 doi:10.20944/preprints202208.0150.v1

Article

Novel Black Pepper Antinematode Compounds Produced by *Bacillus velezensis* RB.EK7 Conversion of Organic Wastes

Thi Huyen Trang Trinh [1], San-Lang Wang [2,3]*, Van Bon Nguyen [4]*, Tu Quy Phan [1], Manh Dung Doan [4], Thi Phuong Hanh Tran [1], Thi Huyen Nguyen [4], Thi Anh Hong Le [5], That Quang Ton [6] and Anh Dzung Nguyen [4]*

[1] Department of Science and Technology, Tay Nguyen University, Buon Ma Thuot 630000, Vietnam; tth-trang@ttn.edu.vn (T.H.T.T); phantuquy@ttn.edu.vn (T.Q.P); ttphanh@ttn.edu.vn (T.P.H.T.)
[2] Department of Chemistry, Tamkang University, New Taipei City 25137, Taiwan; sabulo@mail.tku.edu.tw (S.L.W)
[3] Life Science Development Center, Tamkang University, New Taipei City 25137, Taiwan
[4] Institute of Biotechnology and Environment, Tay Nguyen University, Buon Ma Thuot 630000, Vietnam; nvbon@ttn.edu.vn (V.B.N); dmdung@ttn.edu.vn (M.D.D); nthuyen@ttn.edu.vn (T.H.N); nadzung@ttn.edu.vn (A.D.N);
[5] Institute of Tropical Biology, Vietnam Academy of Science and Technology, Ho Chi Minh City 700000, Vietnam; anhhongbi@yahoo.com (T.A.H.L)
[6] Faculty of Chemistry, University of Science, Ho Chi Minh City 700000, Vietnam; ttquang@hcmus.edu.vn (T.Q.T)
* Correspondence: sabulo@mail.tku.edu.tw (S.L.W); nnvbon@ttn.edu.vn (V.B.N); adzung@ttn.edu.vn (A.D.N);

Abstract: *Bacillus veleznesis* RB.EK7 was recently found as a potent rhizobacterial strain for effective management of black pepper root-knot nematodes. This work aimed to produce, purify, and elucidate the chemical structures of antinematode compounds (ANCs). Concerning cost-effectiveness and environmental issues, this study used organic wastes for the bioproduction of ANCs. Among various substrates, shrimp shells powder was the most suitable carbon/nitrogen source to produce ANCs. The fermentation process for enhancement of antinematode activity was investigated. The targeting ANCs were purified from the fermented culture broth, and their structures were elucidated. Two active compounds were thymine (**1**) and hexahydropyrrolo [1,2-a]pyrazine-1,4-dione (**2**). Notably, for the first time, these purified compounds showed potential and moderate anti- *J2* nematodes and anti-eggs hatching, respectively. The docking study results indicated that the potent antinematode effect of these compounds may be possibly due to the inhibition of the targeting enzyme acetylcholinesterase. The data of this work suggest that organic waste SSP can be potentially reused for the production of thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione with promising use for the management of black pepper nematodes.

Keywords: black pepper; *Bacillus veleznesis*; root-knot nematodes; antinematodes compounds; organic wastes; microbial fermentation; thymine; hexahydropyrrolo [1,2-a]pyrazine-1,4-dione

1. Introduction

Black pepper is one of the important industrial crops with high economic value for export. Its product, peppercorn, has been considered a common daily spice and is the most widely traded spices reaching around 20% of all spices traded commercially [1-2]. This crop is widely planted in Vietnam, Indonesia, India, and Brazil. Of these countries, Vietnam is the largest producer and exporter of peppercorns, with approximately 40% of 546,000 tons worldwide produced [3]. However, the cultivation of this spicy plant faces various pathogen diseases, including the root–knot nematode [4], and one of the major nematodes that seriously damages black pepper is the *Meloidogyne incognita* species [5].

Up to now, many methods have been searched for management of root–knot nematode including using chemical nematicides, biological control using beneficial microbes, chemicals, and cultivars in cotton in a semi-arid environment, co-cultivation with various plants, destroying infected plants, and resistant planting materials [6-10]. Chemical ne-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

maticides are still the most effective means to manage nematodes. However, the long-term use of nematicide compounds such as carbamate and organophosphorus has led to increased nematode resistance, including a lack of effective field control and environmental pollution. Thus, there is a constant search for a new source of nematicidal compounds [6].

Nematicides may be obtained from chemical synthesis or natural sources such as plants and microbial fermentation [5,6,11]. The use of nematicides from chemical synthesis may cause environmental issues, reducing the quality of agro-products and the soil microbial diversity, as well as increasing the resistance of nematodes [11,12,13]. Thus, to reduce the toxicity and other problems, various natural compounds have been investigated for the potential management of nematodes [12]. Among the natural resources, the identification and production of ANCs from microbes has received much attention since they may be produced on an industrial scale for available demand [5]. In current microbial fermentation science, the aspect of reusing organic wastes for the cost-effective production of valuable compounds is an emerging research topic [14-17].

Considering the green and effective treatment of root–knot nematodes, in our earlier study, we screened for the beneficial microbes with antinematode effect in the Central Highlands of Vietnam [18] and *Bacillus veleznesis* RB.EK7 was found as a novel potent anti-nematode bacterial strain. In this work, the antinematode activity of *B. veleznesis* RB.EK7 was significantly enhanced via optimization of culture conditions using organic wastes as the C/N source for fermentation. The major ANCs were extracted, purified, and their chemical structures were identified. The docking study was also performed to investigate the molecular interaction of the ANCs toward their target protein – enzyme acetylcholinesterase.

2. Materials and Methods

2.1. Materials

Rhizobacterial strain *B. velezensis* RB.EK7 was obtained from our previous work [18]. Eggs of *Meloidogyne* sp. were isolated from their galls of the nematode on sick black pepper plants cultivated in Buon Ma Thuot city, which were then used for the preparation of *J2* nematodes. Shrimp shells powder (SSP) was obtained from Shin-Ma Frozen Food Co. (I-Lan, Taiwan), and some other organic wastes, including cassava residue waste (CRW), peanut oil processing by-product (groundnut cake, GC), and soybean residue waste (SRW) were obtained in Buon Ma Thuot city, Dak Lak province, Vietnam. Yeast extract, peptone, and Silica gel (Geduran® Si 60, size: 0.040-0.063 mm) were purchased from Sigma Chemical Co. (St. Louis City, MO, USA), and some solvents used in this work were from Sigma Aldrich.

2.2. Methods

2.2.1. Production of Antinematode Compounds by Bacillus velezensis RB.EK7 Fermentation

Screening Organic Wastes as C/N sources for fermentation: Various organic wastes, including soybean residue, shrimp shell powder, cassava residue (CR), and groundnut oil processing (GOP), and a commercial medium (yeast extract/peptone = 5/3) were used as C/N (0.8%) sources for fermentation. The salt composition of the medium included 0.6% KH_2PO_4 and 0.4% $(NH_4)_2SO_4$. The culture medium was sterilized by autoclaving at 121°C for 15 min. Fifty milliliters of the medium in a 250 mL Erlenmeyer flask was inoculated with 1 mL bacterial seed and fermented at 37°C in a rotary shaker operating at 150 rpm for 72 h. The supernatant was harvested by centrifugation at 10,000× g for 15 min and then used for estimation of antinematode activity. The shrimp shell powder was found to be the most suitable substrate to produce ANCs and was thus further used for further experimentation.

The effect of some culture conditions on antinematodes compounds production: To enhance antinematodes activity, some factors, including initial pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0), cultivation temperature (29, 31, 33, 35, 37 and 39 °C), shaking speed (0, 100,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

150, 200, and 250 rpm), and cultivation time (24, 48, 72, 96, and 120 h) were examined for their effect. The supernatant was harvested by centrifugation at 10,000× g for 15 min and then used for estimation of antinematode activity. The following experiments were designed based on the optimal conditions achieved from previous experiments.

