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Dehalogenase enzyme activity of *Bacillus* sp. D1 isolated from pharmaceutical waste

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Abstract. Dichloromethane (DCM) is widely used in the pharmaceutical industry as an intermediate solvent and active reagent. Most of the pharmaceutical industry waste contains of DCM which is a toxic compounds in the environment. Microorganism plays an important role in maintaining environmental ecosystems by degrading organic pollutants. Dichloromethane degrading microorganisms are known have dehalogenase enzyme activity. *Bacillus* sp. D1 isolated from pharmaceutical industry waste was used in this study. This study aims to determine the activity of *Bacillus* sp. D1 dehalogenase enzyme in various incubation time and pH condition. This research is an experimental study that used a completely randomized design with three replications. *Bacillus* sp. D1 was cultured in the mineral salt medium (MSM) added by DCM in rotary shaker 130 rpm until 7 days incubation. Evaluation of enzyme activity was carried out at various incubation times and pH conditions using the Bergman method. The best enzyme activity was 0.285 U/mL reached at 48 hours of incubation. The highest dehalogenase enzyme activity was 0.913 U/mL at pH 9. The activity of dehalogenase enzyme from *Bacillus* sp. D1 optimum at alkaline condition.

Keyword: dehalogenase, *Bacillus* sp, dichloromethane

1. Introduction

The development of new industries and the expansion of existing industrial production in Indonesia has increased. One of the industries that have elevated is the pharmaceutical industry. The pharmaceutical industry is an industry that produces a therapeutic product for humans as well as animals. The increasing necessary for medicine in Indonesia has led to an increase in the pharmaceutical industry and this has affected the surrounding environment. Processes and activities conducted the pharmaceutical industry is very diverse, depending on the resulting product. Each of the pharmaceutical industry produces waste differently with different characteristics [1]. As much as 80% of pharmaceutical waste containing organic solvents [2]. Organic solvents are used to dissolve the pharmaceutical industry of intermediate compounds and reactive reagents.

Bacillus sp. D1 in this research was obtained from the isolation of pharmaceutical waste. The industry wastehas high content of organic solvents such as dichloromethane. The concentration of organic solvents in waste reached 6500 ppm. Dichloromethane (CH₂Cl₂, DCM) is carcinogenic compounds, highly toxic, volatile, and water soluble. DCM is a toxic pollutant in the environment [3]. According to [4], the mechanism of DCM biodegradation is carried out by producing dehalogenase enzyme by methylobacteria in aerobic conditions. The enzyme is derived from the cytoplasm and is encoded by the *dcmA* gene. Dehalogenase is a group of glutathione S-transferase. According to [5], methylobacteria use formaldehyde produced during DCM degradation for energy sources. *Bacillus* sp. D1 inoculated in the media containing dichloromethane with a concentration of 6500 ppm. The enzyme activity of *Bacillus* sp. D1 has not been tested for variations in incubation time and pH. Based on the description above, it is necessary to test the enzyme activity of *Bacillus* sp. D1 on variations in incubation time and pH.



2. Materials and Method

2.1. Microorganism and culture conditions

The bacteria used was *Bacillus* sp. D1 which has been isolated from pharmaceutical waste. *Bacillus* sp. D1 is inoculated in the mineral salt medium (MSM) containing 6500 ppm dichloromethane. The MSM was composed of K_2HPO_4 (0.5 g/L), $Na_2HPO_4 \cdot 12H_2O$ (0.5 g/L), $(NH_4)_2SO_4$ (0.5 g/L), $MgSO_4$ (0.5 g/L), $CaCl_2 \cdot 12H_2O$ (0.001 g/L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L), and yeast extract (1 g/L).

