Bioactive compound analysis and antioxidant activity of endophytic bacterial extract from Noni fruits (*Morinda citrifolia* L.)

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Abstract. Endophytic bacteria have been recognised as a microorganism that has an important role as the producer of bioactive compounds. In the literature, endophytic bacteria from Noni fruits (*Morinda citrifolia* L.) with their antioxidant activities have been rarely studied. This research aimed at isolating endophytic bacteria associated with Noni fruits to investigate their antioxidant activity, to identify potential endophytic bacteria with 16s rRNA and to detect bioactive compounds. Based on morphological characteristics of bacterial colonies, six endophytic bacteria were isolates from Noni fruits. The isolates were observed and evaluated in terms of their antioxidant activities using 1,1-Diphenyl-2-picryl-hydrazyl radical scavenging activity. Results of this study discovered supernatant from isolate ACP3 to have the highest 1,1-Diphenyl-2-picryl-hydrazyl free-radical scavenging activity up to 68.90%. In addition, molecular identification conducted by poly chain reaction amplification on 16s rRNA gene showed isolate ACP3 belong to *Staphylococcus* sp, with 100% sequence similarities. Looking at GCMS analysis, ethyl acetate fraction from the secondary metabolites of isolate ACP3 contained pyrazine alkaloids, which have been known as a bioactive compound. Based on these results, the ACP3 strain was considered as a good and sustainable resource for natural antioxidants.

1. Introduction

Recent researches have begun to scientifically explore and investigate the potentials of various rarely observed medicinal plants. Among others, Noni plant, particularly its fruits (*Morinda citrifolia* L.), has been widely used in traditional medicine. Technically, the fruit contains various active compounds, e.g. L-Asperuloside, Alizarin, Aucubin, Anthraquinone (dioxaanthracene) and Pyrazine [1], that offer antibacterial and antioxidant characteristics. However, harvesting those active compounds required huge amount of biomass. Recently, the problem has been approached by utilising endophytic bacteria within plant tissue. Bacteria as such are microorganism living in internal tissue of living plant without causing any negative direct effect to the tissue. The advantage indicates a mutualistic symbiosis between endophytic bacteria and their host plant. The use of bacteria as the source of biological products may then simplify technical process and reduce production cost, resulting in products with lower prices. Besides, the ability of endophytic bacteria in producing secondary metabolites corresponding to their host plant opens an opportunity to use them for producing tissue-specific secondary metabolites.
So far, virtually no work has been conducted to investigate the uses of active compounds produced by endophytic bacteria isolated from Noni fruits as an antioxidant. Therefore, it would be interesting to observe endophytic bacteria isolated from Noni fruits as a producer of natural active compounds with antioxidant characteristics. This study aimed at isolating endophytic bacteria that potentially produce antioxidant compounds, analysing optimum incubation time of endophytic bacteria to produce the compounds, and discovering the type(s) and incubation time of endophytic bacteria from isolate(s) with high potentials to produce the antioxidant compounds. Then, this study also attempted to identify the types of bioactive compounds, which were produced during the cultures of endophytic bacteria that showed antioxidant characteristics.

2. Materials and Method

2.1. Isolation of endophytic bacteria

In this study, noni fruit was obtained from farm of BALLITRO, Bogor. The fruits were surface sterilized following the protocol by Liqin’s group [2] with some modification. Samples were cleaned in tap water then with sterile distilled water followed by shaking the fruits with 70% ethanol for 10 minutes, 2.5% sodium hypochlorite for 10 minutes, and 70% ethanol for 10 minutes, followed by three times rinses in sterile deionised water. The surface sterilised material was cut into 1 × 1 cm pieces and placed on the surface of nutrient agar plates supplemented with Nystatin to suppress bacterial growth and incubated at 37 °C for 3 days. For sterility check, 100 µl of the last washed water was plated on nutrient agar plates and incubated at 37 °C for 3 days to observe growth of any microorganism. Well isolated colonies were re-streaked on fresh agar plates and maintained in agar slants then stored at 4 ºC for further use.