2.2.2. Purification and Identification of Antinematodes Compounds and their Chemical structures

The culture supernatant (CS) of strain RB.EK7 was harvested by centrifugation at 10,000× g for 15 min. The CS was evaporated at 50°C to dried powder (199 g). Then, it was extracted by solid-liquid with solvent systems, including n-hexane, chloroform, ethyl acetate, ethyl acetate: methanol (90:10), ethyl acetate: methanol (60:40), and methanol. The extracts were obtained and evaporated under low pressure to recover the solvent, obtaining high grades. The crude extract was used to evaluate anti-nematode activities. The extracted phases of ethyl acetate: methanol (90:10) showed high nematicidal activity and mass. Thus, this extract was further used for the isolation of the ANCs.

The extracts with chloroform and ethyl acetate were mixed and further separated via a silica gel opened column and eluted with CH_2CL_2- MeOH (95:5, V/V) to obtain compound **1** (60.2 mg). The extract of ethyl acetate: methanol (90:10) was loaded onto a silica gel opened column and eluted with CH_2CL_2- MeOH (9:1, V/V), CH_2CL_2- MeOH (5:5, V/V), and MeOH to obtain five subfractions. From subfractions 5.4, compound **2** (28.1 mg) was isolated. The chemical structures of these active compounds were identified based on nuclear magnetic resonance (NMR) and mass spectra analysis and compared to those of the reported compounds in the previous studies. The purity of these purified compounds was confirmed by ultra-high-performance liquid chromatography (UHPLC). The extraction and purification process is summarized in Figure 1.

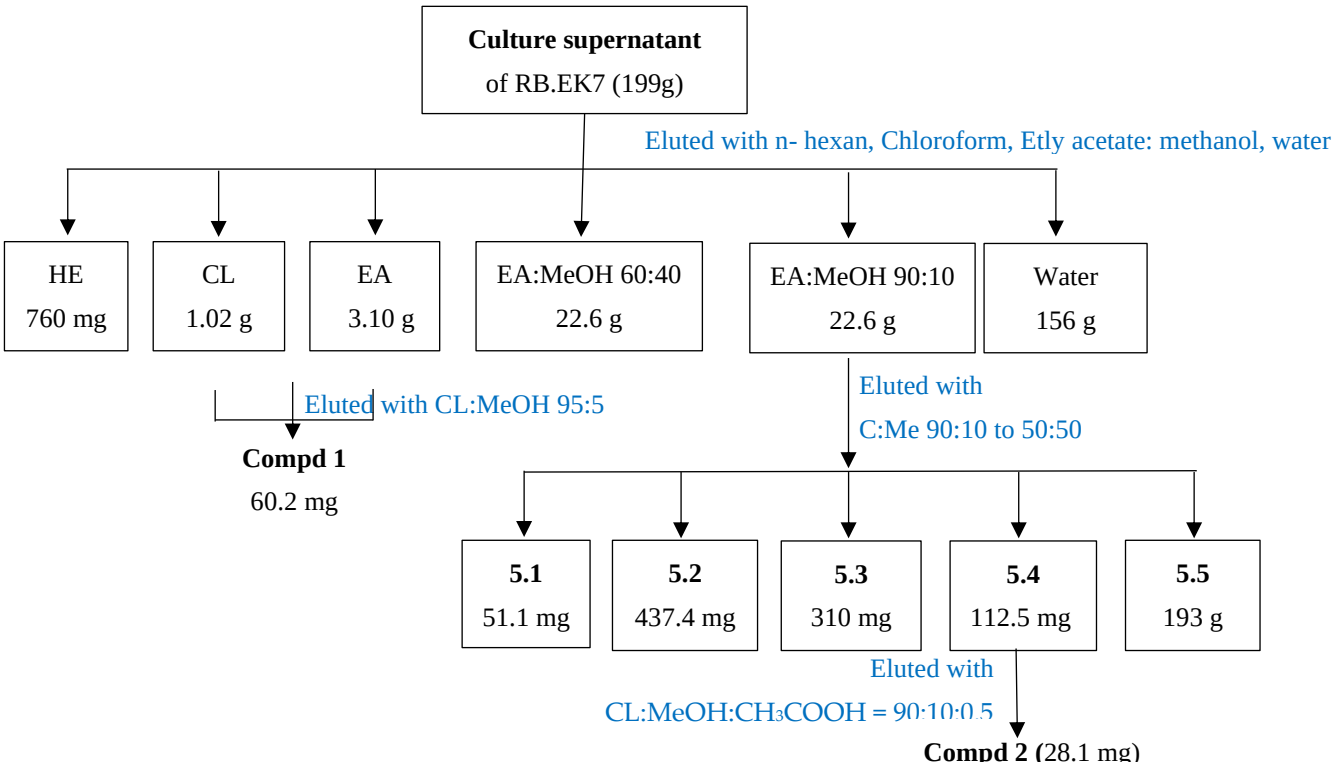


Figure 1. The scheme of extraction and purification of antinematode compounds. HE: hexane, CL: chloroform, EA: ethyl acetate, MeOH: methanol. .

2.2.2. Assays for Biological Activity

The antinematicidal assays were done following the methods presented in our earlier publications [5,19]. The seek black pepper roots were collected from Buon Ma Thuot, Dak Lak province, Vietnam, and the eggs and *J2* nematodes were prepared according to

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

the method of Khan *et al*. [20]. The process for the preparation of nematode eggs and *J2* root-knot nematodes from the sick black pepper roots is illustrated in Figure 2. The samples, including culture supernatants and compounds dissolved in DMSO at various concentrations, were tested for their nematicidal activity via the effect on J2 nematodes and egg-hatching, then the activity was expressed as the anti-J2 inhibition efficiency (%) and egg-hatching rate (%).

The anti-J2 nematode effect: Two hundred microliters of sample solution (200 µL) were mixed with 100 µL sterile distilled water containing 30 individuals of *J2* nematodes in a 1 mL - Eppendorf tube and kept at 20°C for 24 h. The number of immobilized nematodes was counted under a stereoscopic microscope Olympus SZ5 and used to estimate the activity. All the tests were carried out in triplicates.

The egg-hatching inhibitory effect: Two hundred microliters of the sample solution were mixed with 100 µL sterile distilled water containing 200 nematode eggs in a 1 mL - Eppendorf tube, and this mixture was kept at 20°C for 3 days. The number of hatched eggs was counted based on *J2* nematodes using a stereoscopic microscope Olympus SZ5. All the tests were carried out in triplicates.

