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Isolation and selection of siderophore-producing bacteria from roots of Simadu pineapple *(Ananas comosus)* in Subang District, West Java

*Isolasi dan seleksi bakteri penghasil siderofor dari akar nanas (*Ananas comosus*) Simadu di Kabupaten Subang, Jawa Barat*

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Abstrak

Bakteri mampu menghasilkan siderofor umtuk mengkelat besi dalam lingkungan dan digunakan oleh tanaman sebagai kofaktor dalam pembentukan klorofil untuk pembentukan glukosa. Penelitian ini bertujuan untuk mendapatkan bakteri penghasil siderofor tinggi dari akar nanas varietas Simadu di Kabupaten Subang, Jawa Barat. Bakteri diisolasi dari akar nanas, kemudian diseleksi dengan membedakan morfologinya dan diidentifikasi sebagai penghasil siderofor pada media Chrome Azurol S (CAS). Dari eksplorasi ini diperoleh 10 isolat bakteri (M1 sampai dengan M10) yang mampu menghasilkan siderofor. Isolat bakteri M7 memiliki kemampuan menghasilkan siderofor tertinggi. Isolat M7 teridentifikasi sebagai bakteri Gram negatif. Hasil analisis pohon filogenetik berdasarkan sekuensing 16S rDNA menunjukkan isolat ini termasuk genus Providencia. Dibandingkan dengan Providencia vermicola*, isolat standar dari InaCC yang berasal dari akar* Curcuma zedoaria*, M7 menunjukkan produksi siderofor yang lebih tinggi pada media LB pada kondisi aerobik.*

[Kata kunci: Isolasi bakteri, Providencia, akumulasi siderofor, akar nanas Simadu Subang]

Abstract

Bacteria can produce siderophores for chelating iron in the environments and are used by plants as an ingredient cofactor in building chlorophyll for glucose production. This study aimed to obtain high siderophore-producing bacteria from the roots of pineapple var. Simadu, in Subang District, West Java. Bacteria were isolated from the pineapple roots, then selected by differentiating their morphology and identified as producing siderophores with *Chrome Azurol S* (CAS) media. From this exploration, 10 bacterial isolates (M1 to M10) capable of producing siderophores were obtained. Bacterial isolate M7 had the highest siderophore production ability. M7 isolate was identified as a Gram-negative bacterium. The results of the phylogenetic tree analysis based on 16S rDNA sequencing showed this isolate belongs to the genus *Providencia*. Compared to the *Providencia vermicola* as a reference isolate from InaCC derived from the roots of *Curcuma zedoaria*, M7 showed higher siderophore production in LB media under aerobic conditions.

[Keywords: Bacteria isolation, *Providencia*, siderophore accumulation, Simadu Subang pineapple root]

Introduction

Plants have a system to absorb iron in the form of Fe^{2+}, only grass species that produce phytosiderophores can bind Fe in the form of Fe^{3+} (Ariga *et al.,* 2014). In plants, Fe must bind to another compound for it is translocation, because Fe^{2+} as soluble is harmful to plant body, and Fe^{3+} cannot be mobilized because of its low solubility. Citric acid are involved in Fe translocation in the plant body (Kobayashi *et al.,* 2019). Citric acid levels significantly correlated with the expression of some Fe and S deficiency induced genes on tomato (Vigani *et al.,* 2018). The element Fe has an

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important relationship in the activity of converting citric acid to isocitrate. Aconitase (ACO) requires many Fe-S groups because Fe-S is unstable in ACO, so it must be replaced many times by other Fe-S groups. The result of isocitrate changes in the Krebs cycle is alpha-ketoglutarate which after being reacted with the amino acid glutamate (glutamic dehydrogenase or oxaloacetate-glutamate transaminase) will become glutamic acid which is the raw material for making chlorophyll (Lushchak *et al.,* 2014; Panda *et al.,* 2014; Rout & Sahoo, 2015).

