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Comparison of Calcium Carbonate Precipitation (CCP) and Yeast Urea (YU) Culture Media on Hydrocarbonoclastic Bacteria Endospores Formation

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ABSTRACT

Some of hydrocarbonoclastic bacteria species are able to form endospores and potentially to be applied as additional ingredients to strengthen concrete structures. Bacteria endospores are highly resistant in extreme environment such as inner part of concrete which has pH of 13. The aim of this study was to compare the formation of hydrocarbonoclastic bacterial endospores on CCP and YU media. Bacteria isolates were *Bacillus* JA1, JB2, AK4, SU1, Lysinibacillus JB2 and Sporosarcina JA4. Isolates were cultured in CCP and YU media for 24 hours. Generated endospores from these media were stained using malachite green and observed under microscope. Bacteria vegetative cells were removed from culture media through cell lysis using a shock treatment at 70 °C utilize thermostat waterbath. Viable endospores were observed after incubation for 7 days. Our study showed all isolates are able to form endospores. Bacteria isolates produced more endospore in YU media than CCP media. Lysinibacillus JB2 produced highest amount of endospores (4.9×10^6 in CCP media and 5.4×10^6 in YU media) and its endospores also showed the best viability after heat shock treatment for 20 minutes.

***Key words**: CCP-medium, Endospore, Temperature; Yu-medium*

Introduction

Concrete is the most commonly used of construction material, that has the advantage of high compressive strength, long durability, compatible reinforcement, and can be form as desired (Seifan *et al.*, 2016). A concrete before cast have pH 10-13 and temperature of 70 °C, which are extreme environment. Several studies have been using hydrocarbonoclastic bacteria to strengthen concrete structure so bacteria are able to adapt in pH and high temperature. Hydrocarbonoclastic bacteria can form endospore, which are able to survive in extreme environments such as alkaline conditions in concrete materials (Talaiekhozan *et al.*, 2014). Sporulation started in response to environment particular physiological signals extreme (Sella *et al.*, 2014).

Bioconcrete is containing hydrocarbonoclastic bacterial endospores if there is a crack, water and oxygen would have to enter and can activate an endospore, that could trigger formation of calcite ($CaCO_3$) through the enzymatic urease mechanism (Xu *et al.*, 2018). The process of $CaCO_3$ precipitation is a part of metabolism of hydrolysis of urea (CO(NH2)2) to ammonium (NH_4^+) and carbonate (CO32-) (Sahoo *et al.*, 2016). Calcite formed will fill cracks so that closed back (Luhar *et al.*, 2015).

Several hydrocarbonoclastic bacterial were isolated from the limestone mountains of East Java, which was Bacillus JA1, JB3, AK4, SU1, *Lysinibacillus*

JB2 and *Sporosarcina* JA4. These bacteria are able to form endospores (Zulaika *et al.,* 2019). In this research, the production and endospores viability of hydrocarbonoclastic bacteria cultured on CCP and YU media will be compared.

Materials and Methods

Microbial cultures

The cultures used were *Bacillus* JA1, JB2, SU1, AK4, *Lysinibacillus* JB2 and *Sporosarcina* JA4 originated from Jaddih hill, Bangkalan, Akbar cave, Tuban, and Suci, Gresik lime hills area, East Java, Indonesia (Zulaika *et al.,* 2019).

Production Endospores in CCP and YU Media

Composition of CCP media per liter; 20g urea, 2.12g $NaHCO_3$, 10g NH_4Cl, 3g Nutrient Broth, 30mM $CaCl_2$ (Wei *et al.,* 2015). A colony in cultured was inoculated into 20 ml CCP media, incubated on a rotary shaker (100 rpm, 24 hours) at room temperature. The formation of endospores was observed by malachite green staining, characterized by green round organelles in the terminal cell (Pungrasmi *et al.,* 2019). Endospores formed calculated by hand tally counter. The same was done in YU medium with a per liter composition: nutrient broth 3 g, $NaHCO_3$ 2.12 g and urea 10 g (Harley *et al.,* 2002).

