UTILIZATION OF SOYBEAN RESIDUE BY PRODUCT FOR BIOPRODUCTION OF 1-HYDROXYPHENAZINE - A POTENTIAL FUNGICIDAL COMPOUND

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ABSTRACT

1-Hydroxyphenazine (HP) is a heterocyclic nitrogenous compound which reported various potential bioactivities. The current work aimed to utilize soybean residue by-product (SRBP) as a C/N source for the biosynthesis of HP via fermentation and investigated its potent inhibition against some plant pathogen fungi. The novel medium for effective HP production by *P. aeruginosa* TUN03 was investigated comprising 1.75% C/N source (SRBP/Tryptic Soy Broth (TSB) ratio of 8/2), 0.05% $FeSO_4$, and 0.05% K_2HPO_4 to obtain a high yield of HP at 19.23 µg/mL in a small flask. HP was pathogen using a 14 L-bioreactor and achieved higher productivity (32.01 µg/mL) in a shorter fermentation time (10 h) compared to its fermenting at a small scale in flask. In anti-fungal tests, this compound showed potential inhibition against *Fusarium oxysporum* F10 with a high inhibitory value of 65% and moderate effect against *Gongronella butleri* F07 (35%) and *Fusarium incarnatum* F13 (30%). HP was further evaluated for its effect against spore germination of *F. oxysporum* F10 and showed high inhibition value of 75%. The result of this work suggested that SRBP is a suitable C/N source for the production of HP with potential use a fungicidal agent.

Keywords: *soybean residue by- product, 1-Hydroxyphenazine, bioreactor, anti-fungal activity.*

1. INTRODUCTION

Phenazine - a nitrogenous and heterocyclic compound has the formula of $(C_6H_4)_2N_2$. More than 100 phenazine compounds have been found in nature and their chemical structures are formed from the basic chemical structure of phenazine (Cimmino A. et al., 2012). Almost all the natural phenazine derivatives are biosynthesized via microbial fermentation, and more than 50 phenazines were reported being produced by *Pseudomonas* species (Liu H. et al., 2007), of which *Pseudomonas aeruginosa* is a major phenazines-producing bacterial strain, as such this bacterium has been extensively used in the biosynthesis of phenazines.

In various earlier reports, commercial media were used as a C/N source for *P. aeruginosa* fermentation to produce phenazines, and the culture processes were mainly performed on a minor scale of flasks (Nguyen T.H. et al., 2022a). Concerning eco-friendly and cost-effective production, we established the fermentation process using some organic by-products, such as squid pens (Nguyen T.H. et al., 2022a), cassava residue (Phan T.Q. et al., 2024), and groundnut cake (Nguyen V.B. et al., 2023). In this study, soybean residue by-product (SRBP) was considered used for fermentation.

Soybean is a species of legume, which is native to East Asia, widely grown for harvesting its edible bean. Soybean has been widely used for oil extraction, production of soymilk, and other products (Riaz M.N. et al., 2023). soybean residue by-product (SRBP) is the main by-product from oil extraction and soymilk manufacturing with a reported highest residue yield of nearly 90% (Sun Y. et al., 2021). This residue was found rich in protein (16.1-33.4%), fat (8-22.3%), carbohydrates (under 53.6%), and total minerals (0.2-5.3%) in a survey by Shuhong Li et al 2013. Thus, SWRP has been utilized for many purposes. In this work, SWRP was used as a C/N source for fermentation to produce *1*-hydroxyphenazine (HP) – a phenazine compound with potential fungicidal effects.

To date, phenazines have been reported for their potential fungicidal effects against pathogenic fungi in human (Zahraa J.J. et al., 2017; Sudhakar T. et al., 2017), and toxigenic fungi in food (Zahraa J.J. et al., 2017; Mohamed N.F.H. et al., 2020; Hina S. et al., 2021). Notably, phenazines showed inhibition against various plant pathogenic fungi, including *Botrytis cinerea, Alternaria alternate, Fusarium graminearum, Fusarium oxysporum, Magnaporthe grisea, Rhizoctonia solani, Sitophilus oryzae, Macrophomina phaseolina, Colletotrichum capsici, Colletotrichum gloeosporioides, Comastoma falcatum, Phaeosphaerella theae* based on our recent survey (Nguyen T.H. et al., 2022b). Recently, HP was found as a potential fungicide against *Fusarium oxysporum* with the anti-mycelial growth and anti-