2.2. Enzyme production in the various of incubation time and pH conditions

The pure culture of *Bacillus* sp. D1 was grown in the nutrient broth. After 24 hours, the Optical Density (OD) of the culture was measured and adjusted to 0.5 value at wavelength 600 nm. A total of 2 mL culture was inoculated in 48 mL of production medium (MSM contains of 6500 ppm of DCM). The culture was incubated at a shaker incubator at 130 rpm at room temperature for 7 days to produce the enzyme. Samples were evaluated every 24 hours until 168 hours for bacterial growth and the activity of dehalogenase enzymes. The best value of enzyme activity in the variation of incubation time were tested to pH variations. The pH was adjusted to 5, 6, 7, 8, 9 using HCl and NaOH.

2.3. Evaluation of bacterial growth

The growth of bacteria was evaluated by the total plate count (TPC) method. 1 mL of the culture was taken and diluted until certain dilution in the NaCl solution (0.85%). The last three dilutions, as much as 1 mL, were placed on the petri dish and poured with nutrient agar medium. The obtained data was used to create a microbial growth curve during in the DCM medium.

2.4. Extraction of enzyme

Crude enzyme was obtained from the culture of *Bacillus* sp. D1 in the production medium. The culture was centrifuged at 7000 rpm for 30 minutes to separate cells with supernatant. Cells were washed twice using Tris- HNO_3 (pH 9). The cells were resuspended using 1.2 mL of Tris- HNO_3 (pH 9). The obtained suspension was treated with an ultrasonic generator at 9000 rpm for 30 minutes at cold temperature. The cell-free supernatant was obtained by centrifuge at 14000 rpm for 30 minutes. The cell-free supernatant contains of crude enzyme.

2.5. Measurement of enzyme activity

Measurement of enzyme activity was conducted using [6] method. A cell-free supernatant was added by pH 9 Tris- HNO_3 solution and DCM organic solvent with ratio of 1:1. The mixed sample taken as much as 100 μ L was mixed with 2 mL of 0.25M ferric ammonium sulfate in 9M nitric acid and 2 mL mercuric thiocyanate in 95% ethanol. The mixed solution was incubated for 5 minutes until the color changes to yellow-reddish. Absorbance of the mixed solution was measured at a wavelength of 460 nm. Enzyme activity was calculated using the following formula (1).

$$\text{Enzyme activity (U/mL)} = \frac{(\text{Absorbance of test} - \text{absorbance blank}) \times (\text{total volume})}{(\text{incubation time}) \times (\text{enzyme volume})} \quad (1)$$

3. Results and Discussion

Dichloromethane (DCM) is an organic solvent which have a simple chemical chains, but DCM is toxic for living organisms. Microorganisms using dichloromethane have enzymatic mechanisms for their growth. The ability of microorganism to break down the DCM depends on the ability of microorganisms to use the substrate as a carbon source. This research describes the ability of *Bacillus* sp. to grow and use DCM as a substrate. The mechanism of substrate use was observed with the number of colony and their enzymatic activity. The enzyme activity of this research using crude enzyme dehalogenase.

Effect of dehalogenase enzyme activity in variations of incubation time

Bacillus sp. D1 have an enzymatic activity to degrade dichloromethane. Enzymes that are able to break down dichloromethane compounds are dehalogenase enzymes. The results showed that there were dehalogenase enzyme products produced in growth of *Bacillus* sp. D1. Increased incubation time

causes increased enzyme activity. The effect of the incubation time variation in the activity of dehalogenase enzymes is shown in Table 1 and Figures 1.

Table 1. Average of dehalogenase enzyme activity in variations incubation time

Incubation time (hours)	Average of TPC log <i>Bacillus</i> sp.	Average of dehalogenase enzyme activity (U/mL)
24	$14,2 \pm 1,91$	$0,15 \pm 0,05$
48	$20,6 \pm 1,98$	$0,28 \pm 0,10$
72	$20,5 \pm 1,90$	$0,57 \pm 0,04$
96	$20,3 \pm 1,97$	$0,44 \pm 0,13$
120	$20,3 \pm 1,95$	$0,38 \pm 0,07$
144	$14,0 \pm 1,95$	$0,39 \pm 0,02$
168	$13,7 \pm 1,91$	$0,35 \pm 0,10$