Since Fe plays an important role in chlorophyll formation, young leaves in most plants should contain at least 50 ppm on a dry weight basis of Fe. Plants will face chlorosis if they have Fe below 30 ppm (Hochmuth, 2017). Injection of ferrous sulfate into plant stems can solve iron deficiency, but this procedure is expensive, and the wound of injection can increase the risk of bacterial infection (Archer *et al.,* 2022). To overcome this problem, Fe (III)-chelate fertilizers such as iron ethylenediamine di-ohydroxyphenylacetate (Fe-EDDHA) are applied to the soil (Klem-Marciniak *et al.,* 2021). Although applying fertilizer to the soil is preferable to injection, this practice is also very expensive and must be repeated every year because iron chelates easily dissolve in the soil or are leached from the root zone, resulting in very low nutrient efficiency (Schenkeveld *et al.,* 2012). At present, to overcome the lack of Fe in the leaves, a solution of iron sulfate ($FeSO_4$) is sprayed onto the leaves. This method is much more effective than adding ferrous sulfate fertilizer to the soil because it is directly absorbed by the leaves (Johnson, 2016; Ozturk *et al.,* 2019). However, if these sulfate ion compounds reach the soil and react with hydrogen ions carried by water, the sulfate will turn into sulfuric acid which can kill microorganisms needed by plants in the soil (Husson, 2013; Zhou *et al.,* 2018)

Healthy plants usually have a healthy rhizosphere which are dominated by useful microorganisms. The microorganisms help the plant to make nutrient in the soil easy to be uptake. The using of bio-fertilizer is relatively more simple, and low operational cost than chemical fertilizers utilization (Naseer *et al.,* 2019). However, bacteria are the most easily inoculated and identified biological agents in the manufacture of biofertilizers compared to fungi, due to their fast growth.

Subang pineapple *(Ananas comosus)* is a type of Smooth Cayenne variety which is famous for its production of Simadu pineapple. It is called Simadu pineapple because it tastes sweeter than ordinary pineapple on the same hectare of land with more water texture and is not acidic (Rahmithasuci *et al.,* 2014). On one hectare of land consisting of 40,000 pineapple trees, only 2-6 Simadu Subang pineapples were found (Annisa, 2021). There is a potential for bacterial growth in the roots of the Simadu Subang pineapple, which can make the plant absorb Fe better than other pineapple plants on the same hectare of land than other pineapples. The purpose of this study was to obtain high siderophore-producing bacteria from the roots of Pineapple Simadu, Subang Regency, West Java.

Materials & Methods

The materials used were root samples from Simadu Subang pineapple, Nutrient Agar, Luria Bertani Broth, Luria Bertani Agar, Chrome Azurol S media, distilled water, HCl, $FeCl_3.6H_2O$, HDTMA, tetrazolium chloride, $FeCl_3.5H_2O$, ethyl acetate, ammonium molybdate reagent, and $CuSO_4$. The primer used for bacterial identification was 16S (27F and 1492R) using the services of PT. Indonesian Genetics Science. *Providencia vermicola* as reference isolate was purchased from InaCC-LIPI, Cibinong-Bogor.

Simadu pineapple root bacterial isolation

A total of 5 g of roots were taken, then washed with sterile distilled water, and dried on a Petri dish that had been covered with filter paper. The roots were then soaked in sterile water for 1 min, soaked in 70% alcohol for 1 min, and rinsed with sterile water 3 times. The root sample was then dried with sterile tissue. The root tissue is then ground with a mortar. Take 1 ml of the milled solution, put in 9 ml of sterile distilled water, and make the dilution 10^{-1}-10^{-6}, for 0.1 ml of bacterial suspension was taken from a dilution of 10^{-2}-10^{-6} and planted in a Petri dish containing NA media, spread plate method and incubated at 28°C for 24 hours. Each colony that grows periodically is purified by the streak plate method (Sudewi *et al.* 2020). Each colony was refreshed in Nutrient Agar media and Luria Bertani liquid media, then stored in 25% glycerol stock, and frozen in a freezer at -80°C (Arora & Verma, 2017).

Identification of Simadu pineapple root bacteria

Identification is done by experiencing the morphology of bacterial colonies, including colony shape, elevation, margin, and color of bacteria (Senthilkumar *et al.,* 2021).

Gram stain

The Gram staining stage begins with making a smear preparation and then fixing it on fire. Given a solution of crystal violet for 1 min and washed with distilled water, then given Lugol's solution for 1 min and 95% alcohol blanching solution for 10-20 s and washed with distilled water. Finally, the safranin solution was given for 15 s and washed with distilled water, then dried with filter paper, then observed with a microscope using a magnification of 10x100 (Jain *et al.,* 2020).