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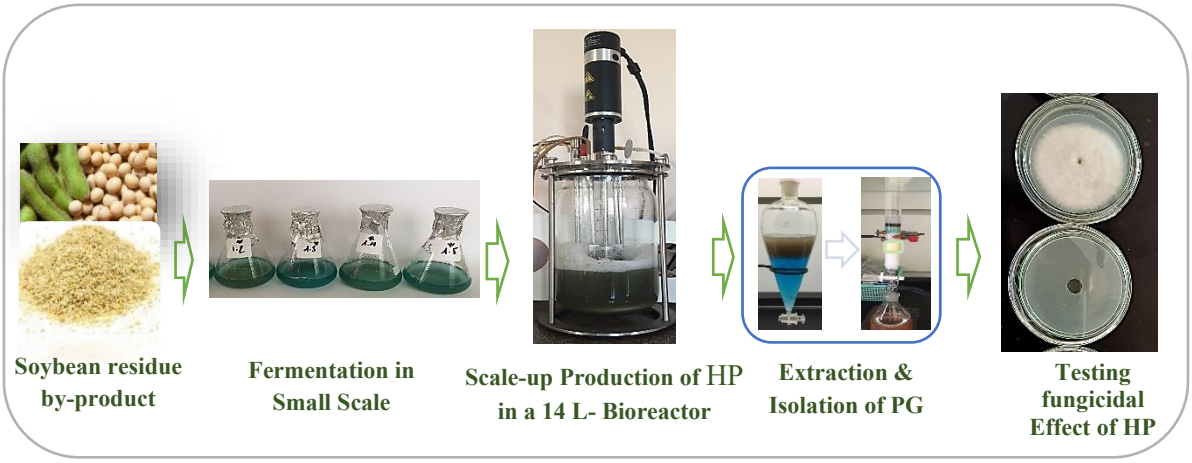
spore germination activity of 68.7% and 98.4%, respectively (Phan T.Q. et al., 2024). In this study, we evaluated the potential fungicidal effects of HP against some plant pathogenic fungi collected in the Central Highland of Vietnam.



Scheme 1. The steps and contents for performing experiments in this work

2. MATERIALS AND METHODOLOGY

2.1. Study contents

- Establishment of the fermentation process for the production of 1-hydroxyphenazine.

- Purification of 1-hydroxyphenazine from the culture medium.

- Assessment of the potential fungicidal activity of 1-hydroxyphenazine.

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

2.2. Materials

P. aeruginosa TNU03 was isolated from the soil of Dak Lak province of Vietnam in the previous work (Nguyen T.H. et al., 2022c). Silicagel (Geduran® Si 60, size: 0.040-0.063 mm) was purchased from Merck Sigma Chemical Co. (St. Louis City, MO, USA). Some plant pathogenic fungi were obtained from our earlier report (Nguyen D.N. et al., 2021).

2.3. Methodology

2.3.1. The effect of SRBP/TSB ratio and their concentration added in medium on HP yield

- The fermentation process: SRBP was combined with TSB at several ratios (SRBP/TSB, w/w) of 5/5, 6/4, 7/3, 8/2, 9/1, and 10/10 then used as C/N sources for cultivation. 30 mL culture medium in a 100 mL-flask containing 1% C/N source, 0.05% K_2HPO_4, and 0.05% $FeSO_4$, initial pH 7 was fermented by *P. aeruginosa* TUN03 at 30°C with a shaking speed of 150 rpm for 3 days (this fermentation process is denoted by *). The supernatant was harvested by centrifugation at 10,000× *g* for 15 min and used to determine the target compound (HP) concentration.

- Various concentrations of C/N source (SRBP/TSB at the ratio of 8/2), including 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0% were prepared in a liquid medium containing 0.05% K_2HPO_4 and 0.05% $FeSO_4$ with initial pH 7, then were fermented using the above protocol (*) to investigate the suitable C/N concentration.

- Scale-up production of HP in a bioreactor system: bacterial strain TNU03 was pre-cultivated in culture broth TSB in some 500 mL flasks at the temperature of 30 °C for 1.5 days, then a total of 400 mL of fermented medium (bacterial inoculum) was added to the reactor containing 4.6 L of the newly investigated culture medium containing 1.5% C/N source (SRBP/TSB at the ratio of 8/2), 0.05% K_2HPO_4 and 0.05% $FeSO_4$, initial pH 7.0 The fermentation was performed at 30°C, 250 rpm, and dissolved oxygen of 1.2 vvm for 12 h, and the HP produced by TNU03 was determined every 2 h.

