ENHANCEMENT OF PRODIGIOSIN PRODUCTION FROM SOYBEAN RESIDUE BY- PRODUCT VIA FERMENTATION TECHNOLOGY

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SUMMARY

Prodigiosin (PG) is a red pigment compound originating from *Serratia marcescens*. Recently, this compound has been extensively studied for its production process and investigation of its potential bioactivities. However, most previous studies used commercial nutrient broth as a carbon/nitrogen (C/N) source for cultivation in small-scale flasks. Regarding the cost-effective and green production, we reused soybean residue by-product (SRBP) for the biosynthesis of PG on a large scale using a 14-L bioreactor system and reported its potent anti-nematode activity. The experimental results revealed that *Serratia marcescens* TNU2 produced PG at the highest yield (5700 mg/L) under fermentation conditions: 6 L of liquid medium containing 1.75% C/N (SRBP/casein at the ratio of 8/2), 0.05% $MgSO_4$, 0.1% K_2HPO_4, with an initial pH of 7.0, and the fermentation was performed at 27.5°C in 10 h. On the activity tests, the purified PG at the tested concentration of 1 mg/mL demonstrated a potential nematicidal effect against root-knot nematode on both *J2* nematodes (100%) and eggs hatching (87.43%) at 1 and 3 days after treatment with PG, respectively. This study suggested that SRBP is a good C/N source for the bioproduction of PG with potential use for managing root-knot nematodes.

Keywords: *Soybean waste , prodigiosin, bioreactor, Serratia marcescens, root-knot nematode.*

1. INTRODUCTION

Prodigiosin (PG), a red pigment that belongs to the prodiginine family is a metabolite of various bacteria. Of these, *Serratia marcescens* is a major producer of PG (Wang, S.L. et al. 2020). PG possesses numerous biological effects such as anticancer, antibacterial, algicidal, antioxidant, immunosuppressant, anti-Alzheimer, anti-inflammatory, antiparasitic, and insecticidal activities. This pigment is also commonly used in food colorants, cosmetics, textiles, candles, and solar cells (Nguyen, T.H. et al. 2021).

Various potential bioactivities and applications of PG have resulted in a dramatic increase in the investigation of PG production via microbial fermentation. However, in most of the previous works, commercial media were used as C/N sources for cultivation, such as yeast malt, casein, tryptone yeast, yeast extract, tryptone soy, nutrient broth, glycerol, glycerol-tryptone, peptone-glycerol, and Luria/Bertani broth (Nguyen, T.H. et al. 2022a). For lower cost production of PG, some nontraditional media, including sesame seed, cassava, sesame oil, crude glycerol, peanut oil, corn steep, coconut oil, peanut seed, copra seed, and the complexes of mannitol/cassava, and mannitol/corn steep have been used as C/N sources for fermentation (Chenqiang, L. Et al. 2019; Giri, A.V. et al. 2004; Wei, Y.H. et al. 2005). Some processed by-products and wastes have also been utilized for fermentation to produce PG in some previous works (Bhagwat, A.; Padalia, U. 2020; Sumathi, C. Et al. 2014; Aruldass, C.A. et al. 2014). Moreover, PG has also been mainly studied for bioproduction in a minor scale of flasks (Nguyen, T.H. et al. 20).

Concerning bioproduction of PG, we reported the production of this secondary metabolite from soybean waste via *Serratia marcescens* conversion. This pigment compound was scale-up produced using a 14-L bioreactor system and also investigated in its potential antinematicidal activity in this study.

2. THE STUDY CONTENT AND METHODOLOGY

2.1. Study contents

- Establishment of the fermentation process for production of prodigiosin in flask.

- Scale-up of PG production in a bioreactor system.

- Assessment of the potential antinematicidal activity of prodigiosin.

The contents and experimental stepts of this study were illustrated in the Scheme 1.

