. 2028 [39], respectively. α-tocopherol, homogentisic acid, and berberine chloride were used as the standard compounds for the DPPH radical scavenging activity, anti-NO, and acetylcholinesterase inhibition assays, respectively.

#### 3.2.5. Docking Study Protocol

The docking simulations were conducted following some typical steps [56,57,67–69]:

- *Preparation of AChE enzyme before docking performance:* The structure data of AChE (DOI:10.2210/pdb1GQR/pdb) was obtained from Worldwide Protein Data Bank. The protein and its 3D protonation were prepared by using the functionality of MOE QuickPrep (Figure 7d) based on the positions of ligand within 4.5 Å and the presence of important amino acids. All of the water molecules were removed before the recreation of enzymic action zones. The active zone (binding site, Figure 8a) of the ligands on the target protein was determined using the site finder function in MOE.
- *Preparation of ligands (inhibitor compounds) before docking performance:* The structures of the inhibitor compounds (ligands, Figure 7a,b) were prepared using ChemBioOffice 2018 software. The optimized structures of the ligands prepared using the MOE system with optimization parameters of Force field MMFF94x; R-Field 1: 80; cutoff, Rigid water molecules, space group p1, cell size 10, 10, 10; cell shape 90, 90, 90; and gradient 0.01 RMS kcal $mol^{-1}$ $A^{-2}$ [56,69].
- *Docking performance between ligands and protein enzyme:* The docking simulation calculations were performed on the ligands of cation-PG, neutral-PG, and neutral-BC and towards the target enzyme protein (AChE) using MOE-2015.10 software. The out-put included amino acid compositions in the binding site on AChE, docking score (DS), Root Mean Square Deviation (RMSD), interaction types (bonds), amino acids interacted with by the ligands as well as the distances of the linkages.

## 4. Conclusions

This current work achieved the enhancement of PG production from *Serratia marcescens* CC17 using discarded marine shrimp heads in the fermentation process. PG was produced with a high yield level (6310 mg $L^{-1}$) in large scale production (6.75 L per pilot) by CC17 in a novel designed medium containing 1.5% C/N source (SHP/casein = 9/1), 0.02% $K_2SO_4$, and 0.025% $Ca_3(PO_4)_2$ with initial pH 7.0, and fermentation was performed at 28 °C for 8 h. The purified PG demonstrated moderate antioxidant and effective anti-NO and acetylcholinesterase inhibitory activities. The acetylcholinesterase inhibitory activity was further evaluated via a docking study, and there were better binding energy scores and more interactions with various prominent amino acids in the biding site on AChE than for Berberine chloride, a commercial AChE inhibitor. The results of this study suggest that SHP is a good material for the cost-effective production of PG via microbial technology and that PG is a potential candidate for anti-NO and anti-Alzheimer's drugs.

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