2.3.2. The purification and evaluation of fungicidal activity of HP

HP was isolated from the culture broth in a bioreactor according to the protocol previously presented in our report (Nguyen T.H. et al., 2022c). Its GCMS and HPLC profiles were conducted following the method reported by Nguyen V.B. et al. (2023).

The fungicidal activity was tested via mycelial growth inhibition and fungal spore germination inhibition, which were presented in detail in our previous work (Nguyen T.H. et al., 2024).

2.3.3. 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 the production of HP.

The effect of SRBP/TSB ratio and their concentration added in medium on HP yield:

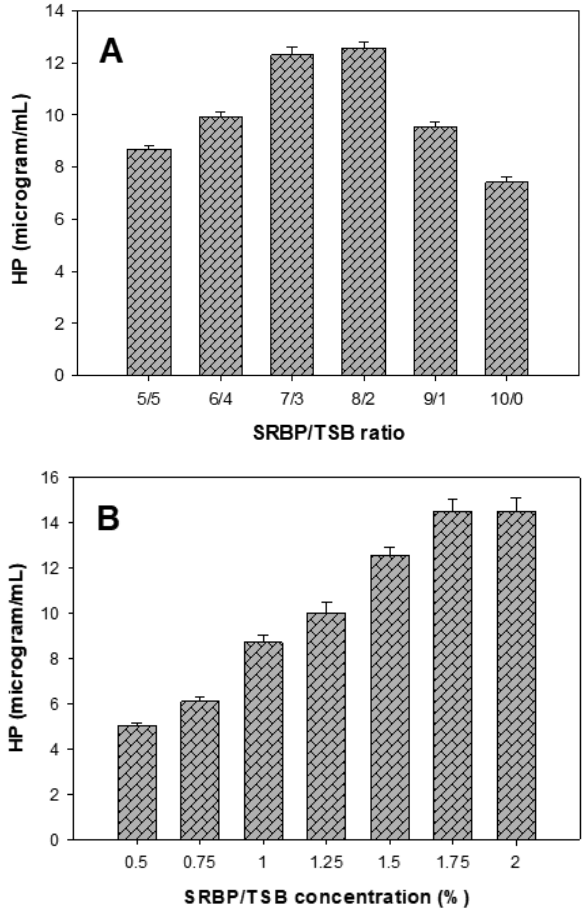
TSB was used as free protein for adding into the culture medium. SRBP was combined with this free protein at several ratios and used as a C/N source for fermentation. As shown in Figure 1A, the SRBP/TSB ratios of 7/3 and 8/2 gave the high content of HP (12.3-12.5 µg/mL) in the fermented medium. For cost-effectiveness, the ratio of 8/2 was chosen for further investigation to determine the most suitable C/N source concentration. The C/N source (SRBP/TSB at the ratio of 8/2) was added into the cultured media with various concentrations of 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0% then used for fermentation by *P. aeruginosa* TUN03. As presented in the Figure 1B, the C/N source at the concentration of 1.75-2% gave the high yield of HP (14.50-14.52 µg/mL) in the fermented media, and 1.75% C/N was chosen as the suitable and cost-effective for fermentation to produce HP.

Phenazine compounds were reported to numerous bioactivities and as such received much attention for their production. In many previous reports, commercial media like King's B, King's A, tryptone, peptone, and nutrient broth for fermentation and phenazines were produced at the content of 5.2-33.0 µg/mL (Elbargisy R.M. 2021; Devnath P. et al., 2021; Ozdal M. et al., 2019; Barakat K.M. et al., 2015; Ozdal M. 2019). For the lower cost of bioproduction of phenazines, several low-cost organic materials such as corn, cottonseed, sweet potato, soya bean, groundnut, peat moss, and taro leaves were investigated for phenazines producing by fermentation, however, these compounds were synthesized at low productivity under 4 µg/mL mL (DeBritto S. 2020; El-Fouly M.Z. et al., 2015).