Selection of bacterial siderophore production

Preparation of Chrome Azurol S (CAS) solution: 60.5 mg of CAS was dissolved in 50 ml of distilled water and mixed with 10 ml of iron (III) solution (1mM $FeCl_3.6H_2O$ in 10mM HCl). This solution was then added to 20 ml of hexadecyltrimethylammonium bromide (HDTMA) solution (72.9 mg HDTMA in 40 ml of distilled water) and mixed. The CAS solution was mixed with Luria Bertani Agar in a ratio of 9 (LBA): 1 (CAS). The blue CAS agar medium was poured into a Petri dish and allowed to freeze. After being allowed to stand overnight, the bacteria were inoculated on the media to determine the ability of bacteria to produce siderophores. Incubation was carried out for 5-7 days. Bacteria that are able to produce siderophore will change the color of the media from blue to orange (Arora and Verma, 2017).

Growth profile of bacteria

Bacterial preculture was made by inoculating bacteria from NA slanted agar into 20 ml LB liquid media in a 100 ml Erlenmeyer which was incubated at 28-30°C for 24 hours in a rotary shaker (200 rpm) (Nabila and Kasiamdari, 2021). The new culture was then transferred to as much as 2 ml to 200 ml of new sterile LB media (Arora and Verma, 2017). This culture was then incubated at a temperature of 28-30°C, a shaker at 150-200 rpm (de Oliveira *et al.,* 2021), and every 3 hours a sample of 5 ml of bacterial culture was taken, then measured at 600 nm OD, for 24 hours.

Siderophore accumulation of bacteria

A total of 2 ml of the liquid culture were transferred to four 1.5 ml Eppendorf tubes, each filled with 1 ml. The sample was then centrifuged at 10,000 rpm, for 10 min, and the supernatant was taken and reacted with 2 ml of sterile CAS solution (Arora and Verma, 2017). The sample was left for 20 min or until it finished reacting, then the absorbance of the sample was measured using UV-VIS spectrophotometry at OD 630. The siderophore content of the sample is known by calculating Equation 1 (Senthilkumar *et al.,* 2021).

$$\text{Siderophore Unit (\%)} = \frac{Ar - As}{Ar} \, x \, 100 \quad \text{.......... (1)}$$

Where Ar is absorbance reference (CAS solution + media not inoculated by bacteria), As is absorbance of the sample (CAS solution + bacterial inoculated media supernatant).

Bacterial siderophore type test

Catecholate (Arnow's test): The supernatant was extracted with an equal amount of ethyl acetate, then 0.1 ml of 0.5 N HCl was added with 1 ml of cultured extract of ethyl acetate and mixed with 0.5 ml of ammonium molybdate reagent (10 g $NaNO_2$, 10 g $Na_2MoO_4.2H_2O$ in 50 ml DI-H_2O) followed by 1 ml of 1 N NaOH. The development of pink/red color indicates the presence of Catecholate-type siderophores (Senthilkumar *et al.,* 2021).

Hydroxamate (Tetrazolium test): A total of 1 ml of culture supernatant was added directly with 8 mg of tetrazolium salt and dripped with 2 N NaOH. The appearance of a dark red color indicates the presence of Hydroxamate type siderophores (Senthilkumar *et al.,* 2021).

$FeCl_3$ *test:* A total of 1 ml of culture supernatant and 1 ml of 2% ferric chloride solution were mixed, then the absorbance was measured by spectrophotometry at a wavelength of 420–450 nm for the orange color of the ferric hydroxamate complex and 495 nm if the dark purple color of the ferric catecholate complex appeared (Senthilkumar *et al.,* 2021).

Carboxylate test: A total of 1 ml of culture supernatant was mixed with 1 ml of 250 mM $CuSO_4$ and 2 ml of pH 4 acetate buffer and the spectrum was examined at a wavelength of 190–280 nm (Senthilkumar *et al.,* 2021).

Molecular identification of bacteria

Genomic DNA extraction was carried out using PCR primers 16S (27F and 1492R), with PCR products Species Barcoding (~1400bp), and Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005) (Table 1). PCR amplification with 2x My Taq HS Red Mix (BIO-25048), Bi-directional Sequencing. The phylogenetic tree was created using MEGA X software, with the Maximum Likelihood method, Kimura 2-parameter model Bootstrap values of 1000 replicate chromosome complete genome data obtained from the National Center for Biotechnology Information (NCBI).

