Pre-evaluation of urease production by *Bacillus* sp. SK II-5 thermophilic bacteria using agricultural waste as a substrate

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Abstract. Agricultural waste contain nutritive minerals including nitrogen, phosphorus, and potassium which help plants grow faster and increase yields, but when released into the open environment can leads the aquatic environment to eutrophication. Agricultural waste, which is mostly contain urea, can be utilized for the growth media of microorganisms to produce urease. Urease is an enzyme that functions to hydrolyze urea to ammonia and carbonate. Naturally, some organisms can produce urease, one of which is the thermophilic bacteria of the genus Bacillus. The purpose of this research is determine the results of viability test of thermophilic bacteria *Bacillus* sp. SK II-5 using small island agricultural waste as media. The viability test of *Bacillus* sp. SK II-5 is carried out using the streak plate and turbidimetry methods. The results of this research were thermophilic bacteria *Bacillus* sp. SK II-5 can grow at 50ºC and 60ºC which have been tested for cell viability in agricultural waste extract agar media at 37ºC.

1. Introduction
Over the last 50 years, the amount of nitrogen pollution entering aquatic environment that causes eutrophication has increased dramatically. The nitrogen contained in agricultural waste comes from urea fertilizer worldwide. Urea from agricultural waste has high content of nitrogen (46%), high water solubility, compatibility to most fertilizer and high foliar uptake [1,2]. Utilization of urea as growth media in urease production is an alternative to reduce urea pollution.

Urease (urea amidohydrolase, EC 3.5.1.5) is an enzyme that hydrolyzes urea (H₂N-CO-NH₂) to ammonia and carbonate [3]. The hydrolysis process produces a high carbonate and increase environmental pH to induce the precipitation of calcium carbonate (CaCO₃) [3,4]. Calcium carbonate formed can be used as grout material to strengthen the structure and reduce permeability of porous materials such as soil or sand [5,6].

Genus of *Bacillus* is one of the urease-producing bacteria that tolerant at high concentrations of urea and calcium. It has a wide growing temperature range of 5-72ºC and is not a pathogenic bacteria [7,8]. These bacteria can produce urease at high temperatures (thermophiles) and work optimally in a high temperature range. This is advantageous for biogrouting applications because enzymes are more thermostable and can be produced in a shorter period of time [9].

Previous research by Fidarliyan and Nugroho Prasetyo [10], reported that urease can be produced at thermophile temperature (50ºC) with urease activity 1002 U/ml from cow urine waste substrate. However, the urease activity is less optimal when applied to biogrouting. The increase in compressive
strength from the control is only 18%. The utilization of urea substrate in cow urine waste is less than optimal. And affects the performance of result urease activity. Therefore, it is necessary to produce urease from agricultural waste that is high in urea content. However, a preliminary test is needed to confirm the viability of the thermophilic bacteria *Bacillus* sp. SK II-5 which is used on urea media from agricultural waste.

2. Materials and Methods

2.1. Materials

The bacterial cultures used in this study were *Bacillus* sp. SK II-5 which are collection from Microbiology and Biotechnology Laboratory, Department of Biology, Faculty of Science, Institut Teknologi Sepuluh Nopember (ITS) Surabaya. Bacterial growing medium were purchased fro Sigma. Materials used were NB, NA, agricultural waste extract media (NB 0.3 grams; NaHCO$_3$ 0.212 grams; NH$_4$Cl 1 gram; and CaCl$_2$2H$_2$O 0.414 grams dissolved in 100 ml of agricultural waste extract 40%). Agricultural waste from small island (Poteran, Sumenep, Madura, East Java). Instrumental used were Laminar Air Flow (LAF), Temperature Rotary Shaker, UV-Vis Spectrophotometer in the Microbiology and Biotechnology Laboratory, Department of Biology, Faculty of Science, Institut Teknologi Sepuluh Nopember (ITS) Surabaya.

2.2. Methods

2.2.1. Subculture of Bacillus sp. SK II-5

*Bacillus* sp. SK II-5 was subcultured in NA (Nutrient Agar) solid culture media in Petri dishes and NB (Nutrient Broth) pre-enrichment media on Erlenmeyer. Isolate of *Bacillus* sp. SK II-5 was grown in NA media with a composition of 2.8 grams of NA dissolved in 100 ml of distilled water. A loopful of *Bacillus* was streaked on NA medium. Then, the bacteria were incubated at 37ºC or room temperature. The incubation period to grow bacterial colonies is 2×24 hours. Then, the second subculture is done with the same media and condition to grow bacterial colonies. The second subculture was incubated at 37ºC or at room temperature for 2×24 hours to grow bacterial colonies. After that, the subculture is stored in the refrigerator. The inoculum was transferred to 100 ml of NB (Nutrient Broth) media as much as 3 ose. Then, the inoculum is placed in the rotary shaker for approximately 2×24 hours [10].