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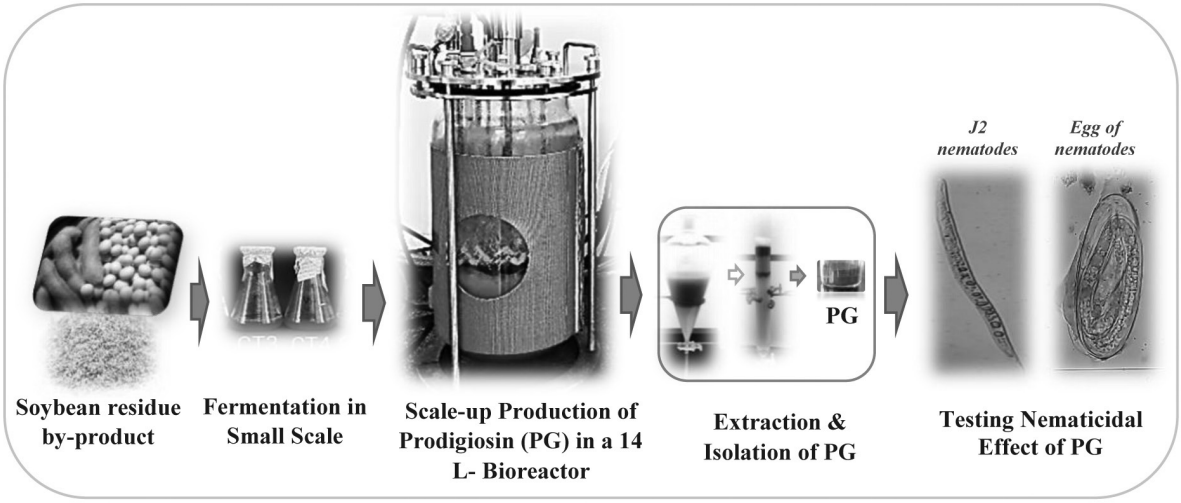
2.2. Materials

Serratia marcescens TNU2 was obtained from our earlier report (Nguyen, et al. 2020). soybean residue by-product (SRBP) was collected in Buon Ma Thuot City. Silicagel (Geduran® Si 60, size: 0.040-0.063 mm) was purchased from Merck Sigma Chemical Co. (St. Louis City, MO, USA). The root knot nematode.



Scheme 1. The contents and stepts for performing experiments of this study

(*Melodogyne* sp.) eggs and J2 were obtained from the roots of the infected black pepper plants in Buon Ma Thuot City.

2.3. Methodology

2.3.1. The effect of soybean waste/casein ratio and their concentration added in culture medium on prodigiosin yield

The fermentation protocol: Casein was used as free protein for adding to the culture medium. SRBP was combined with casein at various ratios (SRBP/casein, w/w) of 5/5, 6/4, 7/3, 8/2, 9/1, and 10/10 and used as C/N sources for fermentation (Nguyen, V.B el al. 2021; Nguyen, T.H et al. 2021). The liquid medium (30 mL in a 100 mL-flask) containing 1% C/N source, 0.015% $MgSO_4$ và 0.03% K_2HPO_4, (initial pH 7) was fermented by *S. marcescens* TNU2 at 30°C with shaking speed of 150 rpm for 2 d (this fermentation protocol is denoted by *). The supernatant was harvested by centrifugation at 10,000× *g* for 10 min and used to determine prodigiosin (PG) concentration.

SRBP/casein at the ratio of 8/2 was used as C/N source for fermentation at its various concentrations (0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0%) using the above protocol (*) to investigate the suitable C/N concentration.

2.3.2. The effect of phosphate and sulfate salts added in culture medium on PG yield

The phosphate salts ($Ca_3(PO_4)_2$, KH_2PO_4, K_2HPO_4, Na_2HPO_4, and NaH_2PO_4) and sulfate salts ($(NH_4)_2SO_4$, K_2SO_4, $FeSO_4$, $MgSO_4$, $ZnSO_4$, and $CaSO_4$) were tested for their effect on PG yiled during fermentation using the above protocol (*).

The phosphate salt K_2HPO_4 at the concentration range of 0-0.2 %, and sulfate salt $MgSO_4$ at the concentration ranges of 0-0.1% were further accessed for their effect on PG yield during fermentation using the above protocol (*).

2.3.3. Scale-up production of prodigiosin in a bio-reactor system

600 ml of bacterial seed were pre-cultured in a 1 L flask at 27.5 °C for 1.50 days and then injected into the fermenter system containing 5.4 L of medium containing 1.75% C/N (SRBP/casein at the ratio of 8/2), 0.05% $MgSO_4$, 0.1% K_2HPO_4, with an initial pH of 7.0, and the fermentation was performed at an initial pH of 7, and fermentation was conducted at 27.5°C in 10 h of cultivation time. Sampling and detection of the PG yield were performed every two hours.