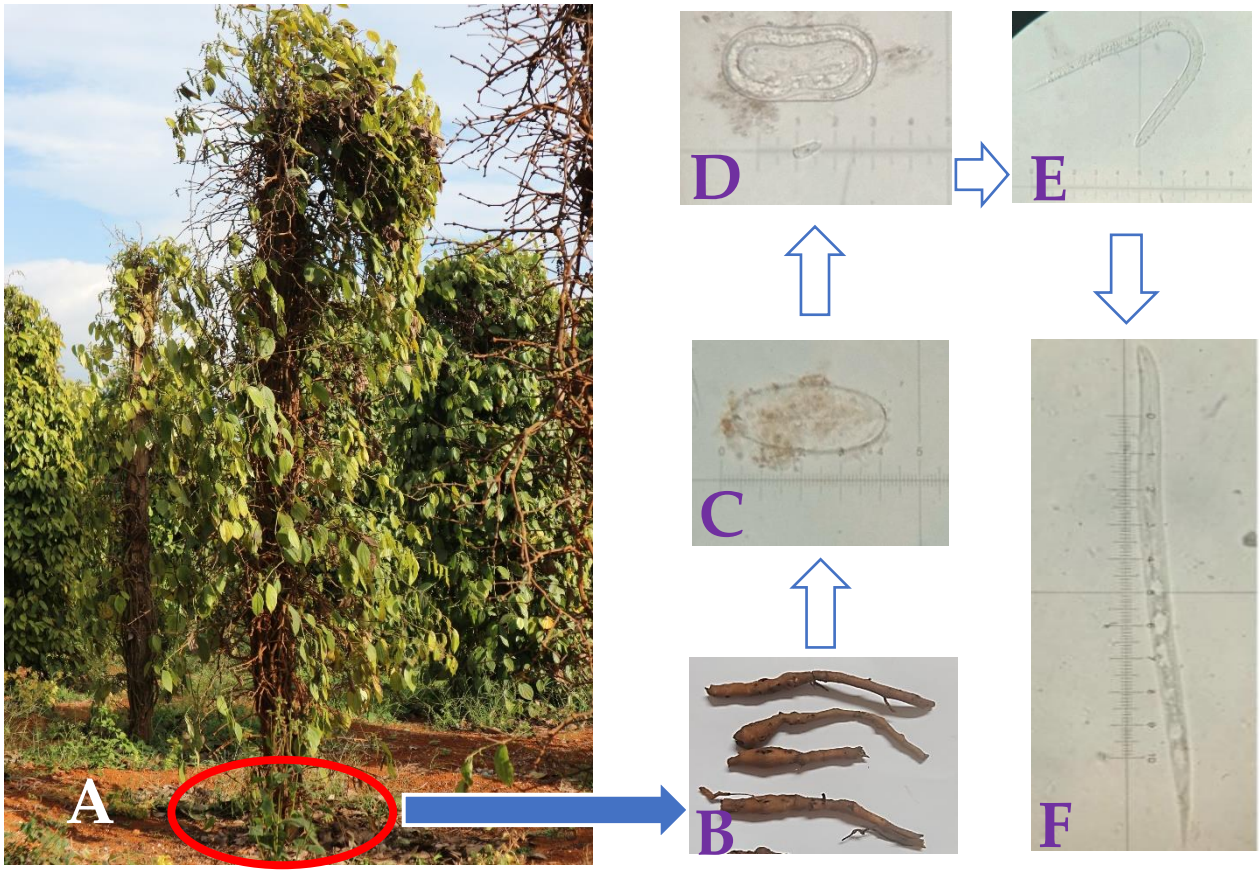


Figure 2. The process for the preparation of nematode eggs and *J2* root-knot nematodes from the sick black pepper roots. The sick black pepper with yellow leaves (A) was chosen for collecting its root-knots (B) which was used for isolation of nematodes eggs (C). The nematode eggs were further incubated for 3–5 days for the eggs to hatch (D), and *J2* root-knot nematodes were obtained (E). The J2 nematodes were immobilized (considered dead nematodes) after treatment with antinematode compounds (F).

2.2.3. Docking Study Protocol

The virtual study protocol was done according to the method presented in our earlier works [14,21,22] with three typical steps as below:

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

① ***Preparation of acetylcholinesterase (AChE) structure and the active sites on AChE:*** The protein structure data of AChE were obtained from the Worldwide Protein Data Bank, then the 3-D was produced by using MOE-2015.10 software. A virtual pH 8 was set to prepare the structures of protein structure. The active sites on AChE were selected using the site finder function in MOE software after removing all the water molecules.

② ***Preparation of ligands (inhibitor compounds):*** ChemBioOffice 2018 software was used for the preparation of ligands structures, which were further optimized using the MOE system with 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−1A−2. A virtual pH 8 was also set for the preparation of the structures of the ligands.

③ ***Docking performance of ligands into the active sites on AChE***: The virtual study was performed on the ligands with AChE using MOE-2015.10 software, and the output data, including Root Mean Square Deviation (RMSD), docking score (DS), interaction types (linkages), amino acid composition, interaction between amino acids with the binding site in AChE, and the distances of the linkages were obtained for analysis.

3. Results and Discussion

3.1. Production of Antinematodes Compounds by Fermentation

C/N sources play an important role in enhancing the yield of secondary metabolites produced by microbes during fermentation [4,5,14,19,22]. To get high productivity of ANCs, various organic wastes, including SR, SSP, CR, and GOP, and a commercial medium (CM, yeast extract/peptone = 5/3) were used as C/N (0.8%) sources for fermentation. The results in (Figure 1A) show that all the supernatants in the media containing organic wastes and commercial medium fermented by *B. veleznesis* RB.EK7 displayed antinematode activity in the range of 50.67-98%. Among these organic wastes, SSP was found as the most suitable C/N source to produce ANCs. The supernatant produced by RB.EK7 using SSP as the sole C/N source for fermentation demonstrated the highest nematicidal effect (98%), while the commercial medium gave an activity of 89.67%, and other organic wastes gave the lower activities in the range of 50.67-73.67%. Thus, the SSP was chosen for further investigation.

As regards effective use of the organic waste SSP for high yield of ANCs, some factors, including the initial pH (5.0-9.0), cultivation temperature (29- 39°C), shaking speed (0 - 250 rpm), and cultivation time (24-120 h) were examined for their effect. Overal, *B. veleznesis* RB.EK7 produced the highest nematicidal activity when it was cultivated in the conditions of pH 6.5-7.0 (Figure 1b), cultivation temperature of 35-37°C (Figure 1c), shaking speed of 150 rpm (Figure 1d), and cultivation time of 72 h (Figure 1e). The bacterial density was also examined during the fermentation; however, there seemed to be no correlation of the culture density with the antinematode activity (the data not shown).

Shrimp shell is one of the most abundant chitinous wastes obtained mainly from by-products of the fishery processing industry [23]. Through microbial conversion, various other bioactive materials such as proteases, chitinase, chitosanases, and oligomers of chitin and chitosan, as well as antioxidants, anticancer, and antidiabetic agents, have been produced from shrimp shells [17,24-27]. However, this is the first report on the utilization of SSP for the production of ANCs via microbial fermentation.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