Malachite green staining: one loop of isolate is scratched on the glass object that has been given distilled water, fixed above bunsen. Then the top preparate was covered with a tissue and dripped with malachite green until all the spreads were flooded. Preparate heated 3-4 minutes at the bunsen. The tissue on the preparate was discarded and rinsed with distilled water, then stained with safranin for ± 1 minute and rinsed again with distilled water. Preparate observed under a microscope with the aid of immersion oil magnification of 1000 times and counted the number of spores formed (Harley *et al.,* 2002).

Shock Treatment Temperature for Endospore Production and Viability

A colony of culture was inoculated into 20 ml YU medium, incubated on a rotary shaker (100 rpm, 24 hours) at room temperature. Furthermore, cells were induced to form endospores by shock treatment at 70°C for 5, 10, 15, and 20 minutes in a water bath. A total of 2 µL of culture was centrifuged (8,000 rpm, 15 minutes), the pellets were endospore. Then the pellets were washed twice with 0.8% (w / v) NaCl (Pungrasmi *et al.,* 2019), dried in an oven at 37°C (24 hours) and stored for 7 days. After storage, the viability was observed by growing the spore in nutrient agar plates.

Results and Discussion

All isolates are able to form endospores on CCP and YU media. With malachite green staining, oval organelles in the cells are green, indicating the formation of endospores (Pungrasmi *et al.,* 2019). In YU medium, the formation of endospores (24 hours incubation) was higher than CCP. *Lysinibacillus* JB2 produced the highest number of endospores compared to other isolates (Figure 1).

Media complexity affects the formation of bacterial endospores. The CCP medium contains urea, $NaHCO_3$, NH_4Cl, Nutrient Broth, and $CaCl_2$ (Wei *et al.,* 2015), while the YU medium contains nutrient broth, $NaHCO_3$ and urea (Pungrasmi *et al.,* 2019). CCP media has a more complete nutritional composition than YU media, so that bacteria are less stressed by extreme

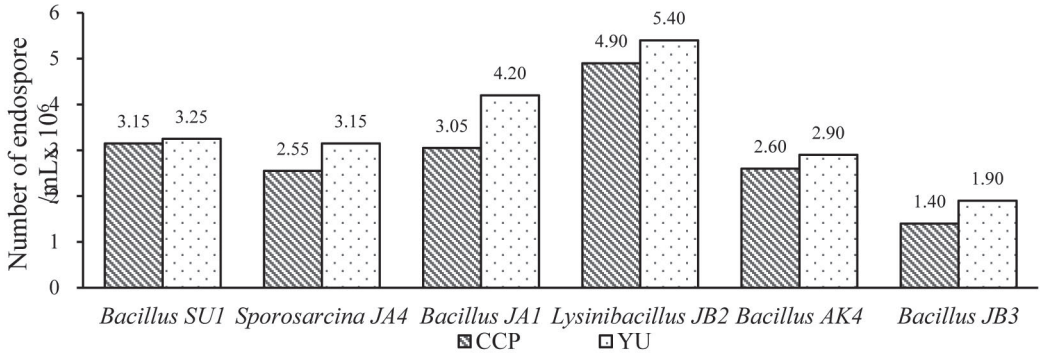

Fig. 1. Production of hydrocarbonoclastic bacterial endospores

Environments and form less endospores. Several extreme environmental factors such as nutrient deficiency, pH or high cell density can trigger the differentiation of vegetative cells into endospores (Sella *et al.*, 2014).

Endospores had been stored for 7 days, after being grown in CCP media showed good viability, indicated by the growth of colonies (Figure 2).

