Article

Utilization of By-Product of Groundnut Oil Processing for Production of Prodigiosin by Microbial Fermentation and Its Novel Potent Anti-Nematodes Effect

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Abstract: This study aimed to reuse groundnut oil processing by-product, groundnut cake (GNC) for the low-cost production of prodigiosin (PG) via microbial technology and to assess its novel potential application for the management of black pepper nematodes. *Serratia marcescens* TUN02 was found as the most active PG-producing strain. Various small-scale experiments conducted in flasks indicated that GNC at 1% may be used as the sole carbon/nitrogen source for cost-effective PG production by fermentation. Notably, no further commercial ingredients and salts are required to supplement into the culture medium of this fermentation. PG was further investigated for scale-up production in a 14-L bioreactor system and PG was produced at high yield (6886 mg/L) with large-scale volume (4 L) in a short cultivation time (10 h). PG was then purified and its nematicidal activity was evaluated and showed effective inhibition of juveniles and egg hatching of *Meloidogyne incognita* species, harmful on black pepper, with low IC50 values of 0.2 and 0.32 mg/mL, respectively. The simple medium containing 1% GNC is the first report of cost-effective biosynthesis of PG, as well as potential in vitro anti-egg hatching activity of PG. These results indicated the potential application of GNC for low-cost bioproduction of PG for promising and novel use in the management of black pepper nematodes.

Keywords: organic wastes; groundnut cake; prodigiosin; *Serratia marcescens*; fermentation; *Meloidogyne incognita*; black pepper

1. Introduction

Management of organic wastes (OWs) is considered to be a major global issue. A significant amount of OW is released per year as processing by-products or wastes of the processing industry. The untreated OWs may cause the serious issue of environmental pollution. However, OWs are often rich in carbohydrates, proteins, lipids, and minerals, and may thus be utilized for various purposes [1]. In current years, the research on recycling OWs for the cost-effective production of bioactive compounds has been an emerging topic [2–7]. In this study, groundnut oil processing by-product was reused for the production of bioactive prodigiosin via fermentation technology.

Groundnut (peanut) is a vital oil, food, and feed crop of the world, native to Central and South America [8]. In 2019, Asia ranked first in the world for total area under groundnut plantation, and Vietnam ranks 15th with 456,513 tons [9]. Groundnut has high oil content,

so it is a principal crop, ranking 4th worldwide as an oil-producing seed [10]. During oil production, a large amount of more than 50% by-product is generated compared to starting material being groundnut cake (GNC) [11]. GNC is still very rich in nutritional ingredients such as protein, carbohydrate, lipid, crude fiber, and minerals [12]. Thus, the recovery and recycling of GNC can avoid waste and provide significant economic value. Most GNC is used as animal food or fertilizer [12]. Moreover, it was used as a substrate for the production of secondary metabolites via microbial fermentation [13–17]. This is an interesting direction that takes advantage of cheap raw materials and can quickly create high-value products in large quantities via fermentation technology.

Recently, prodigiosin (PG) a secondary metabolite produced mainly by *Serratia marcescens* via fermentation has attracted much interest due to its rich biological activities with potential application in many fields such as medicine, agriculture, industry, and environment [6]. Numerous studies concerning PG biosynthesis were reported. However, most of the previous studies used commercial broth for fermentation [18–21]. To lower the cost of PG production, various non-traditional materials, such as corn steep liquor, soybean, peanut, sesame, sesame oil, peanut oil, coconut, coconut oil were evaluated for fermentation [21–23], and some processing by-products and wastes were also utilized [13,14,24–31]. In addition, PG was also mainly investigated for its biosynthesis in a minor scale of flasks.

Considering the issue of cost-effective PG production and environmental pollution, we evaluated organic wastes for PG production in this study. In our previous works, various fishery processing by-products were investigated for PG production [1,2,5,25,26]. In this study, we evaluated the potential use of GNC as a raw and cost-effective substrate for PG production. Large-scale production of PG could be achieved in 14-L bioreactor systems and its novel potential was assessed for agricultural application such as the management of black pepper nematodes in this work.

2. Materials and Methods

2.1. Establishment of Small Scale Production of PG in Flasks

- Methods for determination of nutrient content of GNC: Some major nutrient ingredients of GNC were determined, including total protein [32], total mineral ash [32], mineral elements (by the generation 5 phenom pro and proX SEMs). Content of specific minerals including potassium, magnesium, calcium, and phosphorus was determined by the AA-7000 atomic absorption spectrophotometer (AAS) and spectrophotometric methods for phosphorus [33], total lipids [34], total dissolved sugar [35], and reducing sugar [36].

- Screening potential PG-producing bacterial strain: Several strains of *S. marcescens* obtained from our previous studies including TKU011, TNU01, TNU02, and CC17 [2,5,25,27] were conducted for the tests. Liquid medium containing 1.5% GNC, 0.5% casein, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ at initial pH 7.0 was fermented by these four bacterial strains at 28 °C, 150 rpm (shaking speed) for two days. The fermentation condition was symbolized as (*) to be further used for some experiments. *S. marcescens* TNU02 was chosen as a PG-producing strain for further experiments.

- The effect of supplementary ingredients and GNC concentration on PG production:
    - Effect of vegetable oils on PG production: various vegetable oils including olive oil (OLO), groundnut oil (PNO), brown rice oil (BRO), soybean oil (SBO), coconut oil (CCO), and sesame oil (SSO) were supplemented into the liquid medium containing 1.5% GNC, 0.5% casein, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ at the initial pH 7.0. Thirty milliliters of these experimental media and the control medium (no supplementary oils) were fermented by *S. marcescens* TNU02 strain at the above-mentioned conditions (*).
    - The effect of free protein on PG production: Casein was confirmed as a suitable free protein for enhancing PG production [25]. Thus, casein was added to the culture medium in this study. GNC was mixed with this protein in several ratios of 10/0; 9/1; 8/2; 7/3; 6/4; 5/5 and used as the major C/N source for

fermentation. The culture medium was supplemented with 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and an initial pH at 7.0, then fermented by the TNU02 strain under the above-mentioned conditions (*).

- The effect of GNC concentration on PG production: GNC was used as the sole C/N source for fermentation. Several concentrations of GNC (0.25, 0.5, 0.75, 1, 1.25, 1.5%) was added into the liquid solution containing 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ with an initial pH 7.0. then fermented by TNU02 strain under the above-mentioned conditions (*).

▪ The effect of salts on PG production: This experiment was designed based on previous studies [25,26]. Five types of sulfate salts including $(NH_4)_2SO_4$, K_2SO_4, $FeSO_4$, $ZnSO_4$, and $MgSO_4$ (at 0.05%) were added into the medium containing 1% GNC, 0.1% K_2HPO_4 at an initial pH at 7.0, and fermented by TNU02 strain under the above-mentioned conditions (*). Moreover, five types of phosphate salts including K_2HPO_4, KH_2PO_4, NaH_2PO_4, Na_2HPO_4, and $Ca_3(PO_4)_2$ at 0.1% concentration were also added into the medium containing 1% GNC, 0.05% $MgSO_4 \cdot 7H_2O$ with the initial pH at 7.0, then fermented by TNU02 strain under the above-mentioned conditions (*) for investigation of their effect on PG production.

