Mass spectrometry analysis revealed the production of siderophore from *Klebsiella oxytoca* strain STA01 isolated from sago palm field in Tulehu, Maluku, Indonesia

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Abstract. *Klebsiella oxytoca* STA01 produced the serratiochelin A, an NRPS based siderophore. The strain isolated from the sago palm field in Tulehu, Ambon, Maluku, Republic of Indonesia. Its partial sequence of 16S ribosomal RNA gene has been deposited in GenBank (Accession number MT786342). Its producing siderophore was discovered based on chrome azurol S guide-screening of 12 isolates. One of them showed an orange halo appearance in the chrome azurol S agar plate. The fractionation with 50:50 (v/v) MeOH/water revealed the presence of serratiochelin A. The structure was then determined by MS2 fragmentation.

Keywords. *Klebsiella oxytoca*, Maluku, siderophore, serratiochelin

1. Introduction
The soil is a place for a massive number of diverse microorganisms [1]. In nature, they are living as colonies and provide a different function. The most valuable function is that some microorganisms's adept at transforming nitrogen in the atmosphere into a fixed form that is usable by plants. These heterotroph microorganisms could live either as free-living cells or associated with plants. The examples of plant-associated microbes are *Bradyrhizobium* or *Rhizobium*. They form symbiotic mutualism with legumes [2]. Therefore, the free-living nitrogen-fixing cells, including *Bacillus*, *Azotobacter*, *Clostridium*, and *Klebsiella* [3].

In different conditions, soil microbes also adapt to the environment in order to maintain their fitness [4]. Iron is one of the essential micronutrients of metabolic enzyme and protein regulation [5]. Despite the presence of iron abundant on earth, however, therefore mostly found in insoluble ferric oxides [6]. Thus, the bioavailability of iron in nature is limited. Consequently, many microorganisms excreted chelating agents termed as siderophore [7, 8]. It is a natural product with low molecular weight produced by specific microorganisms that release into the environment to solubilize the iron and facilitating uptake through selective membrane receptors that import the Fe (III)-bound complex [9].

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In the pharmaceutical field, except small peptide form that previously we discovered, a massiliamide produced by Massilia as an example [10]. Siderophore also considered as an important molecule. It can be paired with antibiotics by covalent bonds, resulting in a new modified molecule called sideromycin. Albomycin and salmycin are examples of its molecules. They are inhibiting RNA synthetase and protein synthesis, respectively [11,12]. Therefore, as part of our going effort to investigate natural products from microbial biodiversity in Indonesia, in this study, we designed to isolate bacteria that produce siderophore from the sago palm field in Tulehu, Ambon, Maluku, Indonesia and characterized its releasing molecule.

2. Material and Methods

2.1. Isolation and screening of siderophore producing strain (STA01)
The soil sample was collected from the sago palm field in Salahutu, Tulehu, Ambon, Maluku, Republic of Indonesia (GPS, 3°36’39.6”S 128°19’49.3”E) in March 2018. The soil was collected from a ±20 cm in dept and the mean pH 7.1. The sample was serially diluted and inoculated to grow in solid modified Shipworm basal medium (SBM) without sea salt as described by Han et al. [13] and supplemented with 10% fructose and 2% agar. The colonies were distinguished and pure culture in separate plates after incubation at 30°C for 48 h.

2.2. Qualitative siderophore production
All pure colonies were then grown in an iron-deficient medium of SBM without sea salt and incubated for 48 h with persistent shaking at 140 rpm, 30°C in a Multitron Pro orbital incubator shaker (INFORS HT, Bottmingen, Switzerland). All broth containing pure strains were then extracted using Amberlite® XAD4 (SupeIco-Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The crude extract of each strain was then tested using the chrome azurol S agar test. The production of siderophore by each strain was qualitatively determined using chrome azurol S assay, as described by Schwan and Neiland [14]. Orange halos appearance of test extract on blue agar is indicative of siderophore excretion.

The crude extract from 12 L broth was then fractionated using Sephadex® LH-20 to generated five fractions (A: ddH2O, B: MeOH), fraction A (100 % A), B (75% A), C (50% A), D (25%A), and E (100% B). All fractions were then tested on chrome azurol S agar to predict the presence of siderophore.

2.3. DNA Extraction, 16S rRNA Amplification, and Sequencing
Genomic DNA of strain STA01 was extracted using HiYield® Genomic DNA Mini Kit (bacteria) (Süd-Laborbedarf GmbH, Saarland, Germany). The 16S rRNA genes were amplified by PCR using universal bacteria primers 27 F and 1492 R [15]. The PCR mix contained PCR buffer, dNTPs, MgCl2, and Taq polymerase. The protocol conditions included an initial denaturation period at 94°C for 60 s that covering 35 cycles, annealing at 63°C for 60 s, and extension at 72°C for 60 s. They were followed by a final extension at 72°C for 10 min. The PCR products were subsequently visualized in a 1% agarose gel electrophoresis and stained with peqGREEN™. The PCR product was then purified by PCR Purification Kit (250) QIAGEN™. The 16S rRNA gene sequencing was done by Eurofins Genomics (Ebersberg, Germany) with the ABI PRISM BigDye™ Terminator cycle sequencing kit (Applied Biosystems, United States), and the same primers were used for amplification, following the protocols provided by the manufacturer.

The forward and reverse sequences from strains were aligned, and the consensus sequences were obtained. MEGA 7.0.16 was employed to align the result sequence. Therefore, the phylogenetic relationships were inferred through a UPGMA with 10,000 bootstrap iterations [16,17]. The evolutionary distances were subtracted using the Kimura 2-parameter method [18]. Other sequences were obtained from NCBI.