Phenazines were also produced by using some organic wastes, such as tea wastewater, waste cheese whey, waste frying oil, olives waste, maize wastewater, sugar beet molasses, craft beer waste, peapods, and the phenazines yield were obtained in the rang of 1.3-58 µg/mL. Of these, craft beer waste was found as the most potential C/N source for fermentation, and phenazines were produced at a high of 21-58 µg/mL according to a recent survey by Nguyen et al. (2023). In this study, soybean residue by-product (SRBP) combined with a minor amount of TSB was first reported as being used as a potential C/N source for fermentation to produce HP with a high yield of 14.52 µg/mL.

Scaling up of 1-hydroxyphenazine production using a 14 L-bioreactor system

In microbial fermentation technology, bioreactor systems are considered valuable quipment to effectively bio-produce secondary metabolites with a high-level yield and reduce the fermentation time (Nguyen, T.H. et al. 2021). In this work, HP was produced in mass using a 14 L-bioreactor system. As illustrated in Figure 1C, HP was synthesized by *P. aeruginosa* TUN03 with a significant amount from 6 h of cultivation. The HP yield was significant enhanced till 10 h (reached the yield of 32.01 µg/mL), and no further HP was produced from 10-12 h of fermentation.



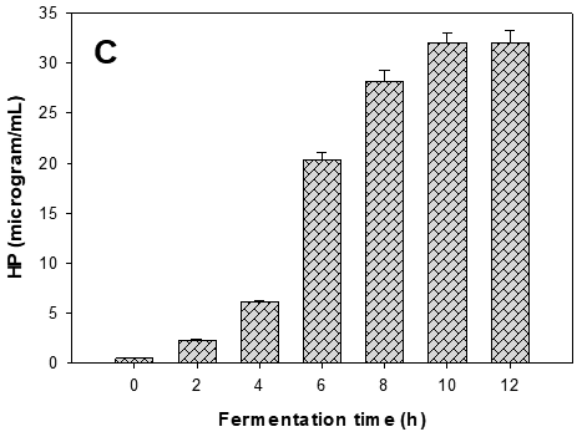


Figure 1. Production of 1-Hydroxyphenazine (HP) via fermentation.

*Note: The effect of SRBP/TSB ratio (**A**) and their concentration added in medium (**B**) on HP yield. Scaling up of HP production using a 14 L-bioreactor system).*

Recently, HP was produced in bioreactor systems using peanut oil processing by-product (Nguyen V.B. et al., 2023) and cassava starch processing by-product (Phan T.Q. et al., 2024) resulting in harvesting high productivity of 35.1 and 36.5 µg/mL, respectively.

3.2. The purification and evaluation of fungicidal activity of HP.

The phenazine compound was isolated and purified from the cultured broth in a bioreactor system using the method presented in our earlier report (Nguyen T.H. et al., 2022c). The purified yellow compound was confirmed as 1-Hydroxyphenazine using GCMS analysis (Figure 2). Based on the HPLC profile of HP produced in this work, it was found to have a high purity (Figure 3) as such it could be used for further study in bioactivity tests.

To confirm the purified compound – HP produced in this study is an active fungicidal compound, various plant pathogenic fungal strains obtained from the previous work (Nguyen D.N. et al., 2021) were used for testing the fungicidal effect of HP. As shown in Table 1, HP shows inhibition against some fungal strains, including *Fusarium solani* F02, *Fusarium solani* F03, *Fusarium solani* F04, *Gongronella butleri* F07, *Fusarium oxysporum* F10, and *Fusarium incarnatum* F13 with the inhibition values in the range of 7-65%. Of those, HP had a weakly inhibition against *Fusarium solani* F02, *Fusarium solani* F03, *Fusarium solani* F04 with an inhibition of 7-16%, moderately effect against *Gongronella butleri* F07 (35%) and *Fusarium incarnatum* F13 (30%). This compound showed the most effect against *Fusarium oxysporum* F10 with a high inhibitory value of 65%. The result of this work suggested HP may be a high potential candidate for management of *Gongronella butleri* F07, *Fusarium oxysporum* F10 and *Fusarium incarnatum* F13.

The fungal spores were reported to play an important role in the pathogenesis of pathogenic fungal strains, in addition, this is also recognized as the most sensitive stage to inhibition, as such, assessing the inhibitory effect of both mycelial growth and spore germination is an effective strategy for the phytopathogenic fungi management (Phan T.Q. et al., 2024). To futher characterize HP as a potential fungicide against *Fusarium oxysporum* F10, the fungal spore germination inhibition of HP was also tested. The result showed that HP demontated better inhibitory effect for spore germination (75%) than inhibition against mycelial growth (65%).