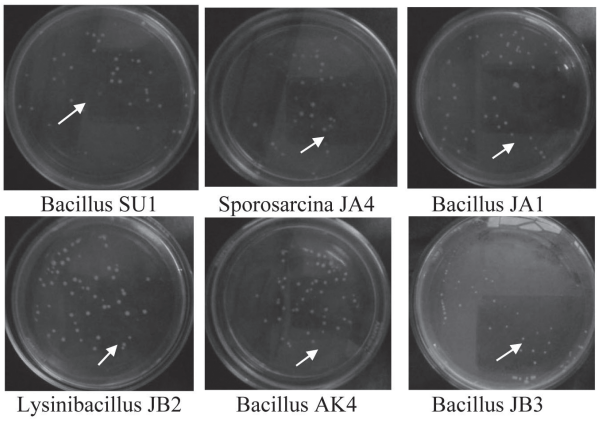

Fig. 2. The viability of endospores that form vegetative cell colonies in CCP-agar media is indicated by arrows

Gram positive bacteria such as genus *Bacillus*, if in extreme environments will form inactive structures called endospores, that could survive for years (Talaiekhozan *et al.*, 2014). The resistance factor of endospore for high temperatures (concrete temperature of 70 °C (Talaiekhozan *et al.*, 2014) involves protection of endospore DNA by small-acid soluble protein, accumulation of divalent cations such as Ca^{2+} and Mn^{2+}, and dehydration of endospore nucleus. In addition, there is a heat resistance role for dipicolinic acid (pyridine-2,6-dicarboxylic acid: DPA), in which divalent cations are chelated in the nucleus of endospores (Kort *et al.*, 2005)

The result of endospore from shock treatment temperature up to 20 minutes, the longer the exposure time, the higher viability. This shows that with an exposure time of 20 minutes, more endospore is formed. *Lysinibacillus* JB2 has the highest endospore viability compared to other isolates (Figure 3).

Some of the factors involved in endospore resistance to high temperatures are DNA saturation with α/β-type SASPs. High temperature causes DNA damage, especially depurination, so there must be protection of endospore DNA against damage. α/ β-type SASP binding has been shown to inhibit DNA

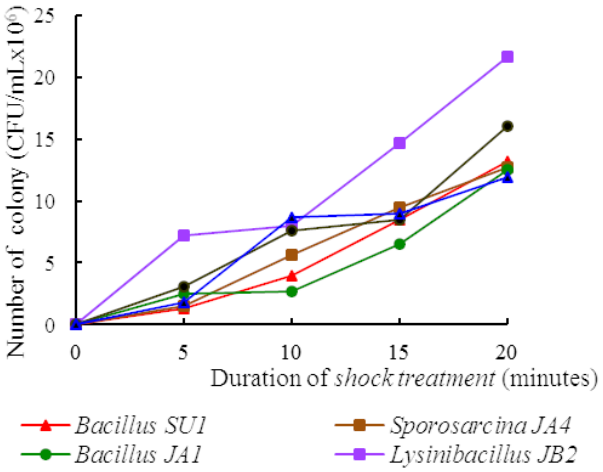

Fig. 3. Endospores viability after 7 days of storage

depurination in vitro. Protection of endospore DNA by binding to SASP type α / β causes saturation of endospore DNA thus providing protection against DNA (Setlow, 2014).

Another factor is the presence of DPA which does not accumulate in the core of endospores, so that the water content does not decrease as much as endospores containing DPA, the lower the water content the higher the resistance of endospores to high temperatures. Low water content causes reduced mobility of protein molecules and increases protein resistance to inactivation temperatures. The presence of DPA in endospores is used as chelating divalent ions. The most common DPA dichelated ions are Ca^{2+} and Mn^{2+}. Another factor is the sporulation temperature, endospores formed at higher temperatures generally have a lower water content than endospores formed at lower temperatures (Setlow, 2014).

Conclusion

All cultured are able to form endospore, the production of endospore in YU media is more than CCP media. *Lysinibacillus* JB2 endospore was the highest than other isolates, which is 4.9 x 10^6 in CCP media and 5.4 x10^6 in YU media. *Lysinibacillus* JB2 endospore also has better viability than other isolates with shock treatment for up to 20 minutes.

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