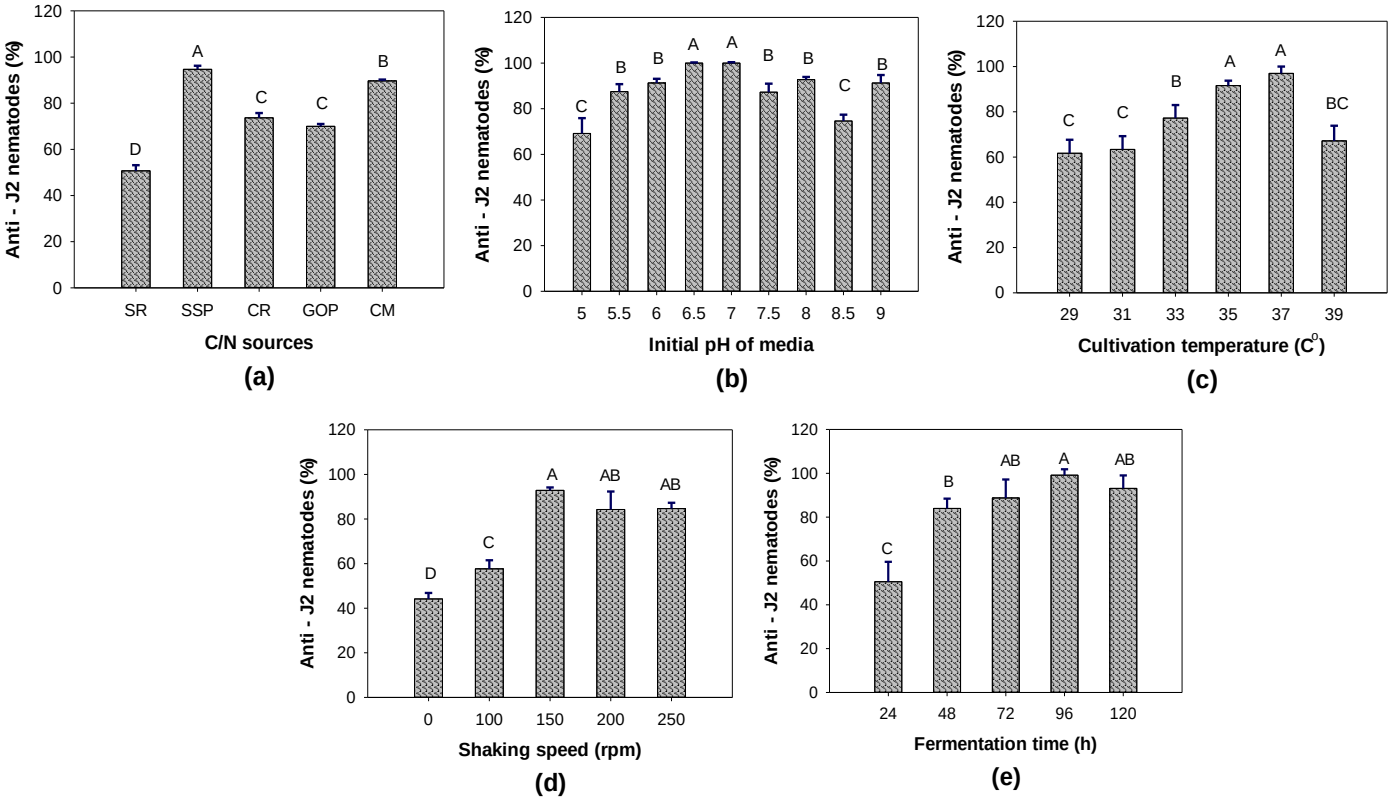


Figure 3. The production of antinematode compounds. The effect of C/N sources (**a**), the initial pH (**b**), cultivation temperature (**c**), shaking speed (**d**), and cultivation time (**e**) on the antinematode activity of supernatants produced by Bacillus veleznesis RB.EK7 was examined.

3.2. Purification and Identification of the Chemical structures of Antinematodes Compounds Produced by Bacillus velezensis RB.EK7

Antinematidic agents may be enzymes or natural compounds with small molecules [1]. Thus, for rapid prediction of the active antinematidic agents for purification, we tested the activity of some enzymes related to the antinematode effect (chitinase and protease) and nematicidal activity of the supernatants produced by *B. velezensis* RB.EK7 (Table 1). The supernatant demonstrated high chitinase activity (4.5 IU/mL), no protease activity, and high anti-J2 nematode activity (93.67%). This result indicated that the nematicidal effect of RB.EK7 supernatant is not due to protease but may be due to the chitinase effect. To elucidate this result, the supernatant was heated to a high temperature (at 100°C for 60 min), then enzyme and ant nematodes effects were tested. After being treated at 100°C, the supernatant did not exhibit any chitinase activity; however, the nematicidal effect still remained high (96.67%). This data provides evidence that the ant nematode agents may not be enzymes (chitinase, protease); thus, the agents of interest may be may be small natural compounds with high thermal stability [2,4]. Considering this, we used the typical method for extraction and purification of active ANCs, including solid-liquid with solvent extraction, separation via an opened silica gel column, and coupling with bioactivity testing. As a result, we could isolate two active compounds. The extraction and isolation of targeting compounds are summarized in Figure 1.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

Table 1. Bioactivities of supernatants produced by Bacillus velezensis RB.EK7 before and after high-temperature treatment .

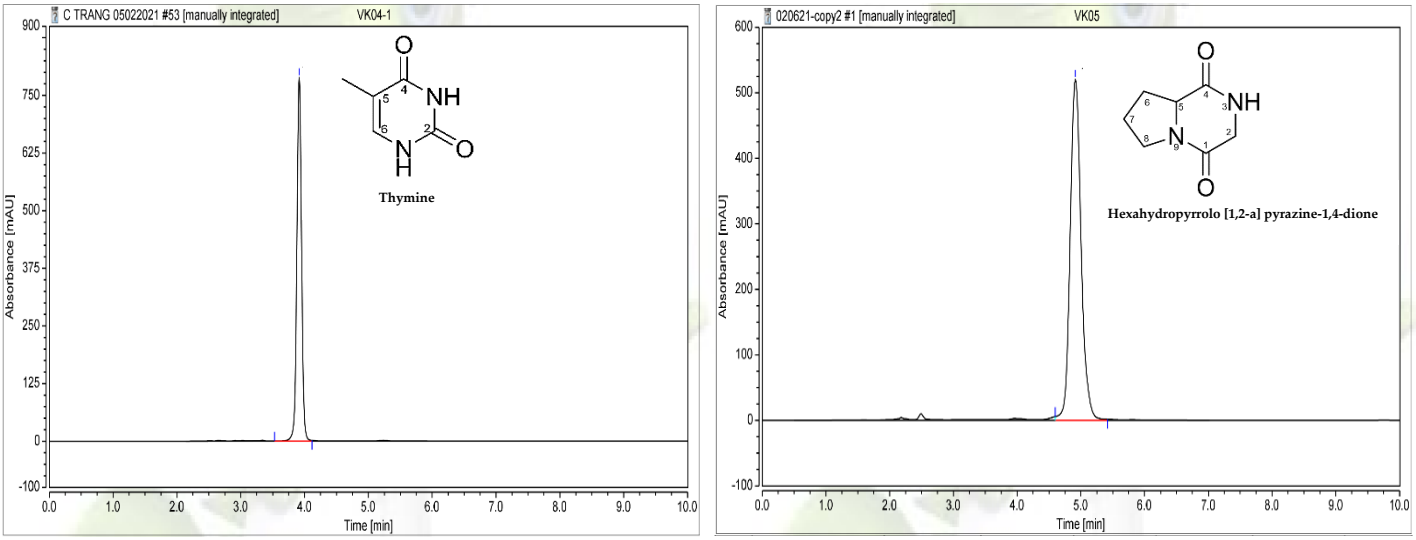
	Chitinase activity (IU/mL)	Protease activity (IU/mL)	Anti-J2 nematodes activity (%)
Supernatants no treated at high temperature	4.5	-	93,67
Supernatants treated at 100C° in 60 min	-	-	96,67

The chemical structures of the two active compounds were identified based on analysis of ^{1}H-NMR, ^{13}C-NMR, HMBC and HSQC, and HR-ESI-MS spectra. The compounds were identified as thymine (**1**) [28], and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione (**2**) [29].

The compound thymine (**1**) was obtained as a white amorphous powder. ^{1}H-NMR (DMSO-d6, 500 MHz) δH: 7.21 (1H, s), 1.69 (3H, s). ^{13}C-NMR (DMSO-d6, 125 MHz) δC: 165.4, 151.9, 138.3, 108.0, 12.0. HR-ESI-MS spectrum: $[M-H]^-$ at *m/z* 125.0354. The NMR and mass spectra data are presented in the supplementary section (Figure S1 – Figure S3).

The compound hexahydropyrrolo [1,2-a] pyrazine-1,4-dione (**2**) was obtained as a white amorphous powder. ^{1}H-NMR (DMSO-d6, 500 MHz) δH: 8.04 (1H, *s*), 4.11 (1H, *t*, 7.41), 3.98 (1H, *dd*, 16.6, 1.8), 3.52 (1H, *dd*, 16.5, 4.6), 3.36 (2H, *m*), 2.13 (1H, *m*), 1.82 (1H, *m*), 1.82 (2H, *m*). ^{13}C-NMR (DMSO-d6, 125 MHz) δC: 169.7, 164.3, 58.3, 46.1, 44.9, 28.1, 22.2. HR-ESI-MS spectrum: $[M+H]^+$ at *m/z* 155.0815. The NMR and mass spectra data are presented in the supplementary section (Figure S4 – Figure S8).