2.2.2. Test for reconfirmation of Thermophilic Bacteria

This reconfirmation test is used to review or ensure that the *Bacillus* sp. SK II-5 is still classified as thermophilic with a high temperature tolerance test and bacterial cell viability test. Bacterial culture in the agricultural waste extract media (5 ml) transferred to a test tube and incubated at 50º C for 12 hours in a temperature rotary shaker. Then, subculture was transferred into agricultural waste extract agar media and incubated for 2×24 hours. Growing bacterial isolates were selected and tested for tolerance to 60º C for 12 hours. Tolerance test at 12 hours was observed visually and turbidimetry using a spectrophotometer at wavelength of 600 nm. Then, the bacterial cell viability test was carried out by subculture back to agricultural waste extract agar media and incubated for 2×24 hours at 37 ºC. Furthermore, the growth of isolate culture was observed.

3. Results and Discussions

3.1 Viability Test of Bacillus sp. SK II-5 Thermophilic Bacteria

Viable isolates after tolerance test at a temperature of 50ºC and 60ºC in an agricultural waste extract media for 12 hours are shown in Figure 1. The isolate was cultured on agricultural waste extract agar media and incubate at 37ºC for 2×24 hours (Figure 1).
Figure 1. Results of the viability test Bacillus sp. SK II-5 after tolerance test of thermophilic bacteria. (A) Colonies after tolerance test at 50 °C. (B) Colonies after tolerance test at 60 °C

Based on Figure 1 (A) and (B), it shows that Bacillus sp. SK II-5 can grow at temperature of 50ºC and 60ºC on agricultural waste extract agar media. This shows that Bacillus sp. SK II-5 is thermophilic facultative. The thermophilic facultative can grow at a temperature range of 25 ºC to 72 ºC. This bacterium is able to live in the mesophyll and thermophile temperature range. The mesophyll temperature range includes 25ºC to 35ºC while the thermophile temperature range includes temperatures of 35ºC to 72ºC [11,12,13]. This results indicate that bacteria utilize urea from small island agricultural waste and produce urease in mesophyll and thermophile temperature range. Characteristics of Bacillus sp. SK II-5 shown in Figure 1 is translucent cloudy white, irregular and filamentous, oblate margin, and rough surface [11].

3.2 Growth Profile of Bacillus sp. SK II-5
Acclimatization process for Bacillus sp. SK II-5 was carried out in three stages based on variations in the concentration of the production medium. Figure 2 presents the growth profile at the initial stages of acclimatization carried out at incubation temperatures of 28ºC and 37ºC, respectively, with a variation of pH 4.7 and 9.

In Figure 2 it can be seen that the acclimatization of Bacillus sp. SK II-5 enters the half logaritmic phase at 12 o’clock in all temperature and pH treatments with absorbance values or Optical Density (OD) 0.5 until 0.9. The half logaritmic phase is used as a reference for the transfer of culture in the acclimatization stage. This is consistent with the study of Burgess et al. [14], that the acclimatization time is when it has passed the lag phase to half logaritmic.

Furthermore, in Figure 3 we can observe the number of cell densities in the logaritmic phase growth profile in the initial stages of acclimatization carried out at the incubation temperature of 28 ºC and 37 ºC with variations of pH 4.7 and 9.

In Figure 3 it can be seen that the acclimatization of Bacillus sp. SK II-5 enters the half logarithmic phase at 12 hours with cell density reaching 0.5 to 0.9 × 10^8 cells ml. This is in accordance with Boling's research [15], that the level of cell density reaching 10^8 cells/ ml is the optimum amount of density that can be used as a reference for the transfer of culture at the next acclimatization stage. The growth of microbial cells if they have reached cell density of 10^8 cells/ ml can be considered as starter culture.
4. Conclusions
Based on the results of research, it can be concluded that Bacillus sp. SK II-5 thermophilic bacteria can grow at temperature of 50°C and 60°C. This shows that Bacillus sp. SK II-5 is viable thermophilic facultative bacteria that can produce urease from small island agricultural waste.
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