Table 1. Inhibitory effects of HP on some pathogenic fungal strains

Fungal strains	Anti-fungi (%)
Fusarium solani F02	7
Fusarium solani F03	16
Fusarium solani F04	11
Gongronella butleri F07	35
Fusarium oxysporum F10	65
Fusarium incarnatum F13	30

4. CONCLUSIONS

This study achieved finding a novel culture medium comprising 1.75% C/N source (SRBP/TSB ratio of 8/2), 0.05% $FeSO_4$, and 0.05% K_2HPO_4. HP was then scale-up produced using a 14-L bioreactor system resulting in a high HP yield of 32.01 µg/mL. The purified HP demonstrated potential inhibition against *Fusarium oxysporum* F10 (65%) and a moderate effect against *Gongronella butleri* F07 (35%) and *Fusarium incarnatum* F13 (30%). In addition, HP also demontated high effect against *F. oxysporum* F10 spore germination of (75%).

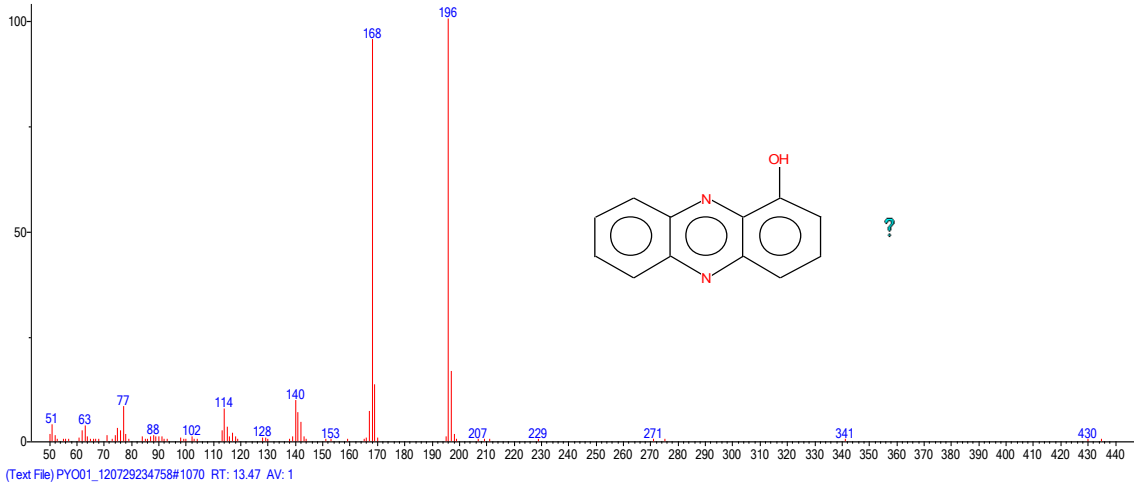


Figure 2. The GCMS profile of 1-hydroxyphenazine

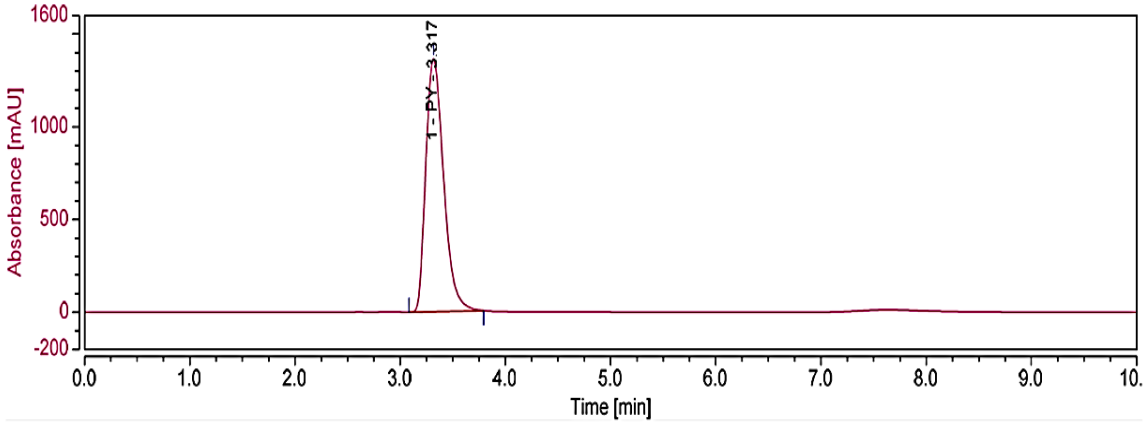


Figure 3. The HPLC profile of 1-hydroxyphenazine purified in this work.