Before the elucidation of chemical structures and bioactivity assessment of these compounds, the purity of these targeting compounds was confirmed using UHPLC. As shown in Figure 4, these two compounds, thymine (**1**) and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione (**2**), appeared as single peaks at the retention time (RT) of 3.915 min and 4.913 min, respectively, and these compounds were also confirmed to be of high purity grade of approximately 100%; as such, these molecules were qualified for further investigation of their nematicidal activity.



(**a**) (**b**)

Figure 4. The HPLC profiles of the two antinematode compounds, including thymine (**a**) and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione (**b**) purified and identified in this study. The compounds were dissolved in methanol, filtered through a 0.22 µm membrane, and 5 µL of each was injected into the HPLC systems (Thermo-Ultimate 3000 UPLC system - ThermoScientific, USA) using a column (Hypersil GOLD aQ C18 column, 150 mm x 2.1 mm, particle size 3 µm, at a temperature of 30°C). The compound thymine was eluted by solvent systems of MeOH (100%)/ammonium acetate (10 mM in water) with a flow rate of 0.8 mL/min, and the UV detection

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

wavelength was 254 nm. The compound hexahydropyrrolo [1,2-a] pyrazine-1,4-dione was eluted by solvent systems of 20% MeOH in water with a flow rate of 0.7 mL/min, and the UV detection wavelength was 210 nm.

Hexahydropyrrolo [1,2-a] pyrazine-1,4-dione is naturally obtained from several microbial strains such as *Streptomyces antioxidans* sp. nov. [30], *S. nigra* sp. nov [31], *S. xanthophaeus* [32], and *B. tequilensis* [33]. However, there is no report on the production of this metabolite from the bacterium *B. velezensis.* Currently, thymine is mainly synthesized chemically by dissolving methyl methacrylate in a solvent of methanol [34]. There is also no literature available on the biosynthesis of thymine from the bacterial genus *B. velezensis.* Thus, these new evidences enrich the production of secondary metabolites from *B. velezensis* and may support the new, valuable, and natural sources of these active compounds for potential applications. In addition, this study is the first to report the reuse of organic wastes for the cost-effective production of these two compounds via microbial fermentation.

3.3. The Antinematodes Activity of the Purified Compounds Produced by Bacillus velezensis RB.EK7

The two purified compounds, thymine (**1**) and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione (**2**), were evaluated for their nematicidal effect. The potential nematicidal candidates are considered to inhibit both *J2* nematodes and egg hatching. Thus, we evaluated the effect of these compounds against both *J2* nematodes and egg hatching. Hemi-pyocyanin, an ANC obtained in our previous work [19], was also used for the comparison, and the results are summarized in Figure 5. In the assays for the antinematode effect, DMSO was used as the negative control and to dissolve the samples. In the negative group, the survival rate of *J2* nematodes was approximately 96.74%, and the egg hatching rate was up to 88%; therefore, DMSO was appropriate for dissolving the samples and a suitable negative control in the experiments [19]. As shown in Figure 5a, all the tested compounds could inhibit J2 nematodes by more than 40%; especially, thymine demonstrated slightly higher activity than the positive compound with the inhibition rate of 68% and 60%, respectively, at the tested concentration of 2.5 mg/mL. At a higher treated concentration (20 mg/mL), hexahydropyrrolo [1,2-a] pyrazine-1,4-dione inhibited J2 nematodes at the rate of 64.2%, while both thymine and hemi-pyocyanine could completely inhibit J2 nematodes at a rate of 99-100%. This result indicated that thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione may be potential and moderate anti-J2 candidates, respectively.

The effect of these purified compounds and hemi-pyocyanin on nematode egg hatching was also detected, and the data are summarized in Figure 5b. At a low concentration (2.5 mg/mL), these compounds demonstrated inhibition against egg hatching, with the rate being less than 57%. This rate was significantly reduced when higher concentrations of compounds, at 5, 10, and 20 mg/mL were used, and the respective egg hatching rates were 40-47%, 23-37, and 15-29%. Similar to anti-J2 nematodes, the thymine and hemi-pyocyanin displayed a potent effect on egg hatching, while the hexahydropyrrolo [1,2-a] pyrazine-1,4-dione showed moderated activity against the egg hatching. Overall, thymine may be suggested as a potential antinematidic agent, while hexahydropyrrolo [1,2-a] pyrazine-1,4-dione was found to be a moderate ANC.

Hexahydropyrrolo [1,2-a] pyrazine-1,4-dione has been shown to have several potential biological activities, including antibiotic activity against various strains of *S. aureus* [33] antioxidant effects [35] and algicidal against *Microcystis aeruginosa* [36]. While data on the biological effects of Thymine is not available, notably, the new biological functions include anti-J2 nematode and anti-nematode egg hatching of these two purified compounds was investigated for the first time in this study. Thus, this study may provide novel potential applications of thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione produced by *B. veleznesis* RB.EK7.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

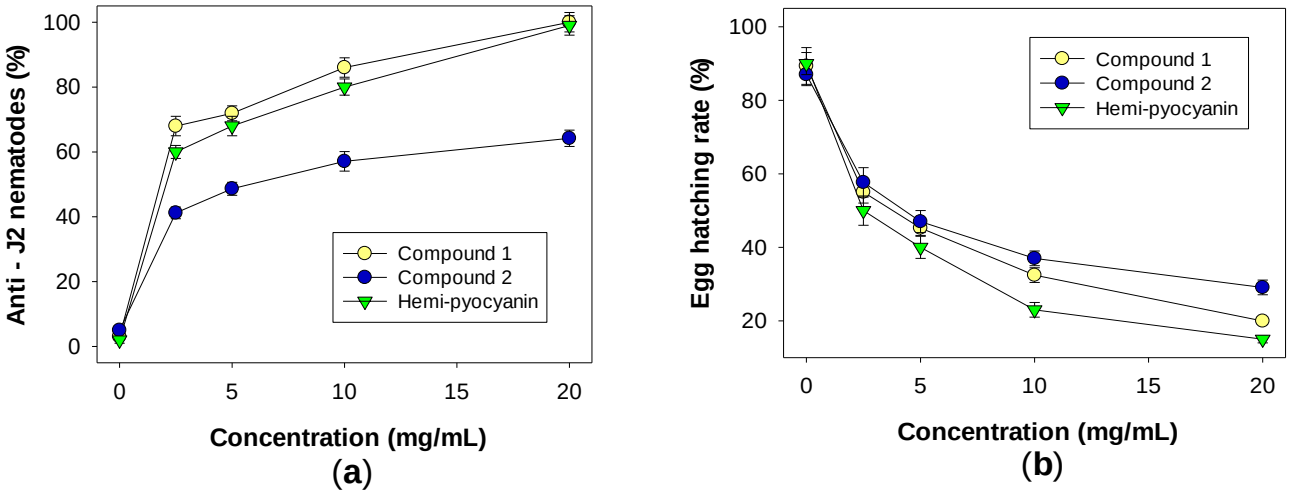


Figure 5. The nematicidal effects of purified thymine (compound 1), hexahydropyrrolo [1,2-a] pyrazine-1,4-dione (compound 2), and Hemi-pyocyanin (an antinematodes compound used as the positive control). The anti – J2 nematodes effect (**a**) and the effect of these compounds on nematode egg hatching rate (**b**).