2.4. MS-based-identification of siderophore excreted by strain STA01
A positive CAS assay-fraction was then subjected to High-resolution electrospray ionization- time-of-flight mass spectrometry (HR-ESI-TOF-MS) of Daltonix maXis 4G which acquired at the Department of Organic Chemistry, University of Tübingen, Germany. It is coupled with RP-HPLC using Reprosil 3 µm C18 100 Å, 10 x 3.3 mm, and linear gradient starts from 90% to 0% of A (A: ddH2O, B: Acetonitrile,
both solvents containing 0.01% formic acid) for 30 min and held constant of 100% B for 10 min. The MS data subsequently analyzed using Bruker Compass DataAnalysis 4.4 SR1(x64).

3. Results and Discussion

3.1. Strain characterization and taxonomical position
Twelve strains have been isolated from the sago palm field in Tulehu, Ambon, Maluku, Indonesia, using a modified SBM medium. Based on characteristic physical properties, none of them belongs to Actinobacteria. Among them, the rod-shaped cell form was showed domination (10/12 strains). We termed all the strain as STA01 to STA12. The distinct siderophile-producing strains were screened out by performing qualitative chrome azurol S assay. Only one pure rode-shaped strain, termed as STA01, showed positive results by forming an orange halo appearance on the chrome azurol S agar test, as shown in Figure 1. The sign indicated the presence of siderophore in broth containing strain. Based on information from 16S rRNA gene sequencing, STA01 has a similarity of 99.8% to K. oxytoca, a gram-negative bacterium.

![Figure 1. Chrome azurol S positive result from the extract of broth containing STA01.](https://www.ncbi.nlm.nih.gov/nuccore/MT786342)

The STA01 sequence of gene-encoded partial 16S rRNA contains 1440 bp and can be concluded as a member of the species of Klebsiella oxytoca based on nucleotide BLAST in NCBI. This isolate has high similarity (99.51%) to the K. oxytoca strain ATCC13182 and 99,44% similar to Klebsiella oxytoca strain NBRC 102593, as shown in Figure 2. Based on 16S rRNA sequences by using MEGA 7 to create Phylogenetic tree with UPGMA method, 10,000 bootstrap test and Kimura 2 parameter to see the evolutionary distance among 18 sequences of genus Klebsiella and one sequence of E. coli ATTC 25922 for out-group showed that STA01 belongs to the group of the genus of Klebsiella. All of the nucleotide sequences are derived from NCBI. The partial sequence of 16S rRNA from K. oxytoca STA01 from this study have been deposited in the GenBank database under the accession number MT786342 (https://www.ncbi.nlm.nih.gov/nuccore/MT786342).
Figure 2. Phylogenetic tree using the UPGMA method based on 16S rRNA gene sequences showing the phylogenetic relationships among the genus Klebsiella. Red arrow indicates the STA01 strain. The percentage of replicate trees shown in the branches used the bootstrap test 10,000 replicates.

3.2. The yield of siderophore containing fraction
The strain with a positive sign from the chrome azurol S agar test then scaled up into 12 L of SBM media (4 x 3L media in 5 L Erlenmeyer). The extraction using Amberlite® XAD4 resin yielded 29 crude extracts. After its fractionation from 29 g crude extract resulting in 44.8, 25.8, 12, 12, and 5.2 % for fraction A, B, C, D, and E, respectively, as shown in Figure 3. The detection of siderophore production of this strain was further confirmed by fractionated the crude and performed chrome azurol S agar test. Only 1 out of 5 fractions showed sign positive outcomes.
Figure 3. Fraction yield of 12 L broth extraction from *K. oxytoca*.

3.3. MS-based-identification of siderophore excreted by strain STA01

In this study, the siderophore-producing bacterium was isolated from the soil sample of the sago palm field and analyzed for the specific structure of siderophore. The fraction C from the culture broth of STA01 has one major peak among the other four peaks detected by UV-Vis detector. However, the yield of fraction C was low. A positive HR-ESI-TOF-MS m/z 430.1615 [M+H]^+ and 452.1432 [M+Na]^+ as shown in Figure 4-A. The prediction formula was C_{21}H_{24}N_{3}O_{7} (Δ = 0.2 ppm, rdb 12.0) and had a high similarity of mass to serratiochelin A.

The confirmation of its planar structure could be explained by the MS2 fragmentation pattern, as shown in Figure 4-B. Five fragmentation MS2 were matched with the fragmentation structure from the generated structure of serratiochelin A. The UV absorbance of this structure maximum at 220 nm (Figure 4-C).

Serratiochelin (A) has been discovered for the first time from *Serratia marcescens* in 1994 [19]. Another publication showed that its siderophore and two additional derivatives (B and C) were isolated from *Serratia* sp. V4 [20]. The newest finding showed that serratiochelin A produced by the bacterial microbiome in mosquito and reduce female *Anopheles gambiae* overall fecundity [21].
Figure 4. Siderophore produced in *K. oxytoca* broth. (A) MS1, (B) MS2 and fragmentation form pattern, (C) UV absorbance and the structure of serratiochelin A

4. Conclusion

We have isolated a species of *Klebsiella* from sago palm soil and talented in producing siderophore. The 16 S rRNA phylogeny revealed that isolated strain, an STA01, belonged to *K. oxytoca*. Furthermore, MS2 analysis of fraction C that contains 50:50 MeOH and water led to the identification of serratiochelin A. Its NRPS-based siderophore was discovered for the first time in 1994 from *Serratia marcescens*. Additionally, we report this is the first *Klebsiella* producing siderophore of serratiochelin A. In the future, the application of serratiochelin paired with antibiotics will be one of the promising molecules in order to combat the multidrug-resistant pathogen.
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