2.2. Screening of the endophytic isolates for antioxidant properties

For selecting the potent endophytic bacteria with antioxidant activities, all the endophytic isolates were grown in 10 mL of TSB medium for 12 h at 37°C with mild shaking (150 rpm). Then 2% of inoculums was added into 500 mL beaker containing 300 mL of nutrient-broth and incubated for 36 h. Cell free supernatant (CFS) was prepared by centrifugation at 12000 g, 4°C for 15 min [3]. The extract was subjected to solvent-extraction, using ethyl acetate in a separating funnel and solvent was evaporated to obtain the endophytic bacterial extract. All isolates at a concentration of 10 mg/mL were examined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical-scavenging assay to confirm the antioxidant activity and then the supernatants were assayed their antioxidant activity by scavenging DPPH free radical method described with any modification [4]. Endophytic bacteria which showed highest antioxidant activity set as potential isolate.

2.3. Growth of potential isolate acp3

Subculture of potential isolate in the TSB medium was incubated at 37 °C for 12 h with agitation before use. A 300 µL aliquot of potential isolate ACP3 was cultured to a 300 mL TSB medium and incubated at 37 °C with agitation, optical density (600 nm) and antioxidant activity were recorded every 2 h for 36 h.

2.4. Assay for antioxidant activity

The radical scavenging ability was estimated by using adapted 2,2-diphenyl-2-picryl hydrazyl (DPPH) method described previously [5]. Thus, an aliquot of extract (10 µL) was added to 195 µL of ethanolic DPPH (120 µM). The reaction mixtures were pipetted on 96-well microtitre plates and incubated at room temperature for 30 min in the dark and absorbance was measured at 517 nm in triplicate, ascorbic acid was used as the positive control and the percentage of inhibition (% I) of free radicals was calculated as % I = [(A blank−A sample)/A blank] ×100, where, A blank: Absorbance of control, A sample: Absorbance of methanolic solution and DPPH.
2.5. Identification of ACP3 by 16s rRNA analysis

Strains isolate potential was identified using 16S rRNA gene. The genomic DNA was obtained according to manual extraction base on CTAB [6]. With genomic DNA as the template, a portion of the bacterial 16S rRNA gene (±1400bp) was amplified with universal primers (27F 5’-AGAGTTTGATCMTGGCTCAG-3’ and 1492R 5’-TACGGYTACCTTGTTACGACTT-3’). Sequencing process was made at Macrogen, Korea. Furthermore, Sequence of DNA was edited using Chromas Pro software and alignment sequence by DNA MAN software. Sequence result were compared with existing sequence using Basic Local Alignment Search Tools program on National Center for Biotechnology Information Blast [7].

2.6. Extraction of endophytic bacterial (ACP3) metabolites

On the basis of maximum antioxidant metabolites production in TSB medium, it was selected for the solvent extraction process. The CFS of the potent endophytic bacterial isolate ACP3 was subjected to ethyl acetate extraction. ACP3 was inoculated in 300 mL of TSB medium in 1L Erlenmeyer flasks and incubated at 37 °C for 30 h. CFS was prepared by centrifugation at 10,000 g for 10 min and extracted thrice with ethyl acetate. For every 100 mL of CFS, 40 mL of ethyl acetate was added, mixed vigorously and the ethyl acetate layer was separated using separating funnel. The ethyl acetate portion was then evaporated to dryness using rotary vacuum evaporator at room temperature (26±2°C) and stored at 4°C for further experiments [1].

2.7. Analysis of bioactive compound

Extract of secondary metabolite bacteria potential was isolated from ethyl acetate fraction for determination of bioactive compound as antioxidant agent. Chemical analysis was conducted using gas chromatography coupled with mass spectrometry (GCMS Agilent Technologies 7890) equipped with a HP ultra 2 Capillary Column (30 m × 0.20 mm LD, 0.11 µm film thickness). The column temperature, 250°C, carrier gas helium linear gas velocity, 30 cm/sec; split ratio, 1/30, ion source temperature, 230°C and interface temperature, 280°C. The identification of chemicals was performed in comparison with database and confirmed using authentic standard samples [4].