3.4. The Interaction of Antinematodes Compounds Towards the Targeting Protein Enzyme

The novel nematicidal effect of thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione against black pepper nematodes was first reported in this work. Thus, the mechanism action models of these active compounds against nematodes are still unknown. To preliminarily predict the mechanism of action of these compounds, we assessed the molecular mechanism of anti-nematode activity via virtual screening assays [37-41]. The protein AchE is an enzyme commonly used for the inhibition of nematode *M. incognita*, a major species of the genus *Meloidogyne* [38]. Thus, this protein (AchE) was used as the targeting enzyme for the docking study.

In the docking simulations, the RMSD and docking score (DS, binding energy) were considered important parameters to confirm the interaction and whether the binding energy of the ligand toward the protein enzyme is significant. When a ligand (compound inhibitor) interacts and binds to a protein enzyme with the DS and RMSD values of lower than −3.20 kcal/mol and 3.0 Å, respectively, the compound is considered a potential inhibitor [21-22,26,42]. As shown in Table 2, both ligands, thymine, and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione interacted with the protein AchE with RMSD and DS values in the range of 1.02 Å to 1.35 Å, and -6.89 kcal/mol to -7.07 kcal/mol, respectively, significantly less than 3.0 Å and −3.20 kcal/mol. This result indicated that thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione are potent inhibitors of protein AchE; as such, the molecular mechanism action of these two compounds against nematodes may be high because of the inhibition of the protein AchE [38]. For a detailed observation of the interactions between the ligands at the active sites on AchE, the 3D structures of the total enzyme and the ligands at the active sites were examined and are presented in Figures 6 and 7. The output data of MOE (Figure 6) indicates that ligand thymine interacts with AchE at the active sites via creating four linkages (2 H-acceptors, 1H-donor, and 1 pi-H) with some prominent amino acids, including Asp182 (3.20/-0.9/1H-donor), Lys51 (2.99/-2.3/H-acceptor), Asn183 (3.18/-1.7/H-acceptor), and Trp179 (4.30/-1.1/pi-H), while the ligand hexahydropyrrolo [1,2-a] pyrazine-1,4-dione interacts with the protein AchE at the active sites through three linkages (2H-donor and 2 H-acceptor) via connecting with three amino acids at the active sites, including Met175 (3.87/-0.8/H-donor), Phe35 (3.05/-2.8/H-donor), and Lys51 (3.11/-1.3/H-acceptor) (Figure 7).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

Table 2. The docking study results of the interaction of the two ligands with the target enzyme acetylcholinesterase (AchE).

Ligands. (Inhibitors)	Symbol (Ligand-protein)	RMSD (Å)	DS (kcal/mol)	Number of interactions	Amino acids interacting with the ligand [Distance (Å) / E (kcal/mol)/linkage type]
Thymine (TM)	TM-AchE	1.35	-7.07	4 linkages (2 H-acceptors, 1H-donor, and 1 pi-H)	Asp182 (3.20/-0.9/1H-donor) Lys51 (2.99/-2.3/H-acceptor) Asn183 (3.18/-1.7/H-acceptor) Trp179 (4.30/-1.1/pi-H)
Hexahydropyrrolo [1,2-a] pyrazine-1,4-dione (HP)	HP-AchE	1.02	-6.89	3 linkages (2H-donor and 2 H-acceptor)	Met175 (3.87/-0.8/H-donor) Phe35 (3.05/-2.8/H-donor) Lys51 (3.11/-1.3/H-acceptor)

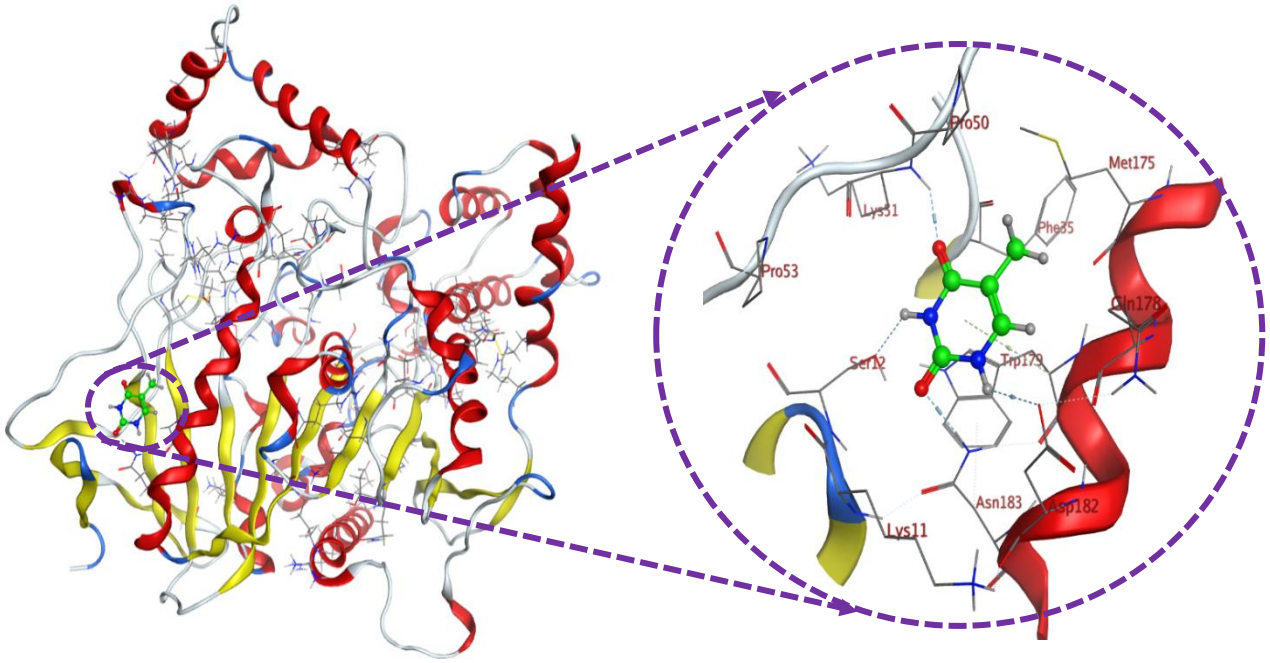


Figure 6. Interaction of thymine with enzyme acetylcholinesterase targeting antinematode effect at the active site.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 August 2022 doi:10.20944/preprints202208.0150.v1

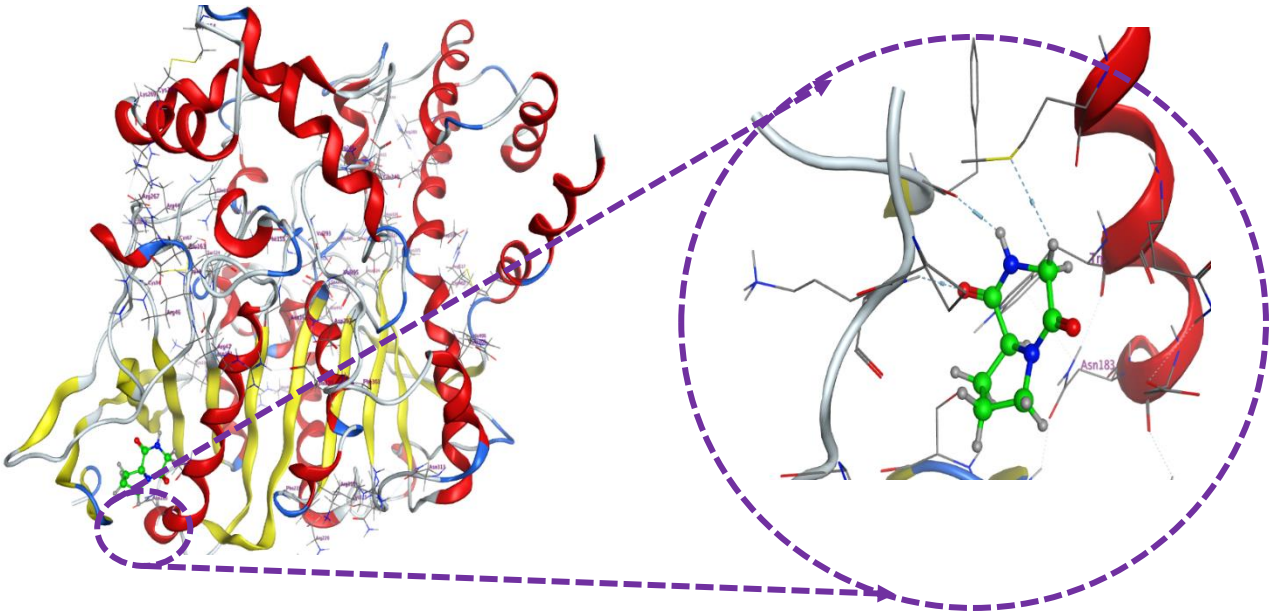


Figure 7. Interaction of hexahydropyrrolo [1,2-a] pyrazine-1,4-dione with enzyme acetylcholinesterase targeting antinematodes effect at the active site.