2.3.4. The nematicidal activity assay

The black pepper eggs and J2 nematodes were prepared according to the method reported by Khan et al (2008). The anti-*J2* nematicidal and anti-egg hatching activities were done following the assay presented in detail in our previous report (Nguyen, T.H. et al. 2022b).

2.3.5. Statistical Analysis

The experimental data 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. RESULT AND DISCUSSION

3.1. Establishment of the fermentation process for production of prodigiosin in flask.

The effect of soybean waste/casein ratio and their concentration added in culture medium on prodigiosin produced by S. marcescens TNU2:

Casein has been found a suitable protein for adding to the culture media to produce PG (Nguyen, V.B. et al. 2020). Thus, this kind of protein was conducted to combine with SRBP at various ratios and used as C/N source for fermentation in this study. As shown in Figure 1a, PG was significantly produced at the high-level yield (1.97-2.35 mg/mL) when SRBP and casein were mixed at the ratios of 5/5, 6/4, 7/3, and 8/2. Regarding a PG production, the SRBP/casein mix in 8/2 proportions was the best choice for further investigation of its suitable concentration. SRBP/casein at the ratio of 8/2 was used as C/N source for fermentation at its various concentrations (0.5-2.0%) and PG was produced at the highest yield of 3.72 mg/mL when C/N source was at the concentration of 1.75% (Figure 1b). In the previous work (Gohil, N. et al. 2021), soybean meal was primarily utilized as the sole C/N source at 2% for fermentation by *S. marcescens* and PG was obtained at a yield of 0.8567 mg/mL (5.19-fold higher than the maximum PG yield achieved in the commercial growth media). In this study, SRBP was used as the major C/N sourced with adding minor casein for fermentation and PG was produced at a high yield of 3.72 mg/mL. This result indicated the potential application of SRBP for PG production via *S. marcescens* fermentation.

The effect of phosphate and sulfate salts added in culture medium on prodigiosin yield:

Phosphate and sulfate salts demonstrated as a significant effect on PG production by *S. marcescens* fermentation (Nguyen, V.B. et al. 2020, 2021). Thus, the culture broth was supplemented with many kinds of phosphate and sulfate salts and was then fermented by *S. marcescens* TNU2 for reaching higher PG yield production. The results showed that K_2HPO_4 was the most suitable phosphate salt (Figure 1c), and its optimal added concentration was 0.01% (Figure 1d). Among various tested kinds of sulfate salts, $MgSO_4$ was found as the most suitable salt for enhancing PG yield (Figure 1e) and its optimal added concentration was 0.05% (Figure 1f).

Based on the reviewed literatures, the phosphate and sulfate salts and their addition levels play an important role in Prodigiosin production by species *S. marcescens* (Wang, S.L. et al. 2020; Nguyen, V.B. et al. 2020, 2021; Nguyen, T.H. et al. 2021, 2022a, 2022b). However, the action of those salts remains unclear.

3.2. Scale-up production of prodigiosin using a 14 L- bioreactor system

In fermentation technology, bioreactor systems are valuable equipment to effectively produce secondary metabolites with a high-level yield in a short fermentation time (Nguyen, T.H. et al. 2021). In this study, a 14 L-bioreactor system was used for scale-up production of PG. As shown in Figure 2, PG was produced with significant mass from 6 h of fermentation and its yield reached a maximum (5700 mg/L) after 10 h of fermentation. Thus, to compare with cultivation in flakes (100 mL), the use of a 14 L-bioreactor system for fermentation results in the production of PG with a higher yield on a larger scale, and with a much shorter cultivation time.

Study on the production of PG via microbial fermentation has received renewed interest that has notably increased in recent years (Nguyen, T.H. et al. 2021, 2022a, 2022b). However, in most of the previous studies, the experiments were conducted in small-scale fermentation in Erlenmeyer flasks to produce bioactive PG, using commercial nutrient broth as C/N source for fermentation. Different from those, PG was studied for larger-scale production in the bioreactor system using organic waste (SRBP) as C/N source.