2.2. Scale-Up of PG Production to a 14 L-Bioreactor System and Extraction of PG from the Culture Broth

Four hundred milliliter of *S. marcescens* TNU01 seeds previously prepared by pre-cultivating in some 500 mL-flasks at 28 °C for 1.5 days was added to a 14 L-bioreactors system (BioFlo/CelliGen 115 containing) containing 3.6 L of culture broth including 1% GNC at an initial pH of 7.0. The fermentation was conducted at 28 °C and 250 rpm, with a dissolved oxygen content of 1.0 vvm. The PG content was measured within 12 h, and the yield of PG produced by *S. marcescens* TNU02 in the bioreactor systems was tested every 2 h.

2.3. Method of Quantification, Purification, and Identification of PG

PG was quantified as discussed in the former report [22] with some modifications. Briefly, 2 mL of methanol was added to 2 mL of culture suspension and mixed with 0.5 mL of 2% $KAl(SO_4)_2 \cdot 12H_2O$. The solution was centrifuged at $1400 \times g$ for 5 min, and 0.5 mL of this supernatant was further mixed with 2 mL HCl/methanol (0.5 N) and then used for detection of optical density at 535 nm (OD535). OD535 was converted to concentration through appropriate calibration using pure PG as standard.

The red pigment compound was extracted and purified according to the method presented in the previous reports [24,25]. The fermented culture broth in the bioreactor system was centrifugated at $10,000 \times g$ for 15 min to harvest the supernatant which was further mixed with ethyl acetate (EA) at a ratio of 1:1 and kept in the funnel for about 3 h with shaking every half hour. The EA layer containing red pigment PG was collected and the crude fluid was concentrated in a rotary evaporator (IKA, Staufen im Breisgau, Germany) at 55 °C under vacuum, then dried to powder (crude PG) at 55 °C in an oven air drier. The crude PG powder was further separated through a silica column and then using an eluted solvent system including methanol/chloroform at ratios changing from 0/10 to 2/8 (*v/v*). The purified PG was dried to a powder by using a rotary evaporator and an oven air drier as above.

The red pigment compound purified in this work was confirmed as PG with a high purity grade via analysis of high-performance liquid chromatography (HPLC). The PG purified in our previous work by the same bacterial strain was also used as a standard compound for comparison [2]. The two samples were dissolved in methanol at 1 mg/mL concentration and then injected (3 μL) into the HPLC system. The analysis conditions were set at a flow rate of 0.8 mL/min for the column and keeping the column at 30 °C for 20 min. PG was detected at the wavelength of 535 nm.

2.4. Anti-Nematode Assays

The anti-nematode effect of the purified PG was evaluated via testing anti-*J2* nematodes and egg-hatching inhibition. The black pepper roots were collected from the sick pepper trees (with the symptoms of yellow leaves) cultivated in Buon Ma Thuot, Dak Lak province of Vietnam, and used for the preparation of eggs and *J2* nematodes.

- Eggs and *J2* root-knot nematodes preparation: eggs and *J2* nematodes were prepared according to the method previously presented by Khan et al., 2008 [37] with some modifications. Black pepper roots collected from the sick trees (symptoms with yellow leaves) were cleaned with water, then the egg masses in the root-knots were isolated using by hand using forceps. Eggs were rinsed with sterile water and then washed with sodium hypochlorite (0.5%), and finally, filtered through a sieve (26 μm pores). The collected eggs were used for the eggs-hatching inhibition assay. A part of these eggs was incubated for 3–5 days to obtain *J2* nematodes which were further used in the anti-*J2* nematode assay. The processes of eggs and *J2* root-knot nematodes preparation are summarized in Figure 1.
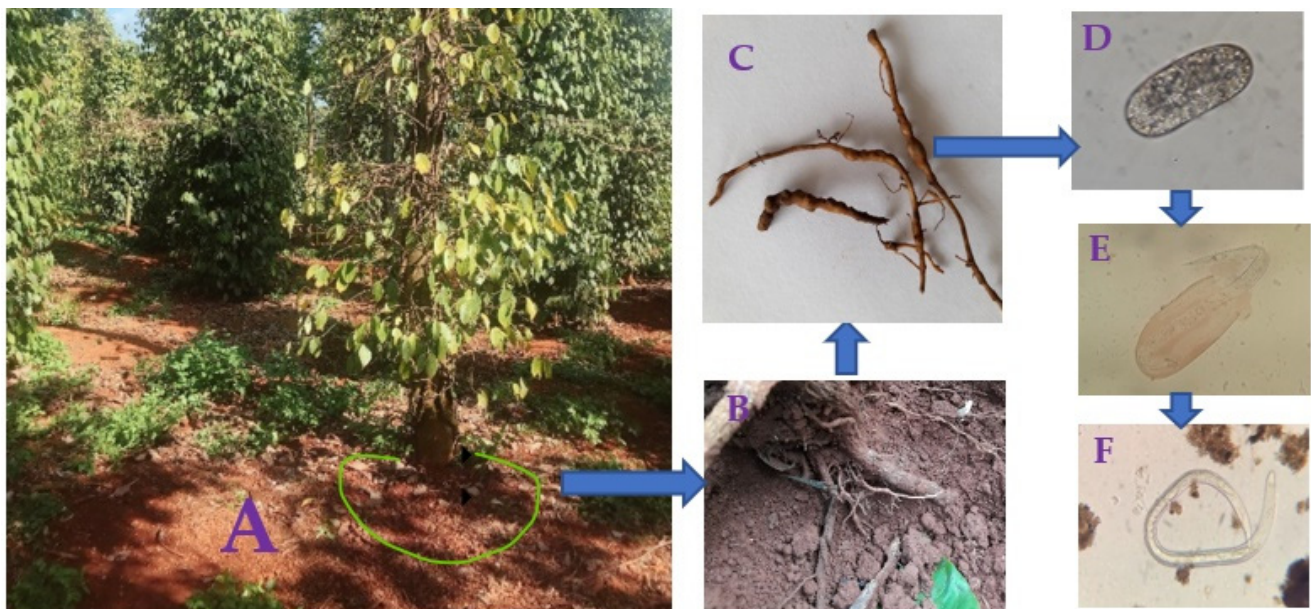
Figure 1. The process of preparation of eggs and root-knot nematodes (*J2*) from black pepper roots. The black pepper with yellow leaves showing the symptom of nematodes infection (**A**) was chosen for collecting the roots (**B**). The roots with knots (**C**) was used for isolation of eggs (**D**) which were further incubated for 3–5 days for eggs hatching (**E**) to obtain *J2* nematodes (**F**).

- The in vitro anti-*J2* nematode assay: 200 μL of sample (PG solution) with various concentrations of 1, 0.75, 0.5, 0.4, 0.2, and 0.1 mg/mL (dissolved in DMSO) was mixed with 200 μL sterile distilled water (containing about 30 individuals of *J2* nematodes) in 96-well culture plate. the mixture was kept at 28 °C for 24 h before counting the immobilized nematodes under stereoscopic microscope Olympus SZ51. The same treatment was given to the control group using DMSO without PG. All the tests were conducted in triplicates.
- The eggs-hatching inhibition assay: 100 μL of PG solution was mixed with sterile distilled water containing 200 nematode eggs, then this mixture was incubated at 28 °C. The hatched eggs were counted (based on *J2* nematodes) after three days of incubation. In the control group, DMSO was used instead of the PG solution. All the tests were performed with three repetitions.

2.5. Statistical Analysis

All the experiments were randomized design. The experimental data on prodigiosin yield, anti-*J*2 nematodes, and the eggs-hatching inhibition were obtained and analyzed via the simple variance (ANOVA) then Duncan's multiple range tests (when the experiment contains ≥6 items that need to be compared) and Fisher's LSD tests (when the experiment contains ≤5 items that need to be compared) at $p = 0.01$ were evaluated. Statistical Analysis Software (SAS-9.4) purchased from SAS Institute Taiwan Ltd. (Taipei, Taiwan) was used for statistical analysis.