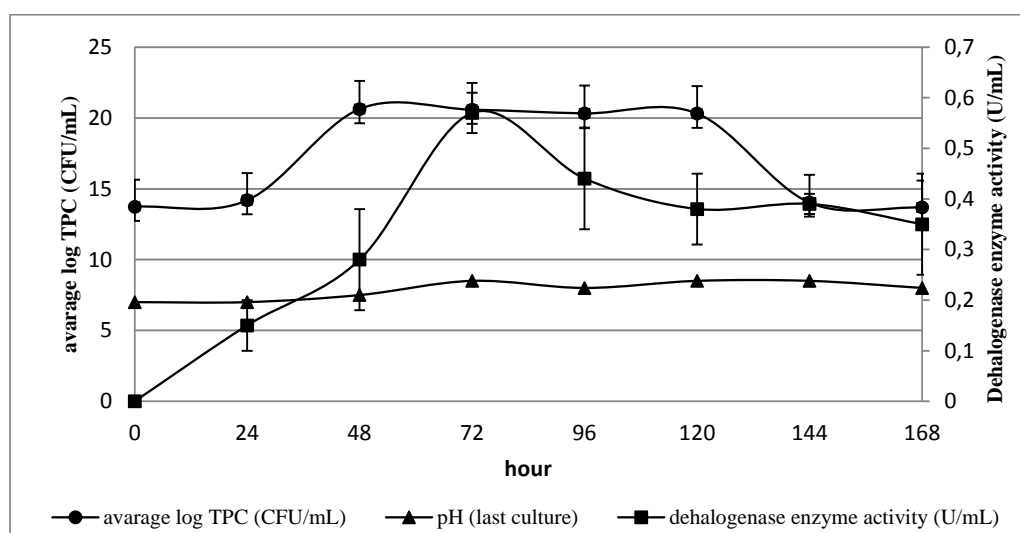


Figure 1. Effect of dehalogenase enzyme activity in incubation time

Figure 1 shows that the enzyme activity increase start from 24 to 72 hours of incubation. The enzyme activity influenced by the growth the bacteria. At 24 to 48 hours of incubation, the growth of *Bacillus* sp. D1 was in logarithmic phase. In logarithmic phase, the nutrient for bacterial growth in culture were still available and abundant, so the cell of bacteria actively divide and the enzyme activity increase. At 72 hours incubation, the bacteria enter to the stationary phase. The number of died cells is same as the number of lived cells.

In addition, increasing enzyme activity can also be affected by changes in the pH of the culture medium. The enzyme activity increases parallel with increasing pH of culture medium, which is 8.5, at 72 hours incubation. The enzyme activity decreases at 96 to 168 hours of incubation. This is associated with growth of bacteria. *Bacillus* sp. D1 entered to the final of stationary phase toward death phase. The nutrient for bacterial growth decreases and the toxic products accumulate in this phase.

Bacillus sp. D1 release the dehalogenase enzyme in the DCM environment, the enzyme can break the bond of halogen compounds on DCM. According to [7], bacteria degrading DCM have a metabolism of dichloromethane to become formaldehyde, formic acid, and carbon dioxide under aerobic conditions. A group of bacteria is capable of breaking dichloromethane into formaldehyde because it was catalyzed by glutathione S-transferase which is induced by the addition of dichloromethane to the media as a carbon source [7]. Dehalogenase is a group of glutathione S-transferase (GST) which catalyzes dichloromethane to formaldehyde with a dehalogenated oxidative

reaction which releases two chloride ions from one mole of dichloromethane molecules. The reactions occurred in the dichloromethane degradation is illustrated as below.



According to [5], methyllobacteria use formaldehyde produced during DCM degradation for energy sources. According to [8], a group of anaerobic bacteria capable of breaking dichloromethane has a metabolism that breaks the dichloromethane into formic acid or acetic acid.

Effect of dehalogenase enzyme activity in variation pH

The optimum value of enzyme activity was 0.57 U/mL at 72 hours incubation time. The optimum value of enzyme activity was tested agains at different pH variations of 5, 6, 7, 8, and 9. The effect of pH variation in the activity of dehalogenase enzymes is presented in the Table 2 and Figure 2.