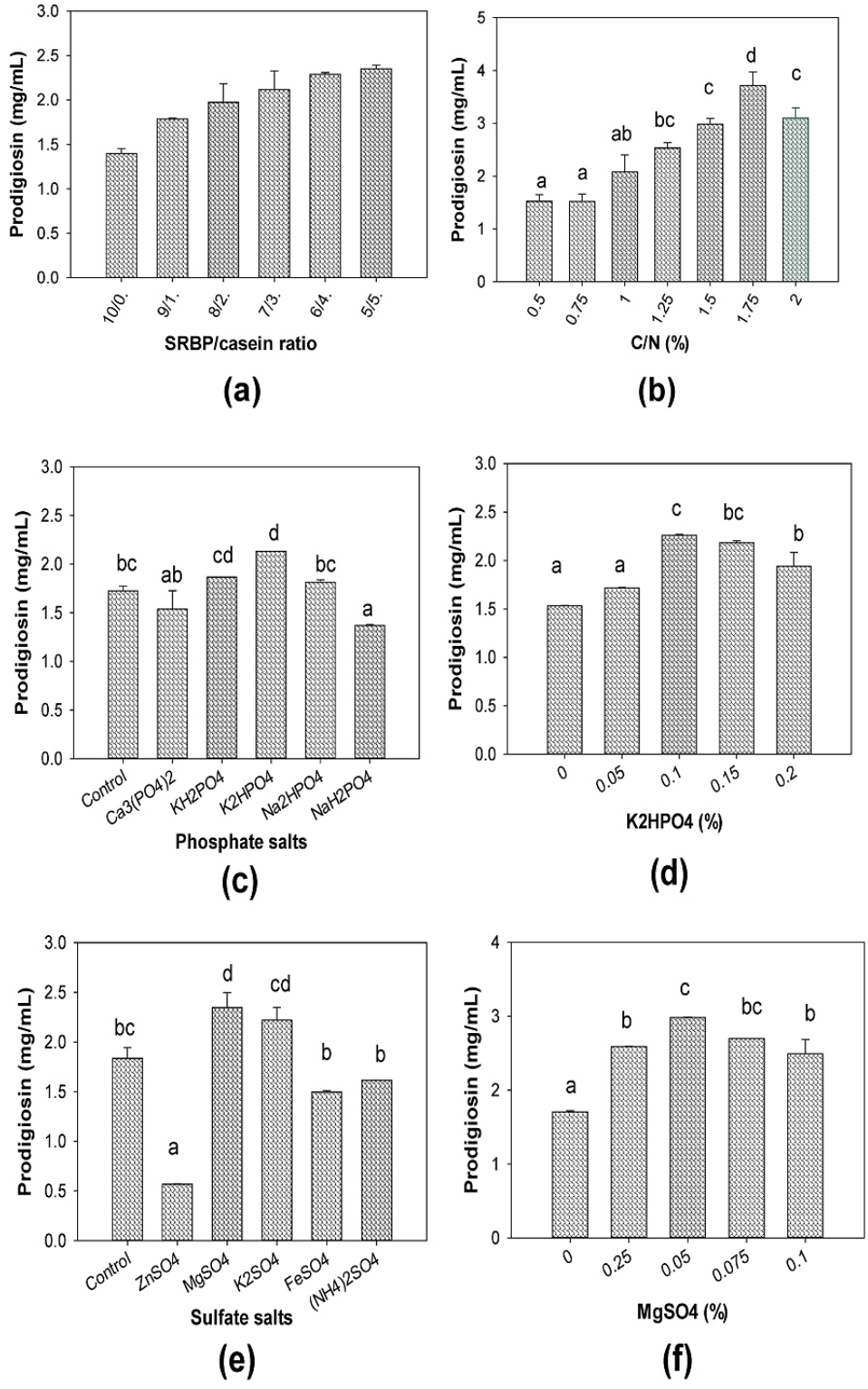


Figure 1. The effect of the SRBP/casein ratio (a), C/N concentration (b), Phosphate salts (c), K_2HPO_4 concentration (d), sulfate salts (e), and $MgSO_4$ concentration (f) on prodigiosin yield production via *S. marcescens* TNU2 fermentation. Phosphate and sulfate salts were not added into the control media (c and e). Standard errors (SE) are shown by the error bars in the figure.

3.3. Evaluation of nematicidal effect of podigiosin purified from the culture broth

PG was extracted and purified from the culture broth using the protocol presented in the previous report of Nguyen, T.H. et al., 2022a and its purity was confirmed by using high-performance liquid chromatography (HPLC) analysis. As shown in Figure 3, the purified PG appeared at a retention time (RT) of 12.28 min, which is approximately similar

to those of PG purified in previous studies (Nguyen, V.B. et al. 2021; Nguyen, T.H. et al. 2021, 2022a, 2022b). In addition, this purified PG appeared as the major single peak, as such, it is qualified enough for biological assaying in the next experiments.

