Isolation of Ureolytic Bacteria of Soft Coral and Their Potential in Microbially Induced Calcite Precipitation (MICP)

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Abstract. Microbial-induced CaCO$_3$ precipitation (MICP) via urea hydrolysis (ureolysis). Application of MICP is performed either by augmenting the site with ureolytic bacteria or by stimulating indigenous ureolytic bacteria. Alternative approaches to promote in situ MICP in soils biostimulation. Biostimulation encourages indigenous urea-hydrolysis bacteria by providing appropriate enrichment and precipitation media; it relies on the natural ubiquity of ureolytic soil bacteria. This study aims to obtain potential bacteria for calcium carbonate precipitation. The research methods used were (1) isolation of indigenous bacteria from soft corals and selection of potential bacteria for calcium carbonate precipitation; (2) morphological identification of selected isolates; (3) characterization of bacterial activity. This research has successfully grown 5 indigenous bacterial isolates from soft corals. One isolate, MICP-36, was able to grow on Marine Agar containing 25 g/L urea. The results MICP-36, was Gram positive and had a prosthetic cell shape. This isolate can induce calcium carbonate deposits of 0.083 g/mL. The test survival of bacteria in concrete showed that MICP-36 was able to live until the 28th day.

1. Introduction

Microbial-induced CaCO$_3$ precipitation (MICP) is an emerging technique use microbes that have activity to precipitate calcium carbonate [1]. The mechanism of action of MICP begins with microbes producing urease enzymes (ureolytic capabilities) to hydrolyze urea (CO(NH$_2$)$_2$) to ammonium (NH$_4^+$) and carbonate (CO$_3^{2-}$). Microbial cell wall which is negatively charged will attract ions from the environment including Ca$^{2+}$. Furthermore, Ca$^{2+}$ ions then react with CO$_3^{2-}$ which causes CaCO$_3$ to be deposited on the cell surface [2]. This occurs in the case of using these microbes to treat cracks in concrete.

There are four important elements that must be considered in the process of calcite precipitation, namely: calcium ion concentration, concentration dissolved inorganic carbon (DIC), pH, and availability of the nucleation cycle. In addition, it is necessary to pay attention to other environmental conditions, such as salinity, temperature, and nutrition. The process of calcium carbonate precipitation is initiated by bacteria producing the urease enzyme to hydrolyse urea CO(NH$_2$)$_2$ produces ammonium(NH$_4^+$) and carbonate (CO$_3^{2-}$) [3].

MICP has shown that microbially released CO$_2$ interacts with the biomineralization solution
favoring carbonate formation. The carbonate combines with the calcium ion (Ca\(^{2+}\)) leading to the precipitation of calcium carbonate (Reaction 1-4) [4].

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\begin{align*}
\text{CO(NH}_2\text{)\text{)}_2 + H_2O & \rightarrow NH_2COOH + NH_3 & (1) \\
\text{NH}_3\text{COOH + H}_2\text{O} & \rightarrow NH_3 + H_2CO_3 & (2) \\
\text{H}_2\text{CO}_3 & \rightarrow HCO_3^- + H^+ & (3) \\
2\text{NH}_3 + 2\text{H}_2\text{O} & \rightarrow 2\text{NH}_4^+ + 2\text{OH}^- & (4) \\
\text{HCO}_3^- + H^+ + 2\text{NH}_4^+ + 2\text{OH}^- & \rightarrow \text{CO}_3^{2-} + 2\text{NH}_4^+ + 2\text{H}_2\text{O} & (5)
\end{align*}
\]

During the biomineralization process, bacteria commonly serve as nucleation sites for the precipitation of calcium carbonate. Urease activity is found in a wide range of microorganisms, i.e. Lysinibacillus fusiformis, Arthrobacter crystallopoietes, and bacteria from the Bacillus group (Bacillus pasteruii, Bacillus sphaericus, Bacillus subtilis, and Bacillus cereus) [5].

MICP is mostly found from locations with high calcium carbonate mineral content such as sea, freshwater, and soils containing lime [6]. Soft coral is one of the creatures that live / grow in the sea. The main component that forms soft coral body structure is lime as evidenced by the discovery of a number of calcareous spicules in their body tissues that are not found in other marine animals [7]. Soft corals are known to have many interactions with marine microorganisms such as bacteria. [8] have succeeded in isolating and characterizing the symbiont bacteria from soft coral Sinularia sp. and survive and tolerate high urea and calcium concentrations [9]. The most important criteria to consider for the selection of a bacterial strain for biomineralization is its ability to synthesize active urease.

2. Materials and methods

2.1. Source of microorganisms

Microorganisms were isolated from soft coral. The first location for soft coral sampling was carried out in the waters of Tanjung Tiram Kendari, Southeast Sulawesi. Sampling was carried out at a depth of ± 3-4 m. The second sampling location is in the waters of Koholifano Island, Muna Regency, Southeast Sulawesi at a depth of approximately 2 meters. Before the sample is taken, first the physical and chemical parameters are measured in the field. The parameters measured were salinity, brightness, temperature and pH of seawater. All of the samples soft coral were taken under sterile conditions, placed in sample bags, and transported to the laboratory aseptically.

2.2. Microorganism MICP isolation

Samples soft coral were blended with marine media in a shaker incubator (24 hours). The samples were serially diluted and plated onto Marine Agar. The plates were incubated at 35 °C for 24-48 hours. After incubation, different colonies with specific morphological characteristics were collected and transferred to Marine Nutrient Broth (NB). In order to isolate the microorganisms from different media contain urea and CaCl\(_2\).