Table 1. Sequence of bacterial 16S rDNA universal primers 27F and 1492R
Tabel 1. Urutan primer universal bakteri 16S rDNA 27F dan 1492R

Name *Nama*	Sequence *Urutan*	Reference *Referensi*
27F	5-AGAGTTTGATCCTGGCTCAG-3′	Wu *et al.,* (2014)
1492R	5-GGTTACCTTGTTACGACTT-3′	Wu *et al,.* (2014)

Results and discussion

Colony characteristics and siderophores production

The colony morphology of 10 isolated bacteria (M1 to M10) is presented in Table 2. All the isolated bacteria gave a cream color and most of them had entire edges, and the other two isolates had circular edges. The elevation of the isolates varied, but was dominated by flat, raised, and umbonate. Meanwhile, most of the bacterial isolates were circular. The combination of shapes, colors, edges, and elevations of the bacteria isolated from the various isolates indicated that the bacterial isolates obtained were different genus. To identify the isolate bacteria, in the next step, Gram staining and molecular identification were carried out on selected isolates that had high siderophore production zone (Senthilkumar *et al.,* 2021).

Table 3 presents the types of siderophores produced by each bacterial isolate. All isolates were able to produce siderophores hydroxamate and ferric hydroxamate with different concentrations. The isolates M1, M2, M8, and M9 produced the highest hydroxamate compared to the other isolates. Meanwhile, isolates M4 and M7 produced the highest ferric hydroxamate compared to the other isolates. Monocot plant seeds such as pineapple can absorb and translocate hydroxamate ligands. The presence of this type of siderophore in the roots will increase the availability of Fe for plants. Hydroxamate type is also able to work stably over a wide pH range (pH 4-10) (Ferreira *et al.,* 2019; Johann *et al.,* 2019).

Table 2. Colony morphology of each isolated bacteria
Tabel 2. Morfologi koloni masing-masing bakteri hasil isolasi

Isolate *Isolat*	Form *Bentuk*	Color *Warna*	Edge *Tepian*	Elevation *Elevasi*
M1	Circular	Cream	Entire	Flat
M2	Spindle	Cream	Entire	Flat
M3	Filamentous	Cream	Lobate	Raised
M4	Circular	Cream	circular	Umbonate
M5	Circular	Cream	Entire	Raised
M6	Spindle	Cream	Entire	Flat
M7	Circular	Cream	Entire	Umbonate
M8	Punctiform	Cream	Entire	Convex
M9	Circular	Cream	Entire	Raised
M10	Circular	Cream	circular	Umbonate

Table 3. Siderophore type of each bacteria isolate
Tabel 3. Tipe siderofor masing masing isolat bakteri

Isolate *Isolat*	Siderophore type *Tipe siderofor*				
	$FeCl_3$		Arnow	Tetrazolium	Shenker
	Ferric hydroxamate	*Ferric catecolate*	*Catecolate*	*Hydroxamate*	*Carboxylate*
M1	+	-	-	++++	-
M2	++	-	-	++++	-
M3	+	-	-	+++	-
M4	+++	-	-	+++	-
M5	++	-	-	++	-
M6	++	-	-	+++	-
M7	+++	-	-	+++	-
M8	++	-	-	++++	-
M9	++	-	-	++++	-
M10	++	-	-	+++	-

Note: Data are represented by the color; *orange (Ferric hydroxamate),* pink/red color (*Catecolate)*, red wine (*Hydroxamate), and* spectrum at a wavelength of 190–280 nm (*Carboxylate).* (++++), high color; (+++), medium color; (++), low color, (+) (very low color). The color of *Carboxylate* test is blue, that cannot read at a wavelength of 190–280 nm.

Catatan: Data disajikan dengan warna; jingga (Ferric hydroxamate), merah muda/merah (Catecolate), anggur merah (Hydroxamate), dan spektrum pada panjang gelombang 190–280 nm (Carboxylate). (++++), warna tinggi; (+++), warna sedang; (++), warna rendah; (+) warna sangat rendah. Warna uji Carboxylate adalah biru, yang tidak dapat terbaca pada panjang gelombang 190–280 nm.