Based on the in vitro bioactivity of ant-J2 nematodes and anti-egg hatching and the virtual study of the two compounds, it suggested that thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione could be a good candidate for management of black pepper nematodes. However, further studies, such as the effect of thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione on *J2* nematodes, egg hatching, microbiota in cultivated soil, black pepper seedlings, black pepper trees in the greenhouse, and in field conditions should be further examined.

4. Conclusions

Almost the organic wastes examined in this study were found as potent substrates for fermentation by *B. veleznesis* RB.EK7 to produce ANCs. Of these substrates, SSP was found to be the most effective in enhancing the production of ANCs during fermentation. Thus, the process of fermentation for the production of ANCs from organic wastes was established under suitable conditions, with the medium containing 0.8% SSP, an initial pH of 6.5-7.0, cultivation temperature of 35- 37 °C, shaking speed of 150 rpm, and cultivation time of 72 h. Two active compounds were isolated from the supernatant and identified as thymine and hexahydropyrrolo [1,2-a] pyrazine-1,4-dione and demonstrated as novel potent and moderate ANCs. The virtual analysis of the molecular mechanism indicated that the potent antinematidic effect of these two compounds may be highly possible due to the inhibition of the target enzyme acetylcholinesterase.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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

Funding: This research was funded by a grant from Tay Nguyen University (T2022- 40CBTĐ) and sup-ported in part by the Ministry of Science and Technology, Taiwan (MOST 111-2320-B-032-001; MOST 110-2320-B-032-001; MOST-110-2923-B-032-001).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tran, T.P.H.; Wang, S.L.; Nguyen, V.B.; Tran, D.M.; Nguyen, D.S.; Nguyen, A.D. Study of novel endophytic bacteria for biocontrol of black pepper root-knot nematodes in the central highlands of Vietnam. *Agronomy* **2019**, *9*, 714.
2. Ravindra, H.; Sehgal, M.; Manu, T.G.; Murali, R.; Latha, M.; Narasimhamurthy, H.B. Incidence of root-knot nematode (*Meloidogyne incognita*) in black pepper in Karnataka. *J. Entomol. Nematol.* **2014**, *6*, 51–55.
3. Pepper (piper spp.), Production/Crops. Food and Agriculture Organization of the United Nations: Statistical Division (FAO-STAT). Available online: http://www.wikiwand.com/en/Black_pepper (accessed on 4 December 2018).
4. Nguyen, V.B.; Wang, SL.; Nguyen, T.H.; Nguyen, T.H.; Trinh, T.H.T.; Nong, T.T.; Nguyen, T.U.; Nguyen, V.N.; Nguyen, A.D. Reclamation of rhizobacteria newly isolated from black pepper plant roots as potential biocontrol agents of root-knot nematodes. *Res. Chem. Intermed.* **2019**, *45*, 5293–5307.
5. Nguyen, T.H.; Wang, S.L.; Doan, M.D.; Nguyen, T.H.; Tran, T.H.T.; Tran, T.N.; Doan, C.T.; Ngo, V.A.; Ho, N.D.; Do, V.C.; Nguyen, A.D.; Nguyen, V.B. Utilization of by-product of groundnut oil processing for production of prodigiosin by microbial fermentation and its novel potent anti-nematodes effect. *Agronomy* **2022**, *12*, 41.
6. Chen, J.; Li, Q.X.; Song, B. Chemical nematicides: Recent research progress and outlook. *J. Agric. Food Chem*. **2020**, *68*(44), 12175–12188
7. Nguyen, V.N.; Kim, Y.J.; Oh, K.T.; Jung, W.J.; Park, R.D. The role of chitinase from *Lecanicillium antillanum* B-3 in parasitism to root-knot nematode *Meloidogyne incognita* eggs. *Biocontrol. Sci. Technol.* **2007**, *17*, 1047–1105.
8. Wheeler, T.A.; Siders, K.T.; Anderson, M.G.; Russell, S.A.; Woodward, J.E.; Mullinix, B.G., Jr. Management of *Meloidogyne incognita* with chemicals and cultivars in cotton in a semi-arid environment. *J. Nematol.* **2014**, *46*, 101–107.
9. Mokbel, A.A. Impact of some antagonistic organisms in controlling *Meloidogyne arenaria* infecting tomato plants. *JOLST* **2013**, *1*, 69–74.
10. Boraha, B.; Ahmed, R.; Hussaina, M.; Phukon, P.; Wann, S.B.; Sarmah, D.K.; Bhau, B.S. Suppression of root-knot disease in *Pogostemon cablin* caused by *Meloidogyne incognita* in a rhizobacteria mediated activation of phenylpropanoid pathway. *Biol. Control* **2018**, *119*, 43–50.
11. Chen, J.; Song, B. Natural nematicidal active compounds: Recent research progress and outlook. *J. Integr. Agric.* **2021**, *20*(8), 2015–2031.
12. Bhandari, G. An overview of agrochemicals and their effects on environment in Nepal. *Appl Ecol Environ Res* **2014**, *2*(2), 66-73.
13. Josef J., Katarína K. Nanopesticides: preparation, targeting and controlled release. London, UK. **2017**, *10*, 81–127.
14. Nguyen, V.B.; Wang, S.L.; Nguyen, A.D.; Phan, T.Q.; Techato, K.; Pradit, S. Bioproduction of prodigiosin from fishery processing waste shrimp heads and evaluation of its potential bioactivities. *Fishes* **2021**, *6*, 30.
15. Venugopal, V. Valorization of seafood processing discards: Bioconversion and biorefinery approaches. *Front. Sustain. Food Syst*. **2021**, *5*, 611835.
16. Hue, H.T.T.; Pradit, S.; Lim, A.; Goncalo, C.; Nitiratsuwan, T. Shrimp and fish catch landing trends in Songkhla Lagoon, Thailand during 2003–2016. *Appl. Ecol. Environ. Res.* **2018**, *16*, 3061–3078.
17. Wang, C.H.; Doan, C.T.; Nguyen, V.B.; Nguyen, A.D.; Wang, S.L. Reclamation of fishery processing waste: A mini-review. *Molecules* **2019**, *24*, 2234.
18. Trinh, T.H.T.; Tran, T.P.H.; Nguyen, T.T.; Tran, T.H.; Nguyen, T.V. The effect of rhizosphere bacteria strains on the growth and resistance ability against nematodes of the nursery black pepper plant (*Piper nigrum* L.). *The 20th National Conference of Vietnamese Phytopathological Society* **2021**, 114-123.
19. Nguyen, T.H.; Wang, S.L.; Nguyen, T.H.; Doan, M.D.; Tran, T.H.T.; Ngo, V.A.; Ho, N.D.; Tran, T.N.; Doan, C.T.; Do, V.C.; Nguyen, A.D.; Nguyen, V.B. Utilization of fishery-processing by-product squid pens for scale-up production of phenazines via microbial conversion and its novel potential antinematode effect. *Fishes* **2022**, *7*, 113.
20. Khan, Z.; Kim, S.G.; Jeon, Y.H.; Khan, H.U.; Son, S.H.; Kim, Y.H. A plant growth promoting rhizobacterium, *Paenibacillus polymyxastrain* GBR-1, suppresses root-knot nematode. *Bioresour. Technol*. **2008**, *99*, 3016–302.
21. Nguyen, T.H.; Wang, S.L.; Nguyen, A.D.; Doan, M.D.; Tran, T.N.; Doan, C.T.; Nguyen, V.B. Novel α-amylase inhibitor hemi-pyocyanin produced by microbial conversion of chitinous discards. *Mar. Drugs* **2022**, *20*, 283.
22. Tran, L.T.; Techato, K.; Nguyen, V.B.; Wang, S.L.; Nguyen, A.D.; Phan, T.Q.; Doan, M.D.; Phoungthong, K. Utilization of cassava wastewater for low-cost production of prodigiosin via *Serratia marcescens* TNU01 fermentation and its novel potent α-glucosidase inhibitory effect. *Molecules* **2021**, *26*, 6270.
23. Nguyen, T.H.; Wang, S.L.; Nguyen, D.N.; Nguyen, A.D.; Nguyen, T.H.; Doan, M.D.; Ngo, V.A.; Doan, C.T.; Kuo, Y.H.; Nguyen, V.B. Bioprocessing of marine chitinous wastes for the production of bioactive prodigiosin. *Molecules* **2021**, *26*, 3138.
24. Tran, T.N.; Doan, C.T.; Nguyen, M.T.; Nguyen, V.B.; Vo, T.P.K.; Nguyen, A.D.; Wang, S.L. An exochitinase with *N*-acetyl-β-glucosaminidase-like activity from shrimp head conversion by *Streptomyces speibonae* and its application in hydrolyzing β-chitin powder to produce *N*-acetyl-D-glucosamine. *Polymers* **2019**, *11*, 1600.
25. Doan, C.T.; Tran, T.N.; Wen, I.H.; Nguyen, V.B.; Nguyen, A.D.; Wang, S.L. Conversion of shrimp head waste for production of a thermotolerant, detergent-Stable, alkaline protease by *Paenibacillus* sp. *Catalysts* **2019**, *9*, 798.
26. Nguyen, V.B.; Wang, S.L. Production of potent antidiabetic compounds from shrimp head powder via *Paenibacillus* conversion. *Process Biochem.* **2019**, *76*, 18–24.