3. Results and Discussion

3.1. Establishment of PG Production on Small Scale in Flask

❖ Determination of the nutrient ingredient contents of the input material for fermentation GNC

Several nutrient components, including protein, lipid, sugar, and salts of the starting materials (GNC) used for fermentation were analyzed, and the data are presented and summarized in Table 1. Based on the analyzed data, this by-product was found rich in protein content (36.01%) and contains a significant amount of residual lipid (9.74%), and a minor amount of total sugar (2.44%). High content of total mineral ash was also obtained 12.02%. The mineral elements of this ash were analyzed via SEM capture. Figure 2 shows that GNC ash contains a diversity of mineral elements as C, O, Fe, K, P, Mg, Ca, Al, and Si. Of these, Ca, Mg, K, and P were found as the major mineral elements in GNC ash. These major mineral elements that play important roles in bacterial growth and development were further quantified by the AAS system, and the content was 0.11%, 0.18%; 0.69%, and 0.38% for Ca, Mg, K, and P, respectively.
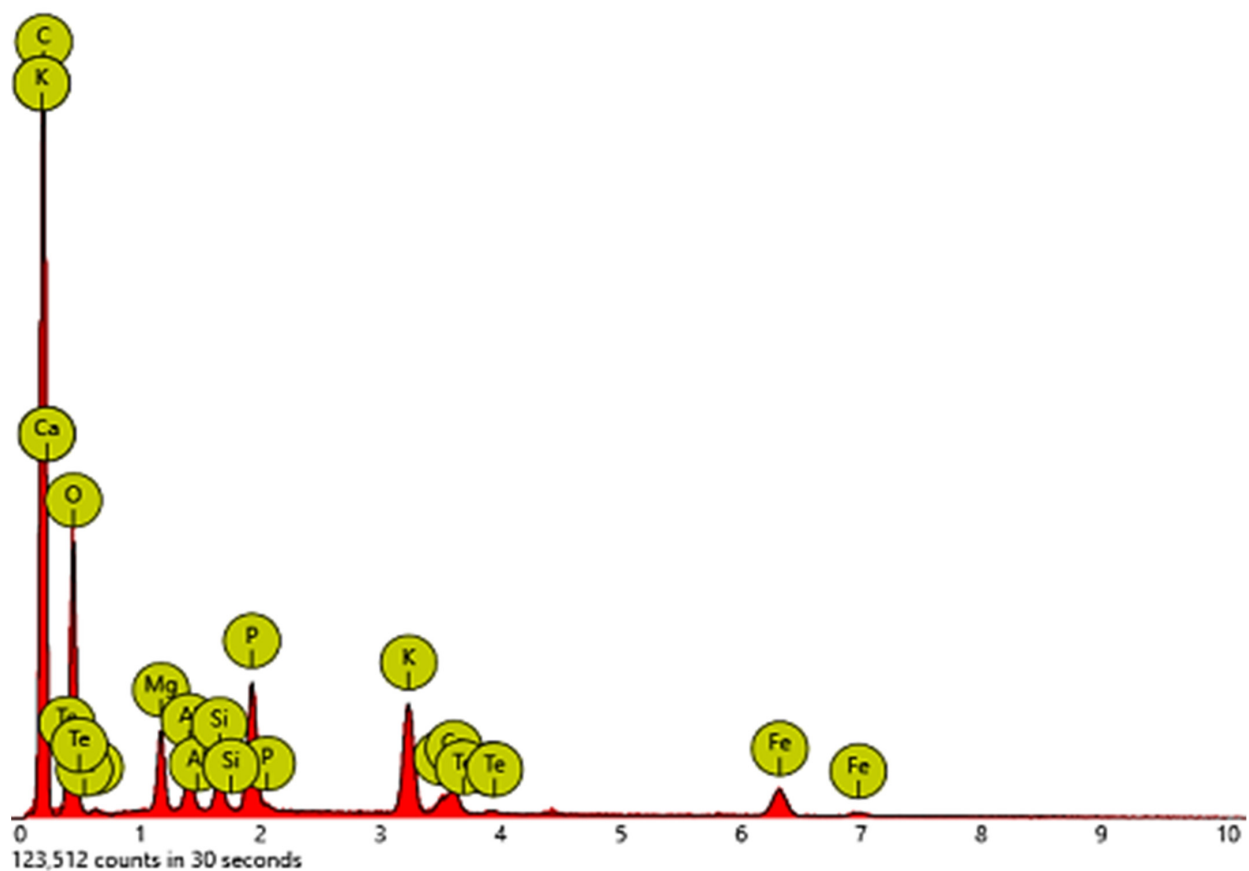
Figure 2. Mineral elements in groundnut cake (GNC) ash. Mineral elements in GNC ash were recorded in five positions by the generation 5 phenom pro and proX SEMs machine and the position of all elements are presented in the above representative picture.

Table 1. The nutrient content of groundnut cake (GNC).

			Content (%)					
Protein	Lipid	Total Ash	Total Sugar	Reducing Sugar	Ca	Mg	K	P
36.01	9.74	12.02	2.44	0.62	0.11	0.18	0.69	0.38

The results of this study are similar to those of some reports that also indicated that GNCs were very rich in protein, minerals, and some other ingredients [12,38], indicating that it is an ideal substrate for bacterial fermentation. Especially, *S. marcescens* can effectively utilize protein as a carbon source by producing lipolytic enzymes [13,21,23]. The experiments also proved that minerals and sugars significantly support PG production [39,40].

❖　Screening potential PG-producing bacterial strains

In our earlier studies, four *S. marcescens* including TKU011, TNU01, TNU02, and CC17 were found as potential PG-producing strains with different yields [2,5,25,27]. In this test, they were used for PG production via GNC fermentation to choose the best strain for further experiments. According to Figure 3, TNU02 presented the highest productivity (2.31 mg/mL) compared to the other strains and was used for all further experiments.
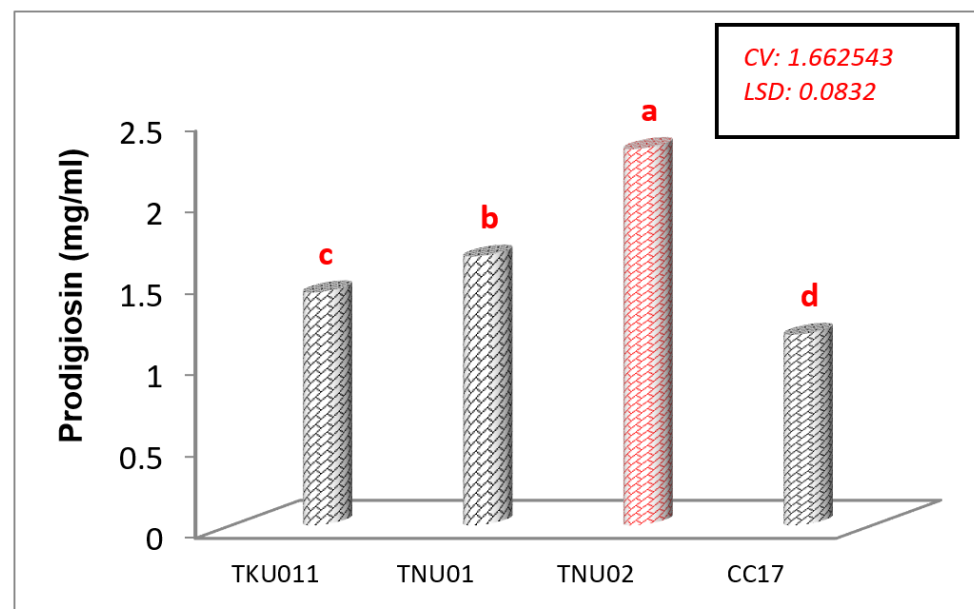

Figure 3. Screening of potential strain for prodigiosin (PG) production. The experiments were conducted in triplicate and data were analyzed via simple variance (ANOVA), and then Fisher's LSD tests at $p = 0.01$ were performed. Values with the different letters are significantly different. LSD—Least Significant Difference; CV—Coefficient of Variation.