Qualitative data of siderophore production is shown in Figure 1. All isolates from Simadu Subang pineapple roots were positive for siderophores accumulation with the presence of an orange zone around the colony. All the bacteria from the Simadu pineapple roots produced siderophores with a high category, seen from the contrast of the orange color around the colony, and its extent. The CAS/HDTMA complex binds strongly to Fe^{3+} to produce a blue color. When strong iron chelators such as siderophores remove Fe^{3+} from the blue complex bond, the color of the medium will turn orange (Senthilkumar *et al.,* 2021). Based on the results of the qualitative test, M7 isolates had the highest siderophore production ability compared to other isolates.

The phylogenetic tree of the M7 isolate is presented in Figure 2. The isolate of M7 have been identified as the genus *Providencia* and have the closest relatives to *Providencia vermicola* (Figure 2a), this bacterium also belongs to the Gram-negative bacterium (Figure 2b). The species *Providencia vermicola* P8438 was found to be the closest relative to isolate M7 and has multiple Mobile Genetic Elements (MGEs) genes, DNA segments that encode enzymes, and other proteins that facilitate the movement of genetic material between bacterial chromosomes. On the *P. vermicola* chromosome P8438, a Nonribosomal Peptide Synthase (NRPS) cluster was also found which functions for siderophore biosynthesis (Lupande-Mwenebitu *et al.,* 2021). The genus *Providencia spp.* Is a Gram-negative bacterium, motile, pathogenic, and mostly associated with urinary tract infections. *Providencia stuartii* and *Providencia rettgeri* species can produce the enzyme urease which is a catalyst for the hydrolysis of urea into NH_3 and CO_2 which are needed by plants (Guerfali *et al.,* 2018; Mao *et al.*, 2018).

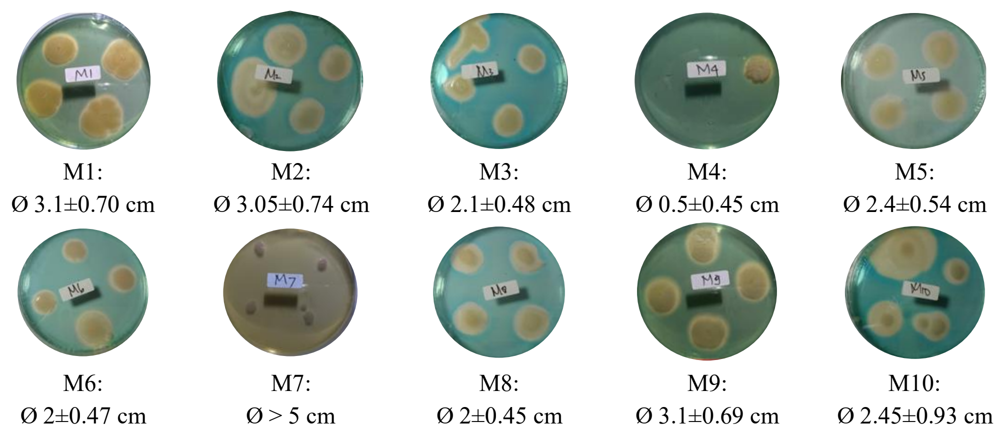

Figure 1. Siderophore production zone by isolate bacteria in a Petri dish for 7 days (Ø = diameter of the orange zone)
Gambar 1. Zona produksi siderofor oleh isolat pada cawan Petri selama 7 hari (Ø = diameter zona oranye)

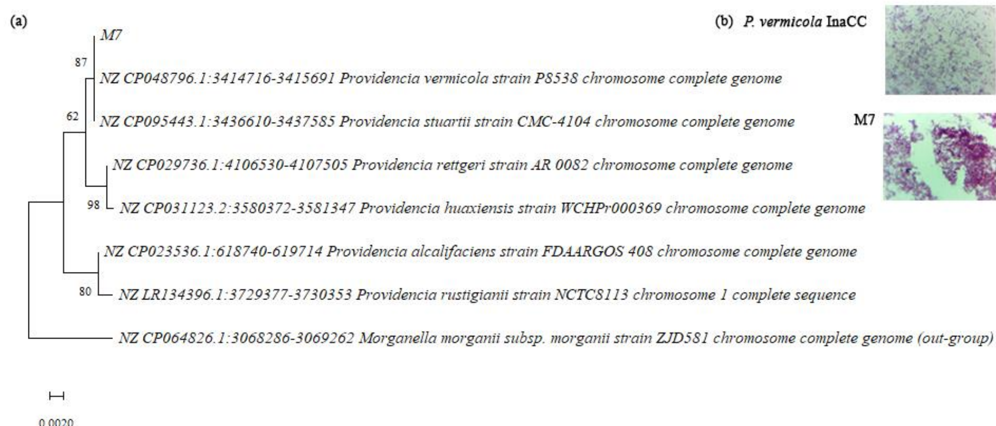

Figure 2. (a) Phylogenetic tree of M7 isolate samples, and (b) Test results of Gram M7 and *P. vermicola* InaCC isolate
Gambar 2. (a) Pohon filogenetik sampel isolat M7, dan (b) Hasil uji Gram isolate M7 dan P. vermicola InaCC