3. Results and Discussion

3.1. Isolation of endophytic bacteria

In this study, the isolation of endophytic bacteria from Noni fruits was conducted by applying surface sterilisation procedures. Six isolates were selected based on morphological characteristics. The grouping of endophytic isolates has suggested based on their morphospecies, i.e. similarity of colony colour, texture surface, edge shape, and growth rate. In addition, various endophytic bacteria were determined by the growth conditions for a host plant, including soil structure, plant age, geographical distribution, time of sampling and types of plant tissue [8].

3.2. Observing antioxidant activity in endophytic bacteria extracts

Besides, this study aimed at identifying the antioxidant potential of endophytic bacteria from Noni fruits. A total of 6 representative endophytic bacteria extracts from inner fruits were tested to investigate their antioxidant capacity in scavenging DPPH radicals. The tests treated cell free supernatant as samples. In general, the composition and distribution of endophytic bacteria community was tissue specific. The order of relative frequency of endophytes colonisation in different tissues was stems > hypocotyls > roots > flowers > leaves. In fact, the antioxidant activity test on the six observed isolates showed all isolates to have the scavenging ability on DPPH radical (Table 1). In the current study, high antioxidant activity rates were detected from the cell free supernatant extract (1 mg/mL) belonging to isolate ACP3, which exhibited a moderate scavenging ability towards DPPH radicals. Isolate ACP3 showed the most potent radical-scavenging activity at 68.90% inhibition value, indicating its characteristics as the most potential antioxidant among observed isolates. The radical-
scavenging activities of these extracts were comparable to ascorbic acid (Vit. C 1 mg/mL) at 87.92% (Table 1), suggesting their high potentials [9].

Antioxidant efficacy cannot be comprehensively predicted with only a single antioxidant assay. Therefore, several methods are necessary to evaluate antioxidant activities. In this study, we evaluated antioxidant activity using DPPH radicals scavenging activity. The DPPH assay is one of the most commonly used methods to evaluate free radical scavenging activity. DPPH is a stable radical that produces a purple solution in methanol. The antioxidant activity in this study was measured by the discoloration to yellow as the stable molecule 2,2-diphenyl-1-hydrazine formed. Antioxidant compounds can scavenge the radicals by donating their hydrogen, and the hydroxyl group of the isolated compounds may play a role in the activity.

Table 1. Inhibition values from antioxidant activity assays

| Isolate ID | Inhibition (%) |
|------------|----------------|
| ACP 1      | 59.20 ± 0.54   |
| ACP 2      | 31.59 ± 0.38   |
| ACP 3      | 68.90 ± 0.34   |
| ACP 4      | 45.14 ± 0.38   |
| ACP 5      | 63.80 ± 0.65   |
| ACP 6      | 39.92 ± 0.45   |
| Vit.C      | 87.92 ± 0.63   |

3.3. Production of secondary metabolite and antioxidant activity

Bacterial growth curve was made for at least three isolates to obtain an optimum time for sampling to test the antioxidant activity. Figure 1 exhibits measurement results of cell density and antioxidant compound production. Increased biomass was shown with a 600 nm OD, which resulted in the increase from 0.1 in after 2 hours, reaching a peak of 1.3 after 30 hours and gradually decreasing to reach 1.1 OD value after 36 hours.

![Figure 1](image.png)

Figure 1. Antioxidant production during the growth of ACP3 at 37 ºC. The optical density (absorbance at 600 nm) and antioxidant activity of culture were measured at the time intervals as indicated. The antioxidant activity expressed as percent inhibition.
Looking at Figure 1, the antioxidant activity of isolate ACP3 was positively correlated with cultivation time in the form of an S-curve. After being incubated for 12 hours, ACP3 entered a logarithmic growth period. Besides, the antioxidant activity of CFS sampled at different time intervals appeared to significantly correlate with the growth of strain over a 28-hour incubation period. The strongest antioxidant activity was observed after 30 hours.