❖　The influence of supplementary compositions added into the culture medium on PG production

Some studies have shown that oil supplements help improve the PG yield [21,22,41]. Thus, some vegetable oils were added into the culture media for evaluating their effect on PG biosynthesis by *S.marcescens* TNU02. Figure 4a indicates that PG was produced with high yields of 2.79 and 2.64 mg/mL in a culture medium with OLO and SBO, respectively. The lowest PG content was only 1.51 mg/mL for SSO. Especially, PNO did not enhance PG yield and gave a low yield of around 1.94 mg/mL. Notably, PG yield reached the highest yield that did not need for the addition of oil (3.13 mg/mL). This finding has not been reported earlier. In the report by Chenqiang et al., among supplement oils, olive oil gave a higher PG yield at about 9.3 fold more than the yield without oil [21]. Ghada et al. [41]

reported that PG production was significantly promoted in the medium containing sesame oil and olive oil. In another research, the two mediums adding 4% soybean oil, and the combination of 4% olive oil and 6% sunflower oil added into the culture media resulted in enhanced PG content in culture broths [22]. Oil supplement strategy improves PG content possibly because lipase from *S. marcescens* hydrolyzes oil substrates to release fatty acids as carbon and energy sources, or the hydrophobicity of oil substrates can create extracellular biosurfactants, that help to facilitate PG production effectively [22]. However, different bacterial strains may require a different level of supplementation of oils. In addition, the input material (GNC) used in this fermentation contains a significant amount of oil of 9.74% (Table 1), as such, further supplementation is not required for *S. marcescens* TNU02 fermentation to induce high production of PG.

(a)

(b)

(c)
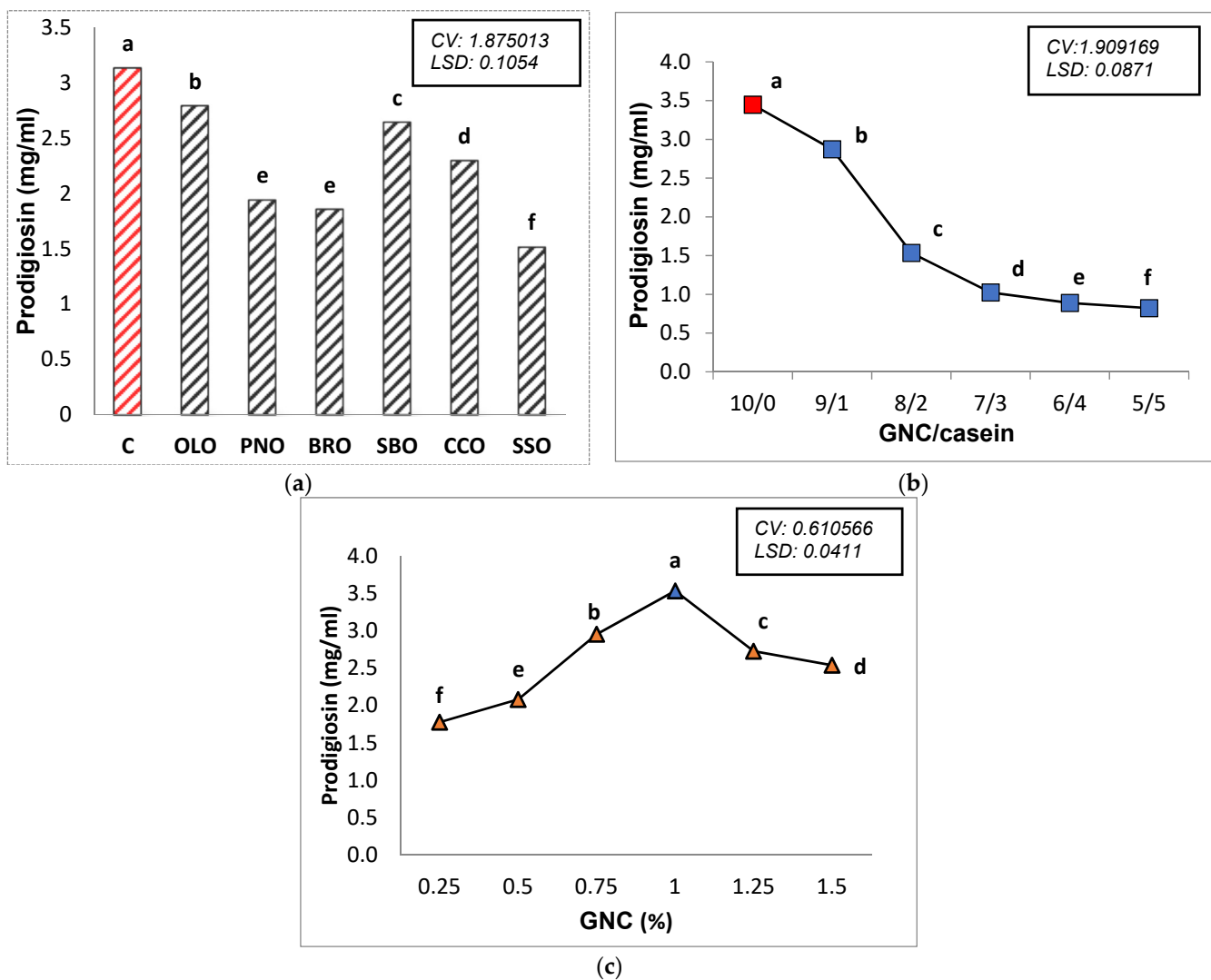
Figure 4. The influence of the culture medium ingredients on prodigiosin production from *S. marcescens* TNU02. Effect of supplements added to the medium. (**a**) Vegetable Oils, including olive oil (OLO), groundnut oil (PNO), brown rice oil (BRO), soybean oil (SBO), coconut oil (CCO), and sesame oil (SSO); (**b**) Casein ratio; (**c**) Substrate concentration. The graph was constructed by Microsoft Excel 2010. All of these experiments were conducted in triplicate and data were analyzed via the simple variance (ANOVA) then Duncan's multiple range tests at *p* = 0.01 were performed. Values in the same figure with the different letters are significantly different. LSD—Least Significant Difference; CV—Coefficient of Variation.

Supplementary free protein (casein) was required to enhance PG production by *S. marcescens* fermentation [13,25]. Thus, GNC was mixed with this protein at several ratios of 10/0; 9/1; 8/2; 7/3; 6/4; 5/5 and used as a C/N source for fermentation. As shown in Figure 4b, the medium-based oral GNC gave the highest PG yield of 3.45 mg/mL. All the experiments with casein supplementation did not enhance PG yield. Different from other previous reports, almost all media using low-cost or by-product materials for fermentation needed to add some sources of commercial-free protein [1,2,13,14,21,25–27,29,42]. In our earlier study, *S. marcescens* TNU02 was used for PG production via fermentation of crab shell powder—this waste contains low protein content (around 5.3%) [43]. Thus, casein was added to the medium with nearly 30% enhancement of PG yield [25], as such the total amount of protein in this optimal medium was around 35.3%. In this study, the GNC used contained high content of protein of 36.01% (Table 1), perhaps this reasonably explains no further requirements of added free protein into the culture broth.