27. Nguyen, V.B.; Nguyen, T.H.; Nguyen, A.D.; Le, T.; Kuo, Y.H.; Wang, S.L. Bioprocessing shrimp shells to rat intestinal α- glucosidase inhibitor and its effect on reducing blood glucose in a mouse model. *Res. Chem. Intermed*. **2019**, *45*, 4829–4846.
28. Available online: http://www.basechem.org/chemical/1290 (accessed on 11 June 2022).
29. Li, Z.; Lin, S.; Liu, X.; Tan, J.; Pan, J.; Yang, H. A freshwater bacterial strain, *Shewanella* sp. Lzh-2, isolated from Lake Taihu and its two algicidal active substances, hexahydropyrrolo [1, 2-a] pyrazine-1, 4-dione and 2, 3-indolinedione. *Appl. Microbiol. Biotechnol.* **2014**, *98*(10), 4737-4748.
30. Ser, H.L.; Tan, L.T.; Palanisamy, U.D.; Abd, M.S.N.; Yin, W.F.; Chan, K.G.; Goh, B.H.; Lee, L.H. *Streptomyces* antioxidans sp. nov., a novel mangrove soil actinobacterium with antioxidative and neuroprotective potentials. *Front Microbiol*. **2016,** *16*(7), 899.
31. Chen, C.; Ye, Y.; Wang, R.; Zhang, Y.; Wu, C.; Debnath, S.C.; Ma, Z.; Wang, J.; Wu, M. *Streptomyces nigra* sp. nov. is a novel actinobacterium isolated from mangrove soil and exerts a potent antitumor activity in vitro. *Front Microbiol*. **2018**, *18*(9), 1587.
32. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Hexahydropyrrolo_1_2-a_pyrazine-1_4-dione (accessed on 12 June 2022).
33. Kiran, G.S.; Priyadharsini, S.; Sajayan, A.; Ravindran, A.; Selvin, J. An antibiotic agent pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro isolated from a marine bacteria *Bacillus tequilensis* MSI45 effectively controls multi-drug resistant *Staphylococcus aureus*. *RSC Advances* **2018**, *8*(32), 17837–17846.
34. Duo, Wenbin, "Synthetic method of thymine", published 2013-08-07, assigned to Tianjin Jude Technology Co. Ltd. Patent code: CN 103232399.
35. Ser, H.L.; Palanisamy, U.D.; Yin, W.F.; Malek, S.N.A.; Chan, K.G.; Goh, B.H.;Lee, L.H. Presence of antioxidative agent, Pyrolo[1,2-a]pyrazine-1,4-dione,hexahydro – in newly isolated *Sterptomyces mangrovisoli* sp. nov. *Front. Microbiol.*, 2015, **6**, 854.
36. Li, Z.; Geng, M.; Yang, H. Algicidal activity of *Bacillus* sp. Lzh-5 and its algicidal compounds against *Microcystis aeruginosa*. *Appl. Microbiol. Biotechnol.* **2014**, *2*, 981–990.
37. Wen, Z.; Shen, Z.H.; Xing, J.H.; Guo, Y.; Xu, T.M.; Peng, W.L.; Liu, X.H. Synthesis and nematocidal activity of novel 1-(3-chloropyridin-2-yl)-3-(trifluoromethyl)-1H-pyrazole-4-carboxamide derivatives. *Chem. Pap.* **2017**, *71*, 921–928.
38. Keerthiraj, M.; Mandal, A.; Dutta, T.K.; Saha, S.; Dutta, A.; Singh, A.; Kundu, A. Nematicidal and molecular docking investigation of essential oils from *Pogostemon cablin* ecotypes against *Meloidogyne incognita*. *Chem. Biodivers.* **2021**, *18*, e2100320.
39. Babu, R.O.; Moorkoth, D.; Azeez, S.; Eapen, S.J. Virtual screening and in vitro assay of potential drug like inhibitors from spices against Glutathione-S-Transferase of *Meloidogyne incognita*. *Bioinformation* **2012**, *8*, 319–325.
40. Kundu, A.; Dutta, A.; Mandal, A.; Negi, L.; Malik, M.; Puramchatwad, R.; Antil, J.; Singh, A.; Rao, U.; Saha, S.; et al. A comprehensive in vitro and in silico analysis of nematicidal action of essential oils. *Front. Plant Sci.* **2021**, *11*, 614143.
41. Emmanuel, B.; Diana, F.; Deisy, X.A.; Philippe, N.; Petitot, A.S.; Lisei, S.M.E.; Erika, V.S.A.; Etienn, G.J.D.; Bernard, M.; Natália, F.M. A chemosensory GPCR as a potential target to control the root-knot nematode *Meloidogyne incognita* parasitism in plants. *Molecules* **2019**, *24*, 3798.
42. Baron, A.D. Postprandial hyperglycaemia and α-glucosidase inhibitors. *Diabetes Res. Clin. Pract.***1998**, *40*, 51-55.
43. Geng, P.; Bai, G. Two novel aminooligosaccharides isolated from the culture of *Streptomyces coelicoflavus* ZG0656 as potent inhibitors of α-amylase. *Carbohydr. Res.* **2008**, *343*, 470-476.