In the literature, antioxidant compound produced by endophytic bacteria consisted of various substances [10]. Antioxidant substances produced by endophytic bacteria are carotenoid pigment [11] surfactin, and also L-asparaginase [12]. Most of the compounds were produced maximally at the end of exponential phase. Thus, the sample for antioxidant activity was collected at the 30th hour (the end of exponential phase). In fact, the result supported a suggestion by Zhao et al., who stated secondary metabolites from bacteria to typically be produced at the end of exponential phase or at the beginning of stationary phase [13]. The death phase of isolate ACP3 began to appear after 36 hours, in which OD_{600} gradually decreased to reach 1.1. On the other hand, secondary metabolites produced by ACP3 isolate was harvested at the early period of exponential phase (after 30 hours of incubation) when cell biomass was at optimum OD (Figure 1). Antioxidant bioactive compound produced during the incubation of ACP3 isolate at 37°C was observed to perform its maximum activity at the stationary phase of growth. In short, this study revealed the endophytic extracts under investigation to have a proton-donating ability and to serve as a free-radical inhibitor or scavenging, potentially acting as a primary antioxidant.

### 3.4. Molecular identification of endophytic bacteria

Furthermore, genomic isolation was conducted by following the method suggested by Sogandi et al., described in the research procedure [6]. Next, the isolated genome was amplified by applying 16S rRNA gene. After purification, sequence of the 16S rRNA gene was determined. After that, obtained partial sequences of the 16S rRNA gene were aligned with base sequences taken from the GenBank (NCBI database). The aligned sequencing of ACP3 isolate showed 16S rRNA gene in the isolate to have ± 1389 bp (base pair). ACP3 isolate showed 100% similarity to *Staphylococcus sp* and was registered to the GenBank as *Staphylococcus sp*, obtaining an accession number of MN068816.1.

*Staphylococcus* was a member of the phylum Firmicutes. These bacteria were endophytic bacteria in plant that are found in maize kernels, grapevine (*Vitis sp.*), and hybrid spruce (*Picea glauca x Engelmannii*). *Staphylococcus* was also found as endophytic bacteria in phytoremediation plant, *e.g.* poplar trees. In particular, *Staphylococcus* found in papaya mesocarp has been recognized to produce amylase, cellulase, pectinase, and xylanase. Allegedly, these bacteria might have acted as a provider of nutritional agents and offered a great potential in improving post-fermentation product, *e.g.* antioxidant [13].

### 3.5. Identification of bioactive compound

The identification of bioactive compounds from ACP3 was conducted by an extracted method using ethyl acetate. In general, ethyl acetate was often used as an extraction solvent with a significant selectivity in the extraction of low-molecular-weight phenolic compounds and high-molecular-weight polyphenols [14]. On the other hand, have reported that ethyl acetate allowed that highest phenolic content and the selective removal of non-phenolic compounds. Therefore, the antioxidant activity of endophytic ethyl acetate extract could be due to the presence of phenolic compounds [4].

Looking at GCMS analysis, a total of 17 secondary metabolites were identified from isolate ACP3, which could be classified into several groups. In details, the highest concentration was for benzyl hexopyrrolol [1,2-A] Pyrazine-1,4-dione with 21.48% content at 31.309 retention time. Besides, detected dominant compounds were Pyrazine compounds and their derivatives with retention times of 11.803 (1.39%), 28.662 (5.59%), 28.682 (1.90%), 28.841 (5.11%), 28.882 (2.97%), and 29.158 (7.94%). In addition, there were other classes of compounds, *i.e.* 2Hidopyran 2 ethoxy 3,4 dihydroy, 2 hydroxy 3,5,5 trimethyl cyclohex-2-enone, 3,5 dyhidroxy 4,4 dimethyl 2,5 cyclohexadien-1-one and Sulfide. Pyrazine itself was a compound consisting of nitrogen-containing heterocyclic
components and was the result of interactions between α-dicarbonyl, while amines were the result of interactions between α-amino groups of amino acids. In fact, amino acid chains, especially nitrogen-containing atoms such as glutamine, asparagine and lysine, had been found as being critical in the formation of pyrazine [15].