The substrate concentration also affects pigment production [16,44], thus, evaluation of this criterion is necessary. Several concentrations of GNC were fermented by *S. marcescens* TNU02 and the results are summarized in Figure 4c. In the medium containing 1% of GNC, the highest yield was found to be of PG at 3.53 mg/mL. This indicator varies depending on the strain of bacteria. In Naik's report, the maximal pigment-producing strain *S. marcescens* CF-53 was observed in 8% of GNC [14]. Bhagwat reported [13] that the GNC at 4% concentration acts as a source of nutrients to increase PG production. However, *S. marcescens* TNU02 just used a little substrate (1%) to create maximal PG content in this study.

The oil and free protein were shown to not affect PG productivity of *S. marcescens* TNU02, so the medium with only 1% substrate of GNC was utilized for assessment of the effect of salts on PG production. The results are presented in Table 2. The highest yield of PG was observed (5.38 mg/mL) when no phosphate and sulfate salts were supplemented into the culture broth. In our previous study, the presence of mineral salts aids in improving the PG yield that differed depending on strains or substrate used for fermentation [1,2,25,26,42]. In addition, the quantity of mineral addition often oscillates very low only around 0.02–0.25% in our previous reports [1,2,25,26,42].

Table 2. Effect of salt addition on prodigiosin production.

Prodigiosin (mg/mL)			
Sulfate		**Phosphate**	
No salts	5.38 ± 0.034 [a]	No salts	5.38 ± 0.034 [a]
No-sulfate	2.12 ± 0.012 [e]	No-phosphate	2.67 ± 0.073 [d]
$(NH_4)_2SO_4$	3.32 ± 0.01 [c]	K_2HPO_4	3.42 ± 0.01 [c]
K_2SO_4	3.02 ± 0.008 [d]	KH_2PO_4	1.89 ± 0.002 [e]
$FeSO_4$	1.94 ± 0.017 [f]	NaH_2PO_4	1.41 ± 0.012 [f]
$ZnSO_4$	0.59 ± 0.015 [g]	Na_2HPO_4	1.94 ± 0.01 [e]
$MgSO_4$	3.37 ± 0.005 [b]	$Ca_3(PO_4)_2$	4.04 ± 0.002 [b]
CV: 0.504726LSD: 0.0355		CV: 1.407352LSD: 0.1041	

The PG content results were mean $\pm$ SD of three replicates. All of these experiments were conducted in triplicate and data were analyzed via the simple variance (ANOVA) then Duncan's multiple range tests at $p = 0.01$ were performed. Values in the same column with the different letters are significantly different. LSD—Least Significant Difference; CV—Coefficient of Variation.

Finally, the experiment highlighted that the designed medium for the highest yield of PG by *S. marcescens* TNU02 fermentation is very simple, containing only 1% GNC. In this study, the GNC used contained a high content of protein of 36.01% (Table 1). Moreover, GNC even contained a high content of lipid (9.74%) and a series of mineral elements. Of these, some major mineral elements, including Ca, Mg, K, and P were detected in a significant amount of 0.11%, 0.18%, 0.69%, and 0.38%, respectively, which may be enough

for *S. marcescens* TNU02 fermentation. Thus, the presence of diversified nutrition makes GNC an ideal material for bacterial fermentation for the cost-effective production of PG.

In current years, the research aspect of recycling wastes and by-products for the cost-effective production of PG has been an emerging topic [1–7,13,14,25,26,42]. As summarized in Table 3, vast arrays of by-product substrates were utilized for fermentation by *S. marcescens*. However, in almost the previous studies, the culture broth required the addition of several commercial ingredients. In our former reports, various marine wastes such as squid pens, shrimp heads, shrimp shells, and crab shells were reused for PG production via fermentation with the addition of some protein and mineral salts for reaching the highest PG yield (3700–5400 mg/L) [2,25,26,42]. Recently, the medium containing cassava wastewater supplemented with casein, $MgSO_4$, and K_2HPO_4 was reported as a potential for PG synthesis [1]. In other reports that used GNC, some ingredients still needed to be added for preparing the optimal medium [13,14]. In this study, although GNC concentration was very low (only 1%), PG production was significantly high (5380 mg/L) on the small flask. Based on this research, we demonstrated the use of groundnut oil processing by-products for the production of the bioactive compound via bacterial fermentation technology. In the next experiment, we scaled up fermentation in the 14 L-bioreactor system using the optimal medium condition on a triangular vase.

Table 3. The medium formulation of several reports using by-product as the main substrate.

Production Bacteria	Main Substrate	Supplement Ingredients	Prodigiosin (mg/L)	References
S. marcescens TNU02	1% groundnut cake	None	5380	In this study
S. marcescens CF-53	8% groundnut cake	None	39,800	[14]
S.marcescens ATCC 13880	4% groundnut cake	2% sucrose	900	[21]
S. marcescens TNU02	Demineralized crab shell powder (de-CSP)	1.6% (de-CSP/casein:7/3), 0.02% $(NH_4)_2SO_4$, 0.1% K_2HPO_4	4510	[25]
S. marcescens TKU011	1.5% squid pen	0.1% K_2HPO_4, 0.05% $MgSO_4$	978	[27]
S. marcescens CC17	Shrimp head powder (SHP)	1.5% (SHP/casein:9/1), 0.02% K_2SO_4, 0.025% $Ca_3(PO_4)_2$	5355	[2]
S. marcescens TNU01	Demineralized shrimp shell powder (de-SSP)	1.6% (de-SSP/casein:7/3), 0.02% K_2SO_4, 0.05% K_2HPO_4	5910	[5]
S.marcescens TNU01	1.75% squid pens powder	0.03% K_2HPO_4, 0.05% $MgSO_4$	3790	[26]
S. marcescens TNU01	Cassava wastewater	0.25% casein, 0.05% $MgSO_4$, 0.1% K_2HPO_4	5202	[1]

3.2. Scale-Up of PG Production to an Automatic Liquid Fermentation System (14 L-Bioreactor) and Extraction of PG in the Culture Broth

Bioreactor systems were effectively used to produce bioactive compounds with large-scale production and reduction of fermentation time [1]. In this work. A 14 L-bioreactor system was used for the fermentation of 4 L of the newly designed culture medium containing 1% GNC by *S. marcescens* TNU02. As shown in Figure 5, PG was produced with the highest productivity (6886 mg/L) at 10 h of fermentation. In the comparison, PG was induced at a larger scale (4 L) with a higher yield (6886 mg/L) in a much shorter fermentation time than that of fermentation in flasks.