Identification of isolates of siderophores-producing bacteria

Table 4 presents a comparison of the qualitative siderophore production of M7 and *P. vermicola* InaCC isolates. In the first 3 hours, both isolates could produce siderophore. After 12 hours, the siderophore produced in M7 isolate increased drastically, much higher than siderophore accumulation in the isolate of *P. vermicola* InaCC.

Figure 3 shows the growth curves and siderophore accumulation by isolate of M7 and *P. vermicola* InaCC. The growth of the M7 and *P. vermicola* InaCC isolates were the same (Figure 3a). Siderophore accumulation by M7 isolate was much higher than that produced by *P. vermicola* InaCC (Figure 3b). The siderophore accumulation of M7 culture increased significantly in the early incubation until 12 h cultivation, and then decreased. The highest production of siderophores of M7 culture was at 80.34%. This value was higher than siderophore accumulation of *Pseudomonas aeruginosa* culture which was only 69.16% (Arora and Verma, 2017). On the other hand, the siderophore accumulation in the culture of *P. vermicola* InaCC was decreased after 3 h cultivation.

Table 4. Siderophores production zones of M7 and *P. vermicola* InaCC isolate
Tabel 4. Zona produksi siderofor isolat M7 dan P. vermicola InaCC

Isolate	Time (hour) *Waktu (Jam)*				
	3	12	15	21	24
	(Ø, cm)				
M7	0±0	1.05±0.04	1.51±0.03	2.53±0.03	4.5±0.42
Control			0±0		
P. vermicola (Ø)	0.6±0	0.6±0	0.6±0	0.6±0	0.6±0
Control					

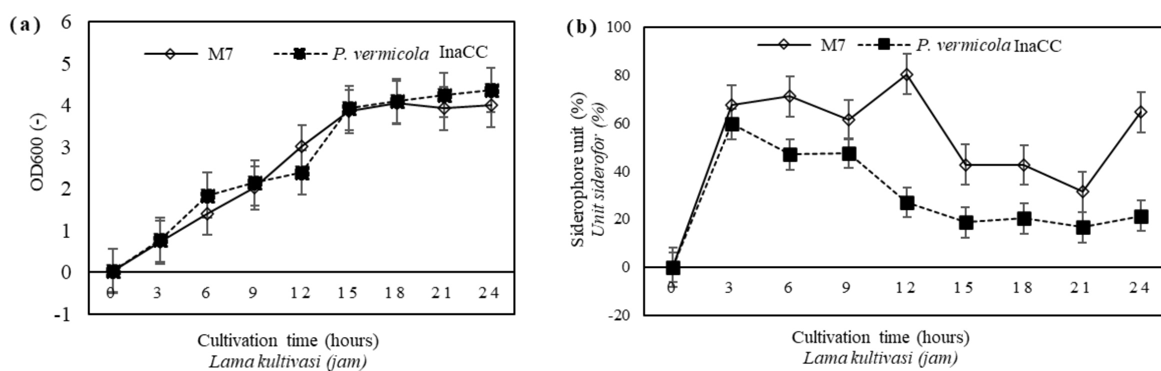

Figure 3. (a) Growth curve and (b) Accumulation of siderophores by isolates M7 and *P. vermicola* InaCC on cultivation time

Gambar 3. (a) Kurva pertumbuhan dan (b) Akumulasi siderofor oleh isolat M7 dan P. vermicola InaCC *terhadap lama kultivasi*

Conclusion

We succeeded in getting ten bacteria isolates producing siderophores from the roots of Pineapple Simadu, Subang. The isolate M7 had ability to produce siderophore of 80.34±0.44%. The types of siderophores produced were hydroxamate and ferric hydroxamate. This bacterial isolate has close to *P. vermicola*, but had a higher siderophore-accumulation ability than *P. vermicola*.

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