ỨNG DỤNG PHỤ PHẨM BÃ ĐẬU TƯƠNG ĐỂ SINH TỔNG HỢP 1-HYDROXYPHENAZINE - MỘT HỢP CHẤT KHÁNG NẤM TIỀM NĂNG

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TÓM TẮT

1-Hydroxyphenazine (HP) là một hợp chất nitơ dị vòng có nhiều hoạt tính sinh học tiềm năng đã được công bố. Mục tiêu của nghiên cứu này là sử dụng phụ phẩm bã đậu tương (SRBP) làm nguồn C/N cho quá trình lên men sinh tổng hợp hoạt chất HP và đánh giá tiềm năng ức chế trên một số loài nấm hại thực vật. Môi trường thích hợp để sinh tổng hợp HP hiệu quả bằng chủng vi khuẩn *P. aeruginosa* TUN03 đã được thiết lập. Thành phần môi trường bao gồm 1,75% nguồn C/N (tỷ lệ SRBP/Tryptic Soy Broth (TSB) là 8/2), 0,05% FeSO4 và 0,05% K2HPO4, hoạt chất HP được sinh tổng hợp với hàm lượng cao (19,23 µg/mL) trong điều kiện bình tam giác trong 12h và đạt (32,01 µg/mL) trong bioreactor 14 L, với thời gian lên men 10h. Kết quả đánh giá hoạt tính kháng nấm cho thấy hợp chất này ức chế mạnh đối với nấm *Fusarium oxysporum* F10 với giá trị ức chế là 65% và tác dụng ức chế tương đối với nấm *Gongronella butleri* F07 (35%) và *Fusarium incarnatum* F13 (30%). HP tiếp tục được đánh giá tác dụng ức chế nẩy mầm bào tử nấm *F. oxysporum* F10 và cho giá trị ức chế là 75%. Kết quả của nghiên cứu này cho thấy SRBP là nguồn C/N thích hợp để sản xuất HP và HP là chất có tiềm năng sử dụng làm tác nhân diệt nấm.

Từ khóa: *Bã đậu tương, 1-Hydroxyphenazine, bioreactor, hoạt tính kháng nấm.*

REFERENCES

Barakat K.M., Mattar M.Z., Sabae S.Z., Darwesh O.M., Hassan S.H (2015). Production and characterization of bioactive pyocyanin pigment by marine *Pseudomonas aeruginosa* Osh1. *Res. J. Pharm. Biol. Chem. Sci*., 6: 933-943.

Cimmino A., Evidente A., Mathieu, V., Andolfi A., Lefranc F., Kornienko A., Kiss A (2012). Phenazines and cancer. *Nat. Prod. Rep*., *29:* 487.

DeBritto S., Gajbar T.D., Satapute P., Lalitha S., Ramachandra Y.L., Sudisha J., Shin-ichi I (2020). Isolation and characterization of nutrient dependent pyocyanin from *Pseudomonas aeruginosa* and its dye and agrochemical properties. *Sci Rep.,* 10: 1542.

Devnath, P.; Uddin, M.K.; Ahmed, F.; Hossain, M.T.; Manchur, M.A (2017). Extraction, purification and characterization of pyocyanin produced by *Pseudomonas aeruginosa* and evaluation for its antimicrobial activity. *Int. Res. J. Biol. Sci*., 2017, *6*, 1–7.

Elbargisy R.M (2021). Optimization of nutritional and environmental conditions for pyocyanin production by urine isolates of *Pseudomonas aeruginosa*. *Saudi J. Biol. Sci*., 28: 993–1000.

El-Fouly M.Z., Sharaf A.M., Shahin A.A.M., El-Bialy H.A., Omara A.M.A (2015). Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. *J. Radiat. Res. Appl. Sci., 8*: 36–48.

Hina S., Sania M., Quratulain S., Muhammad Q.J., Ahmad A (2021). Bio-characterization of food grade pyocyanin bio-pigment extracted from chromogenic *Pseudomonas* species found in Pakistani native flora. *Arab. J. Chem*., 14: 103005.