(A)   (B)   (C)
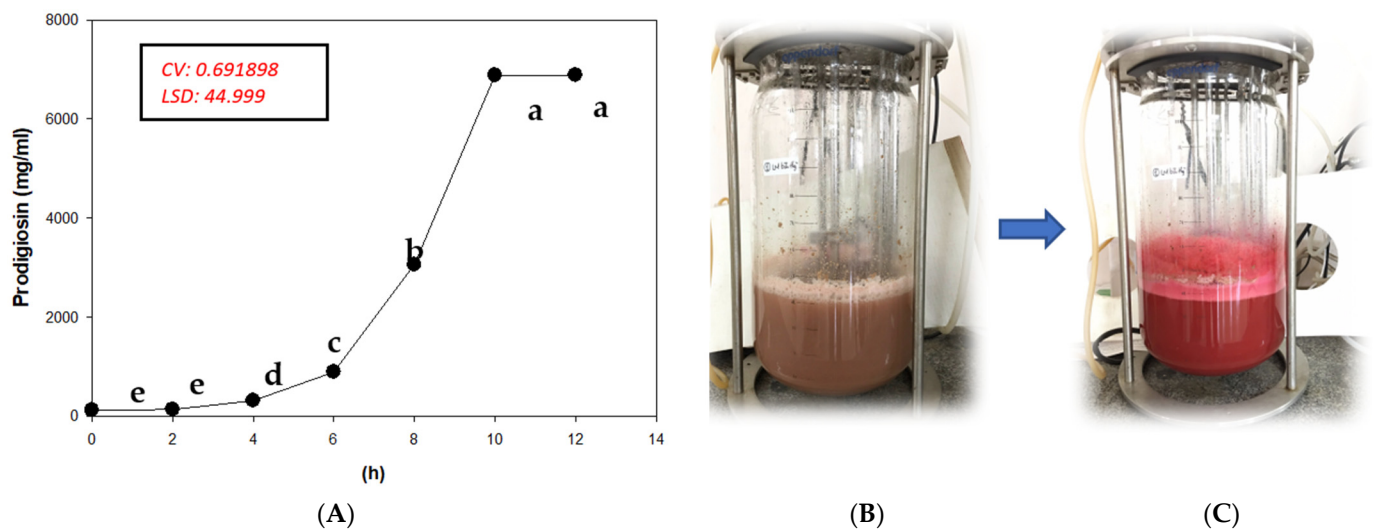
Figure 5. The scale-up prodigiosin production on a 14 L-bioreactor system. Liquid medium (4 L) containing 1% groundnut cake was fermented by *S. marcescens* TNU02 at 28 °C, with a shaking speed of 250 rpm, aeration of 1.0 vvm within 12 h. The recorded data of PG yield produced during the process of fermentation (**A**). The reaction tube of the 14 L-bioreactor system at 0 h (**B**) and 10 h (**C**) of fermentation. The experiments were conducted in triplicate and data were analyzed via the simple variance (ANOVA) then Duncan's multiple range tests at $p = 0.01$ were performed. Values with the different letters are significantly different. LSD—Least Significant Difference; CV—Coefficient of Variation.

Since PG has several potential uses, the number of studies based on PG production has dramatically increased in recent years [1]. However, investigations on PG biosynthesis were in small-scale flasks; only a few studies have reported PG production on large scale [1,2]. Recently, some studies approached scale-up of PG production in different bioreactor systems [1,2,5,25,26,28,45–48]. As summarized in Table 4, PG was produced via fermentation in a bioreactor system with the yield and fermentation time in the range of 50–6886 mg/L, and 8–65 h, respectively. Almost all studies reported the PG production in reactors with true working volume under 7 L except for one previous work reported by Chidambaram et al., (2014) [47], in which production of PG was reported in the largest scale of 50 L of culture medium. However, the PG productivity was not very high (522 mg/L) and the time course of fermentation was up to 20 h. In this work, PG was produced in reactor size (4 L of culture medium) with high PG productivity (6886 mg/L) in a short fermentation time (10 h).

The red pigment compound biosynthesized in a 14 L-bioreactor system was extracted and purified following the assay presented in our early work [24,25], and this purified compound was analyzed by HPLC. As shown in Figure 6, this compound appeared with a single major peak at a retention time (RT) of 12.425 min. The RT of this purified PG is approximately similar to those of PG reported in previous works (RT = 12.283–12.400 min) [5]. For further confirmation, the reference PG obtained from our earlier work [1,2] was also analyzed via HPLC systems at the same running conditions, and showed the RT of 12.392 (data not shown), and corroborates with that of the PG compound isolated in this work. Thus, the red pigment compound was confirmed as PG with a good purity grade, and this purified PG was used for the evaluation of antinematode activity in the experiments that followed.

Table 4. Research for scale-up prodigiosin production in a bioreactor system.

PG-Producing Bacterial Strain	Reactor Size (L)	Fermentation Time (h)	Prodigiosin (mg/L)	References
S. marcescens TNU02	4	10	6886	This study
S. marcescens TNU01	3–7	12	3450	[1,5,26]
S. marcescens TNU02	4.5	8	5100	[25]
S. marcescens CC17	6.75	8	6310	[2]
S. marcescens BS 303 (ATCC® 13880™)	0.935	65	872	[48]
S. marcescens	6.5	52	595	[28]
S. marcescens 02	2.75	20	583	[46]
Chryseobacterium artocarpi CECT 849	50	24	522	[47]
S. marcescens	3	30	50	[45]
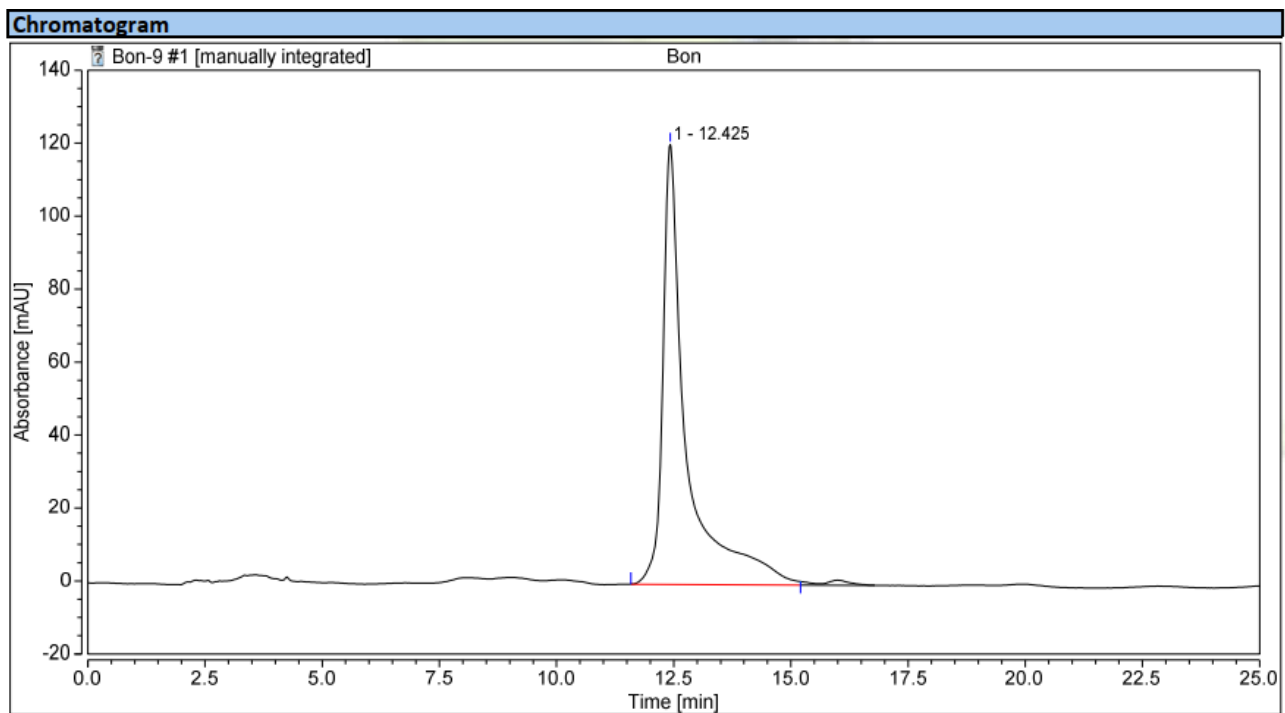
Figure 6. The high-performance liquid chromatography spectrum of prodigiosin produced in this work. The sample was dissolved in methanol at 1 mg/mL concentration and then injected (3 μL) into the HPLC system. The analysis conditions were set at a flow rate of 0.8 mL/min for the column and keeping the column at 30 °C for 20 min. PG was detected at the wavelength of 535 nm.