2.3. Urease activity assay

The isolates were put in a test tube, added 3 mL of urea media, then incubated at room temperature for 2 days. Positive test, if there is a change in the color of the media to pink.

2.4. Estimated Calcium Carbonate assay [10]

Isolates were inoculated in marine broth media containing calcite, a mixture of urea and calcium chloride. Then it was incubated at room temperature for 2 days. Calcite analysis was carried out using the volumetric method, with add 0.1 M HCl to the isolate culture medium, then titrating with 0.1 M NaOH solution.
3. Results and discussion

The main objective of this study was to investigate whether bacteria symbiont coral, can potentially act as a ureolytic. The corals are able to live in marine waters with normal salinity, namely 30-35%; 100% brightness value; temperature 25-30 C, pH 7.50-8.40 (Table 1).

Table 1. Sea water characteristics at the soft coral sampling point

| Sample                  | Characteristics of sea water |
|-------------------------|-----------------------------|
|                         | Salinity (%) | Brightness (%) | Temperature (˚C) | pH |
| Location 1: Tanjung Tiram, Konawe Selatan, Southeast Sulawesi | | | | |
| Soft coral 1            | 30            | 100            | 29              | 7.5 |
| Soft coral 2            | 30            | 100            | 29              | 7.5 |
| Location 2: Koholifano, Muna, Southeast Sulawesi | | | | |
| Soft coral 3            | 31            | 100            | 30              | 7   |

Marine bacteria are known to have many interactions with marine organisms, one of which is coral organisms [11]. The interactions carried out can be in the form of symbiotic and pathogenetic interactions. The results of isolation of symbiont bacteria from soft corals 1 (3 colonies species), soft corals 2 (2 colonies species), and soft corals 3 (4 colonies species).

Table 2 show the different growth at marine media with urea, profiles of the 9 isolate strains from the soft coral. Growth colony at medium with 25 g/L urea was 1 colony species from soft coral 3 which was able to survive. Bacterial isolates symbiont with soft coral 2, there were 2 types of colonies capable of living at a concentration of urea 23 g / L. Whereas at soft coral 3, one symbiont bacterial isolate was live at medium with 36 g / L urea (Table 2). The ability to live on media containing urea is related to the ureolytic activity of the isolates. From the data obtained, it shows MICP-36 isolate has the best ability to hydrolyze urea.

The ability to live isolate strains on media containing CaCl₂. Bacteria symbiont soft coral from location 1, obtained 1 isolate that is able to live on media containing 36 g / L CaCl₂. From location 2, have 1 isolate symbiont soft coral that was able to grow on marine media with 70 g/L CaCl₂ (Table 3). From the experiment obtained, it shows MICP-70 isolate has the best ability to live on media containing 70 g/L CaCl₂.

Table 2. Bacterial isolates on marine media contain urea

| Sample     | Colony species (at Marine Agar Media + Urea) |
|------------|---------------------------------------------|
|            | 15 g/L | 20 g/L | 21 g/L | 22 g/L | 23 g/L | 24 g/L | 25 g/L |
| Soft Coral 1| 3      | 3      | 3      | 3      | 3      | 3      | 3      |
| Soft Coral 2| 2      | 2      | 2      | 2      | 2      | 0      | 0      |
| Soft Coral 3| 4      | 1      | 0      | 0      | 0      | 0      | 0      |

Table 3. Bacterial isolates on marine media contain CaCl₂

| Sample     | Colony species (at Marine Agar Media + CaCl₂) |
|------------|---------------------------------------------|
|            | 20 g/L | 23 g/L | 35 g/L | 36 g/L | 40 g/L | 50 g/L | 60 g/L | 70 g/L |
| Soft Coral 1| 3      | 3      | 3      | 1      | 0      | 0      | 0      | 0      |
| Soft Coral 2| 2      | 1      | 0      | 0      | 0      | 0      | 0      | 0      |
| Soft Coral 3| 4      | 4      | 4      | 4      | 3      | 2      | 1      |
The isolates MICP-36 and MICP-70 were Gram positive (Figure 1). This result is in line with the research conducted by Achal et al. (2010) and Kumar et al. (2013), most MICP potential bacteria are Gram positive.

MICP-36 isolates must have the ability to degrade urea high enough than other soft coral symbiont isolates. The result of urea degradation is the formation of carbonate ions (CO$_2^-$). This carbonate ion will later bind with the Ca$^{2+}$ ion to form a precipitate of CaCO$_3$. So that the stronger the ureolytic power, the more calcium carbonate can be produced.

Determine urease activity, results a red indicator is to detect an increase in pH in the media, because hydrolysis of urea is ammonia. Phenol red in the media can cause color changes according to the pH conditions in the media. If phenol red is in an alkaline media, cause color change to pink (Figure 2).

The results showed that isolate MICP-36 in 30 mL of MA medium was able to produce 2.5 g of calcium carbonate. Isolate MICP-70 in 30 mL MA medium yielded 1.5 g calcium carbonate. The different potassium carbonate produced is due to the different types of isolates and their habitats, thus affecting their characteristics. Isolate MICP-36 is one of the isolated strains with a high potential urease activity for biotechnological application in different aspects.

4. Conclusion
The symbiont microbes that live on soft corals have different types and characteristics. This difference is also due to different aquatic habitats. The soft coral symbiont isolate which had the best urease activity was isolate MICP-36.

Acknowledgements
The authors gratefully acknowledge the financial supported provided by Direktorat Jenderal Pendidikan Tinggi Kementerian Pendidikan dan Kebudayaan.
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