Li S., Zhu D., Li K., Yang Y., Lei Z., Zhang Z (2013). Soybean Curd Residue: Composition, Utilization, and Related Limiting Factors. *International Scholarly Research Notices,* 1: 1-8.

Liu H., He Y., Jiang H., Peng H., Huang X., Zhang X., Linda S.T., Xu Y (2007). Characterization of a phenazine-producing strain *Pseudomonas chlororaphis* GP72 with broad-spectrum antifungal activity from green pepper rhizosphere. *Curr. Microbiol.,* 54: 302–306.

Mohamed N.F.H., Diaa A.M., Sherien M.R.E (2020). Toxicity Evaluation and Antimicrobial Activity of Purified Pyocyanin from *Pseudomonas aeruginosa*. *Biointerface Res. Appl. Chem*., 10(6): 6974 – 6990.

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Nguyen D.N., Wang S.L., Nguyen A.D., Doan M.D., Tran D.M., Nguyen T.H., Ngo V.A., Doan C.T., Tran T.N., Do V.C., Nguyen V.B (2021). Potential Application of rhizobacteria isolated from the Central Highland of Vietnam as an effective biocontrol agent of robusta coffee nematodes and as a bio-fertilizer. *Agronomy,* 11: 1887.

Nguyen T.H. et al (2022a). Utilization of Fishery-Processing By-Product Squid Pens for Scale-Up Production of Phenazines via Microbial Conversion and Its Novel Potential Antinematode Effect. *Fishes,* 7: 113.

Nguyen T.H., Nguyen V.B (2022b). Overview of phenazine compounds biosynthesized by *Pseudomonas aeruginosa* and their bioactivities. *Tay Nguyen journal of Science*, 16 (52).

Nguyen T.H., Wang S.L. Phan T.Q. *et al.* (2024). New record of reusing brewing by-product for biosynthesis of prodigiosin and its novel anti-pathogen fungi via in vitro tests and molecular docking study. *Res Chem Intermed.,* 50: 925–949.

Nguyen T.H., Wang S.L., Nguyen A.D., Doan M.D., Tran T.N., Doan C.T., Nguyen V.B (2022c). Novel α-amylase inhibitor hemi-pyocyanin produced by microbial conversion of chitinous discards. *Mar. Drugs*, 20: 283.

Nguyen V.B., Wang S.L., Nguyen A.D (2023). Bioconversion of a Peanut Oil Processing By-Product into a Novel α-Glucosidase Inhibitor: Hemi-Pyocyanin. Processes, 11: 1468.

Ozdal M (2019). A new strategy for the efficient production of pyocyanin, a versatile pigment, in *Pseudomonas aeruginosa* OG1 via toluene addition. *3 Biotech,* 9: 370.

Ozdal M., Gurkok S., Ozdal O.G., Kurbanoglu E.B (2019). Enhancement of pyocyanin production by *Pseudomonas aeruginosa* via the addition of n-hexane as an oxygen vector. *Biocatal. Agric. Biotechnol.,* 22: 101365.

Phan T.Q., Wang S.L., Nguyen T.H., Nguyen T.H., Pham T.H.T., Doan M.D., Tran T.H.T., Ngo V.A., Nguyen A.D., Nguyen V.B (2024). Using Cassava Starch Processing By-Product for Bioproduction of *1*-Hydroxyphenazine: A Novel Fungicide against *Fusarium oxysporum*. Recycling, 9: 12.

Riaz M.N (2006). *Soy Applications in Food*. Boca Raton, FL: CRC Press. ISBN 978-0-8493-2981-4.

Sudhakar T., Karpagam S., Shiyama S (2013). Antifungal efficacy of pyocyanin produced from bioindicators of nosocomial hazards. *Int J ChemTech Res*, 5: 1101-1106.

Sun Y., Li C., Zhang S., Li Q., Gholizadeh M., Wang Y., Hu S., Xiang J., Hu. X (2021). Pyrolysis of soybean residue: Understanding characteristics of the products. *Renewable Energy,* 174: 487-500.

Zahraa J.J., Anaam F.H., Muthana Â., Nuha F.A., Eman S.A (2017). Bioactivity of pyocyanin of *Pseudomonas aeruginosa* clinical isolates against a variety of human pathogenic bacteria and fungi species. *Int. J. Antimicrob. Agents*, 7(3).