3.3. Novel Anti-Nematode Activity of Purified PG

Root-knot nematode (Meloidogyne sp.) is well-known to destroy many crops, including black pepper [49–51], which is considered the king of spices and is an important industrial crop with high economic and export value. However it is harmed seriously by root diseases [52], e.g., one of the main nematode damage in Vietnam is Meloidogyne incognita species [17]. This nematode is controlled mainly using chemical or biological methods [53]. Biological control is an effective replacement solution because it is cost-effective, safe, and environment friendly [54]. Whereby, using antagonistic creature or inhibition compounds are researched very popularly [55–58]. The research on the use of inhibitors has increasingly attracted attention with many reports focusing on discovering

nematode inhibitors from plant extracts or microbial fermentation [54,58–62]. Especially, the secondary compound of microorganisms has several advantages, efficiently killing nematodes and their ability to be produced at an industrial scale. Recently, the culture broth fermented by TNU02 strains [33] was shown to be efficient in killing black pepper *J2* nematodes of Meloidogyne sp. However, there was no evidence of the effect of purified compound PG toward black pepper *J2* nematodes and their egg hatching. Thus, in this study, we report the anti-black pepper-nematodes activity of PG via testing the resistance of purified PG on both objects *J2* and eggs of Meloidogyne sp.

3.3.1. The Anti-*J2* Nematodes Effect of PG

Meloidogyne sp. *J2* plays an important role in inducing the root-knot symptoms and destroying agricultural plants [63]. So extermination of *J2* development is a vital strategy in controlling this nematode. The anti-*J2* activity is tracked by mortality (%) after 24 h of incubation. As shown in Figure 7, PG was found to efficiently inhibit both *J2* nematodes and egg hatching. After 24 h of PG treatment at the concentration of 0.5, 0.57, and 1 mg/mL, the *J2* mortality was recorded at the great values of 96.7, 100, and 100%, respectively. At a low treated concentration of PG (0.1 mg/mL), the mortality was also higher than 30%. The IC50 value (the concentration of PG which can kill 50% *J2* nematodes) was calculated and PG showed a low IC50 value of 0.2 mg/mL.

The inhibitory effect of PG against several plant-parasitic nematodes was shown in earlier works. Rahul et al. found that this pigment is effective against plant-parasitic nematodes Radopholus similis and M. javanica, with the IC50 values of 83 and 79 μg/mL, respectively [64]. Omnia et al. found that crude PG inhibited tomato M. incognita *J2* with an IC50 value of 31.9 mg/mL [55]. PG was shown to be significantly effective against Caenorhabditis elegans and Heterodera schachtii with IC50 values of 0.127; 13.3 μM [42]. PG also acts against Nacobbus aberrans, Haemonchus contortus, and Panagrellus redivivus with maximum inhibition values up to 88.8, 100, and 100%, respectively [65]. This is the first study to report PG a novel potential candidate for controlling *J2* root-knot nematodes originated from black pepper.

3.3.2. The Inhibitory Effect of PG on Nematode Egg-Hatching

Egg hatchability is a crucial factor that determines the development of the nematode population [66], the egg resistance targeting helps to minimize the damage caused by the nematode. Thus, to evaluate PG as a potential candidate for the management of black pepper nematodes, the egg-hatching inhibition was also tested. The results are presented in Figure 8, according to which, PG also demonstrated potent inhibition against nematode egg-hatch with a maximum activity of 87% at the treated concentration of 1 mg/mL. At low treatment concentration (0.1 mg/mL), the egg hatching rate was recorded at 55%. The IC50 value for egg-hatching inhibition of PG was also recorded at a low concentration of 0.32 mg/mL. The morphology of normal eggs (not treated by PG) and un-hatched eggs (treated by PG) was observed and the findings are presented in Figure 8. The membrane of the egg treated by PG was destroyed and intracellular composition was leaked.