4. Conclusions
The results of this study have discovered endophytic bacteria Staphylococcus sp strain ACP3 (Acc No. MN068816) as a potential producer of bioactive products. During experiments, extracts of endophytic bacterial isolated from Non fruits (Morinda citrifolia) were found to exhibit a significant antioxidant property. Besides, endophytic bacteria might act as an alternative source to produce therapeutic agents and bioactive metabolites, which were high antioxidant agents not easily obtained through chemical syntheses. Hence, this research would serve as a referred work for more comprehensive studies in the future on the chemical and biological characteristics of bioactive natural products produced by these endophytes. Then, ACP3 isolate was revealed to produce pyrazine as a bioactive compound, by which further examinations may focus on the potentials of endophytes to serve as a biological or pharmacological agent.

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References
[1] Sogandi, P Nilasari 2019 Isolation and molecular identification of Endophytic bacteria from Noni fruits (Morinda citrifolia l.) and their antibacterial activity IOP Conf. Ser. Earth Environ. Sci. 299 012020 1-11.
[2] Wu L, Shang H, Wang Q, Gu H, Liu G, Yang S 2016 Isolation and characterization of antagonistic endophytes from Dendrobium candidum Wall and the biofertilizing potential of a novel Pseudomonas saponiphila strain Appl. Soil Ecol. 105 101–108.
[3] Suhandono S, Kusumawardhani M K, Aditiawati P 2016 Isolation and molecular identification of endophytic bacteria from rambutan fruits (Nephelium lappaceum L.) cultivar Binjai, Hayat J. Biosci. 23 39–44.
[4] Chatterjee S, Ghosh R, Chandra N 2019 Production of bioactive compounds with bactericidal and antioxidant potential by endophytic fungus Alternaria alternata AE1 isolated from Azadirachta indica A. Juss PLoS One. 4 1–18.
[5] Gunasekaran S, Sathiavelu M, Arunachalam S 2017 In vitro antioxidant and antibacterial activity of endophytic fungi isolated from Mussaenda luteola J. Appl. Pharm. Sci. 7 234–238.
[6] Sogandi, Apon Z M, I Made A 2019 The Characterization of bacteriocins produced by lactobacillus plantarum strains isolated from traditional fermented Foods in Indonesia and the detection of its plantaricins-encoding genes Indonesian J. Biotechno. 24 1–7.
[7] Tamura K, Peterson D, Stecher G, Nei M, Kumar S 2011 MEGA5 : Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods Mol. Biol. Evol. 28 2731–2739.
[8] Shin T Y, Bae S M, Woo S D 2019 Screening and characterization of antimicrobial substances originated from entomopathogenic fungi J. Asia-Pacific Entomol. 19 1053–1059.
[9] Cui J, Guo T, Ren Z, Zhang N, Wang M 2015 Diversity and antioxidant activity of cultivable endophytic fungi from alpine plants of Rhodiola crenulata PLoS One 3 1–16.
[10] Yun H, Kim D, Gwak W, Shin T 2017 Mycobiology entomopathogenic fungi as dual control agents against both the pest Myzus persicae and phytopathogen Botrytis cinerea Mycobiol. 45 192–198.
[11] Prabhu M, Rao N, Xiao M, Li W, Crampton M C 2017 Fungal and bacterial pigments: secondary metabolites with wide applications Front Microbiol. 8 1–13.
[12] Joshi S R 2015 L-Asparaginase and antioxidant activity of endophytic bacteria associated with ethnomedicinal plants Indian J. Biotechnol. 14 59–64.
[13] Zhao J, Ma D, Luo M, Wang W, Zhao C, Zu Y 2014 In vitro antioxidant activities and antioxidant enzyme activities in HepG2 cells and main active compounds of endophytic fungus from pigeon pea Food Res. Int. 56 243–251.
[14] El-haci I A, Bekkara F A, Mazari W, Gherib M 2013 Phenolics content and antioxidant activity of some organic extracts of endemic medicinal plant Anabasis aretioides Coss & Moq from Algerian Sahara Pharmacogn. J. 5 108–112.
[15] Fu L, Lu W, Zhou X 2016 Phenolic compounds and in vitro antibacterial and antioxidant activities of three tropic fruits: persimmon, guava, and sweetsop Biomed Res. Int. 1-9.