In this study, we assessed the antinematicidal effect and comparison with this activity of purified PG and the crude sample via the assays of anti-*J2* and eggs of black pepper *Meloidogyne* sp. As presented in Table 1, PG demonstrated a high nematicidal effect with J2 nematodes molarity of 100% and IC50 values of anti-J2 nematodes of 0.197 mg/mL. A crude sample was also found showing a potent nematicidal effect with J2 nematodes molarity of 98.2% and IC50 values of anti-J2 nematodes of 0.263 mg/mL. The experimental data also indicated that PG and crude sample are potential anti-egg hatching agents with max mortality and IC50 values of 86.5%, 0.33 mg/mL and 90.2%, 0.27 mg/mL, respectively, after 3 days of treatment.

Recently, some secondary metabolites were produced, isolated and identified from microbes isolated in the central highlands of Vietnam. Some compounds were reported to show nematicidal activity against black pepper nematodes. Hemi-pyocyanin produced by *Pseudomonas aeruginosa* TUN03 fermentation showed moderate effect of anti-*J2* and anti-egg hatching with IC50 values of 0.746 mg/mL and 0.441 mg/mL (Nguyen, T.H. et al. 2022b). The two novel antinematicidal compounds, including thymine and hexahydropyrrolo[1,2-a] pyrazine-1,4-dione were produced by *Bacillus veleznesis* RB.EK7 (Trinh, T.H.T. et al 2022). Thymine showed potent nematicidal activity with 100% mortality of *J2* nematodes and an anti-egg hatching effect of 70.1%, while hexahydropyrrolo [1,2-a]pyrazine-1,4-dione demonstrated moderate ntinematicidal activity with 64.2% mortality of *J2* nematodes and anti-egg hatching activity of 57.9 %.

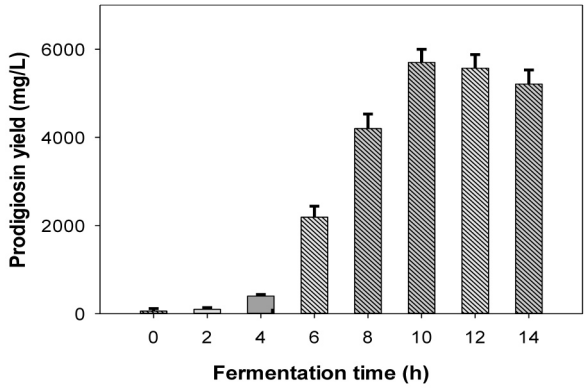


Figure 2. Scale-up production of prodigiosin using a 14 L- bioreactor system. Standard errors (SE) are shown as error bars.