In an earlier study, PG was explored for its inhibition against egg hatching of Radopholus similis and M. javanica [64]. Based on the available literature, the potential of PG against inhibition of Meloidogyne sp. egg-hatching is also a new finding in this study. The experimental data on the anti-nematode effect of PG highlight the novel and potential application of PG on the management of nematodes against black pepper.
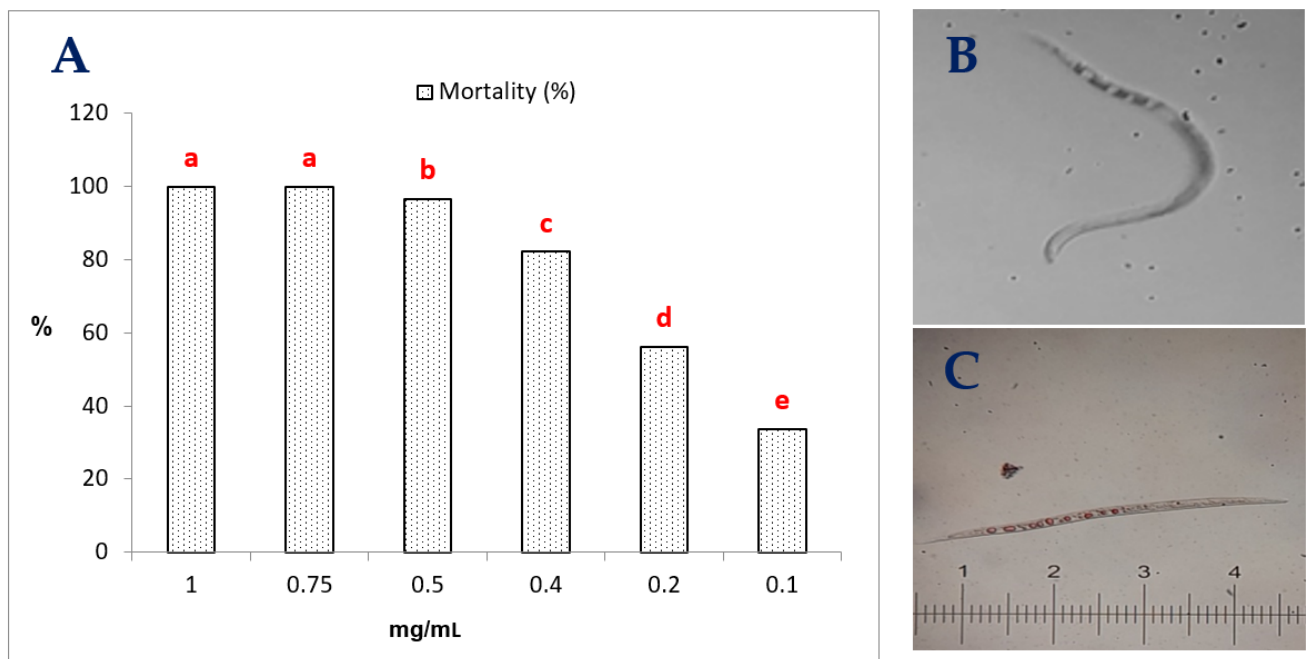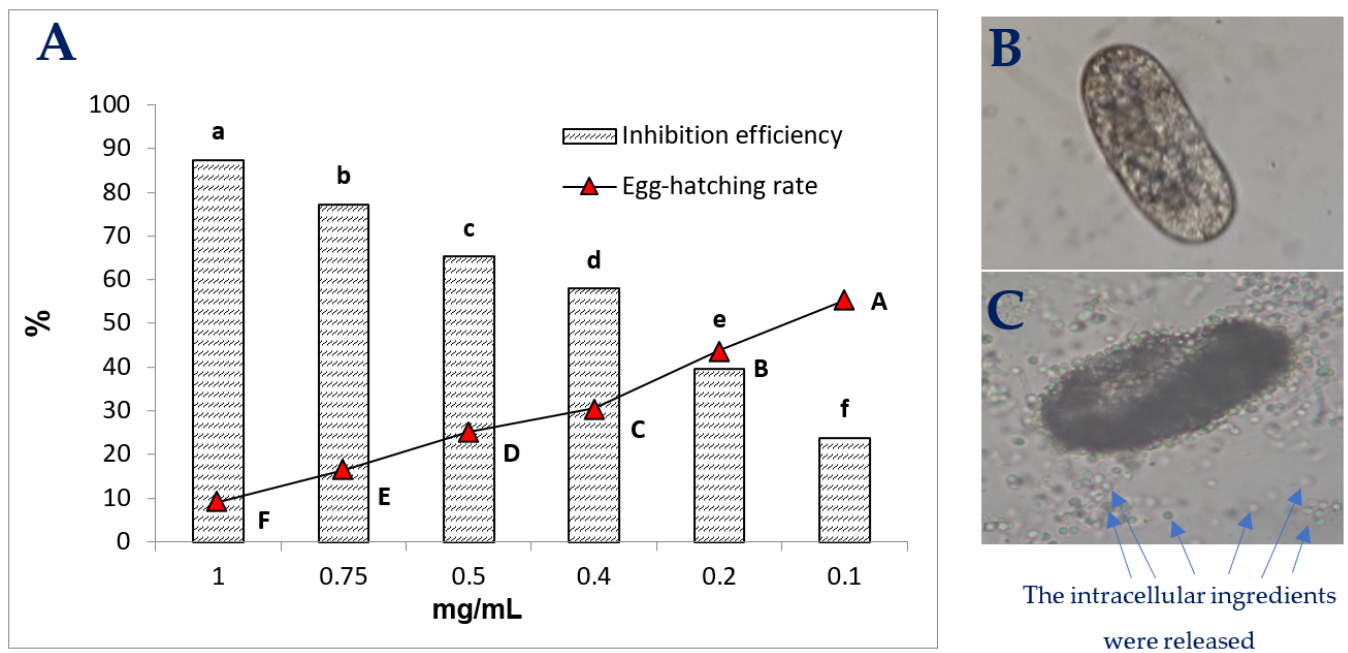
Figure 7. The anti-*J2* nematodes activity of purified prodigiosin. The anti-*J2* nematodes effect of prodigiosin (**A**). The experiments were conducted in triplicate and data were analyzed via the simple variance (ANOVA) then Duncan's multiple range tests at $p = 0.01$ were performed. Values with the different letters are significantly different. The value of Least Significant Difference (LSD) and Coefficient of Variation (CV) were 0.872937 and 1.7646, respectively. The graph bio-activity was constructed by Microsoft Excel 2010. The mobilized *J2* nematodes (**B**) became immobilized *J2* nematodes (**C**) after 24 h of treatment with prodigiosin. The images of coffee nematode eggs and *J2* nematodes were observed and recorded using an Optical Olympus Microscope (Model CH30RF200, Olympus Co., Tokyo, Japan).

Several molecular mechanisms of anti-nematode activity were confirmed via enzyme or protein inhibitor targeting by virtual screening assay [67–71]. Therein, acetylcholinesterase (AchE)—a common enzyme was used for the inhibition of M. incognita (a major species of genus Meloidogyne) [68]. In previous research, we discovered that purified PG expresses potent binding energy (-12.3 kcal/mol) for its interactions with many amino acids (6 linkages) in the biding sites on AChE [2]. Perhaps, this molecular mechanism may explain the strong activity of purified PG in M. incognita inhibition. Nematicides that inhibit egg hatching and juveniles are considered prospective drug candidates [72] and the activity of PG was shown against both targets. Besides nematode resistance, PG has confirmed inhibition on a series of phytopathogens such as bacteria, fungi, and insects [6]. Disease on black pepper is supposedly caused by a combination of nematode and fungal [44]. PG was found to be an effective inhibitor against many fungi harmful to crops, thereby also controlling fungi such as Fusarium oxysporum and Rhizoctonia solani—that cause slow death of the pepper plant [73,74]. Moreover, in vitro tests showed PG to be safe on cell and animal models. PG demonstrates effective inhibition against cancer cells, with no toxicity to normal cells [75], it is also genetically non-toxic, as was determined through Ames and microkernel test [76]. It was also shown to be safe in mice [77]. Especially, there is an important report demonstrating the safety of beneficial soil biodiversity factors when used at high doses of 500 μg/mL [74]. Based on this scientific information, and the experimental results of this study, PG has the potential of being a useful bio-solution to control multi-pathogens and may effectively and safely replace the chemical methods for controlling agricultural diseases. However, for the development of PG as an applicable anti-nematodes agent, further investigations, including the effect of PG on Meloidogyne

sp. J2, egg hatching, soil microbiota, black pepper seedlings, and black pepper trees in the greenhouse and field conditions should be conducted.

Figure 8. The egg-hatching inhibition of purified prodigiosin. The egg-hatching inhibition (%) of purified prodigiosin (**A**). The experiments were conducted in triplicate and data were analyzed via the simple variance (ANOVA) then Duncan's multiple range tests at $p = 0.01$ were performed. Values with the different letters in the same column/line) are significantly different. The value of LSD (Least Significant Difference), CV (Coefficient of Variation) for Inhibition efficiency (%) were 2.5123; 1.657060, respectively and those of egg-hatching rate (%) was 1.7747, and 2.279696, respectively. The graph bio-activity was constructed by Microsoft Excel 2010. The normal nematode egg (**B**) with the membrane around was treated with prodigiosin. After 3 days of treatment, the membrane of the nematode egg was decomposed and released the intracellular ingredients (**C**).

4. Conclusions

In this study, *S. marcescens* TNU02 was screened as the most potent strain for PG biosynthesis from the medium containing groundnut cake (GNC). Groundnut oil processing by-product, GNC, was recognized as a low-cost organic waste for cost-effective PG production via fermentation. Analysis of this input material showed that it possesses very rich nutrition compositions. Various small-scale flask-level experiments have proved that S. marcescens TNU02 produced PG at a high-levels in a very simple medium containing 1% GNC, and no other commercial compositions needed to be added. PG production was also scaled-up in a 14-L bioreactor system and a high yield (6886 mg/L) was obtained in a short fermentation time (10 h). The purified pigment was isolated and its inhibitory effect on potential nematode damaging black pepper crops was assessed. This work suggests that GNC is an organic waste with the potential to be used for low-cost scale-up production of PG for potential application in the management of black pepper nematodes.

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A.D.N., T.H.N. (Thi Hanh Nguyen) and V.B.N.; supervision, V.B.N.; visualization and project administration, V.B.N., A.D.N. and S.-L.W. All authors have read and agreed to the published version of the manuscript.

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