Table 2. Average dehalogenase enzyme activity in variation pH

Time incubation (hours)	Optical density <i>Bacillus</i> sp.	Average dehalogenase enzyme activity (U/mL)
5	0,048 ± 0,02	0,046± 0,01
6	0,165 ± 0,11	0,393± 0,20
7	0,475 ± 0,09	0,097± 0,06
8	0,638 ± 0,03	0,092± 0,09
9	0,697 ± 0,09	0,913± 0,40

Based on Figure 2, enzymes activity increase at pH 9. The activity of dehalogenase enzyme at pH 9 had an activity value of 0.913 U/mL. The increase of bacterial cell density (OD) along with the increase in enzyme activity, it can be concluded that dehalogenase enzyme activity depends on the viability of bacterial cells. Based on the research of [9], DM1 bacteria were able to break down dichloromethane and express the dehalogenase enzymes. Bacteria DM1 is the genus of *Pseudomonas*.

According to [10], one of the important factors in bacterial growth is the pH value. Bacteria need a pH 6.5 to 7.5 for grow optimally. The minimum and maximum pH for bacterial growth are 4 and 9. Based on [11], the effect of pH related to bacterial growth is also related to enzyme activity. Enzymes are needed by bacteria to catalyzes reactions for bacterial growth. If the pH in a medium or environment is not optimal, it will interfere with the activity of the enzyme and eventually interfere with the growth of the bacteria itself. Thus it can be related that pH is one of the factors that have the potential to influence enzyme activity, and is closely related to the active function of the enzyme, solubility of the substrate, and enzyme-substrate bond.

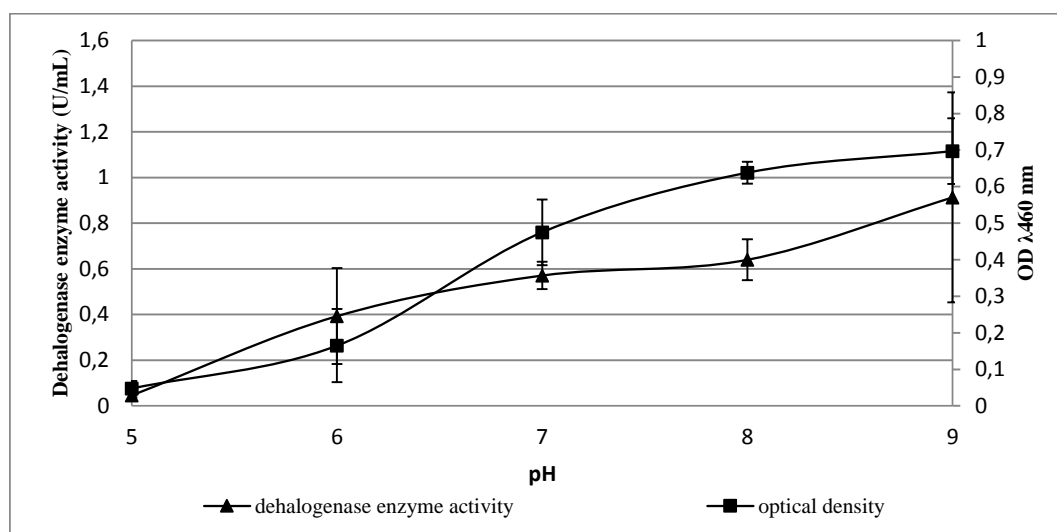


Figure 2. Growth of *Bacillus* sp. D1 and dehalogenase enzyme activity in variation of pH

4. Conclusions

The variation of incubation time affected the activity of dehalogenase enzyme by *Bacillus* sp. D1. The best dehalogenase enzyme activity was 0.57 U/mL at 72 hours of incubation. The variation of pH affects in the growth of *Bacillus* sp. and produced of the enzyme. Dehalogenase enzyme activity continues to increase at pH 9 with an activity value of 0.913 U/mL.

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