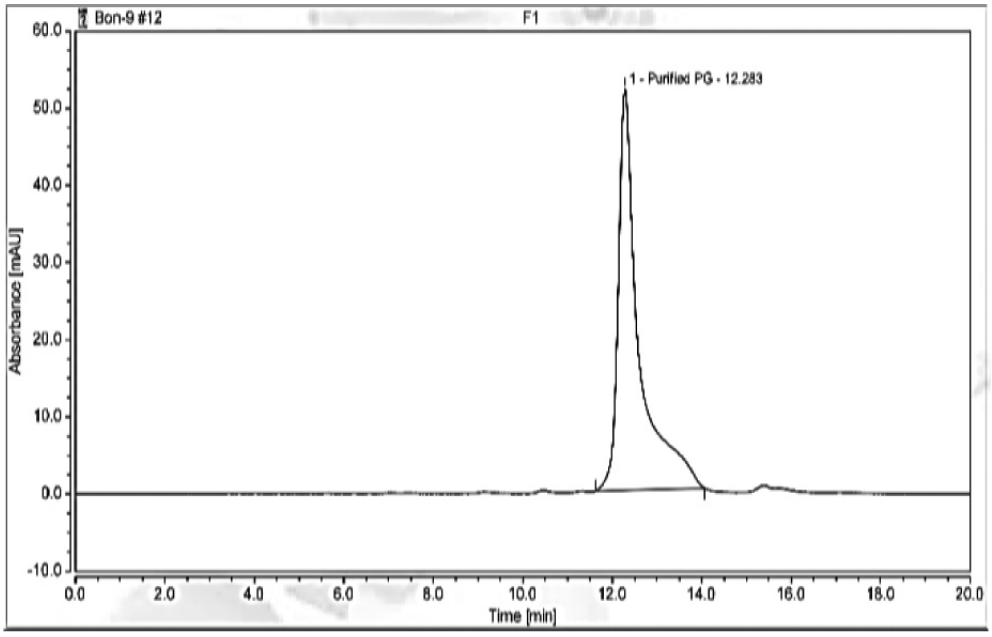


Figure 3. The HPLC profile of prodigiosin purified in this work.

PG was dissolved in methanol at 1 mg/mL concentration and then injected (3 µL) into the HPLC system then separated via a C18 column. 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.

Table 1. The nematicidal effect of purified PG and crude sample

Sample	Anti-J2 nematode		Anti-egg hatching	
	IC50 mg/mL	Max %	IC50 mg/mL	Max %
Prodigiosin	0.197	100	0.33	86.5
Crude sample	0.263	98.2	0.27	90.2

Note: the max inhibition value was recorded at the tested concentration of the sample at 1 mg/mL

4. CONCLUSIONS

PG was scale-up produced using a 14-L bioreactor system with suitable conditions: 6 L of medium containing 1.75% C/N (SRBP/casein at the ratio of 8/2), 0.05% $MgSO_4$, 0.1% K_2HPO_4, with an initial pH of 7.0, an initial pH of 7, 27.5°C in 10 h of cultivation time. This work indicated soybean waste is a potential source for fermentation to produce PG which may be a potentially antinematicidalcandidate.

5. FUNDING

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TĂNG CƯỜNG SINH TỔNG HỢP HOẠT CHẤT PRODIGIOSIN TỪ PHỤ PHẨM BÃ ĐẬU TƯƠNG BẰNG CÔNG NGHỆ LÊN MEN

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TÓM TẮT

Prodigiosin (PG), một hợp chất màu đỏ được sinh tổng hợp chủ yếu bởi vi khuẩn *Serratia marcescens*. Hiện tại, PG đã được nghiên cứu nhiều về các khía cạnh như sinh tổng hợp và đánh giá hoạt tính sinh học. Tuy nhiên, trong vấn đề sinh tổng hợp PG các nghiên cứu trước chủ yếu sử dụng các môi trường dinh dưỡng thương mại làm nguồn cacbon/nitơ (C/N), và các thí nghiệm lên men thường được tiến hành ở quy mô nhỏ (bình tam giác). Hướng tới tính hiệu quả và thân thiện môi trường trong quá trình sản xuất PG, chúng tôi tiến hành nghiên cứu sử dụng phụ phẩm bã đậu tương (SRBP) làm nguồn C/N cho quá trình lên men tổng hợp PG trên hệ thống bioreactor 14 lít và đánh giá tiềm năng kháng tuyến trùng của PG. Kết quả thực hiện cho thấy chủng vi khuẩn *Serratia marcescens* TNU2 tổng hợp PG với sản lượng cao (5.700 mg/L) trong môi trường lỏng gồm 1,75% cacbon/nitơ (SRBP/casein ở tỷ lệ phối trộn 8/2), 0,05% $MgSO_4$, 0,1% K_2HPO_4, pH ban đầu của môi trường 7,0, lên men ở 27,5°C sau 10 h trên hệ thống bioreactor. Kết quả đánh giá hoạt kháng tuyến trùng gây hại tiêu đen của PG ở nồng độ 1 mg/mL cho thấy tỷ lệ gây chết tuyến trùng tuổi 2 là 100% và ức chế trứng nở tới 87,43% sau thời gian xử lý tương ứng 1 ngày và 3 ngày. Kết quả của nghiên cứu này cho thấy phụ phẩm bã đậu tương là nguồn cơ chất tiềm năng cho lên men sinh tổng hợp hoạt chất PG và cũng minh chứng về tiềm năng ứng dụng hoạt chất PG trong quản lý hiệu quả tuyến trùng nốt sưng gây hại trên tiêu đen.

Từ khóa: *Bã đậu tương, prodigiosin, bioreactor, Serratia marcescens, tuyến trùng nốt sưng.*

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