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Biosorption of Metal Ions on Methanol Dehydrogenase Enzymatic Activity of *Bradyrhizobium japonicum* USDA110

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ABSTRACT

This research aims to understand the effect of metal ions bioabsorption which belong on different elemental groups to the methanol dehydrogenase (MDH) enzymatic activity in nitrogen-fixing bacteria *Bradyhizobium japonicum* USDA 110. Ten metal ions with each have 30µM concentration were added to grow *Bradyhizobium japonicum* USDA 110 in 10^{-1} diluted nutrient medium. The MDH activity test showed a similar result between the bacteria grown in medium without metal ions addition (control) and the bacteria were grown in a calcium ion/Ca^{2+} added media. The highest MDH enzymatic activity was shown on the bacteria grown in a magnesium/Mg^{2+} added medium, which showed 0.08 (U/mg) enzymatic activities. The addition of magnesium/Mg^{2+} metal ion accelerates the bacterial growth by 2.6 times and MDH activity by 1.28 times compared to control. The MDH enzyme is essential, especially for bacteria which exist in the soil environment, to adapt to high methanol concentration and to support bacterial anaerobic growth capacity along with plant symbiotic process. Moreover, the MDH activity staining method could also act as pollutant indicators like metal ions and hydrocarbon derivates. This research concluded that metal ions biosorption (calcium/Ca^{2+} and magnesium/Mg^{2+}) are required for bacterial cells reproduction and oxidation of single carbon chain compounds like methanol. The nitrogen-fixing symbiotic bacteria, *Bradyhizobium japonicum* USDA 110 showed high MDH activity after the two metal ions absorption. However, contrary results were shown on vanadium/V^{3+}, manganese/Mn^{2+}, iron/Fe^{3+}, copper/Cu^{2+}, zinc/Zn^{2+}, and aluminum/Al^{3+} absorption, which showed low MDH activity and cells biomass.

Keywords: Calcium, Magnesium, Nutrient medium, Symbiosis

Introduction

Research on methanol addition as energy or sole carbon source on bacterial growth has been done (Delmotte *et al.,* 2009; Chistoserdova, 2011; Skovran *et al.,* 2011). The bacterial ability to mobilize single chain carbon compounds like methanol and methane then further elucidates the carbon cycle in the environment (Kalyuzhnaya *et al.,* 2008; Schmidt *et al.,* 2010). Research on methanol dehydrogenase (MDH) enzymatic activity with the treatment of metal ions addition has also been done (Keltjens *et al.,* 2014; Chistoserdova, 2016; Vu *et al.,* 2016). The researchers showed the effect of metal ions from Lanthanide group to the MDH activity and cell physiology of methylotrophic bacteria. However, in this research we used a different approach as MDH enzymatic activity of *Bradyrhizobium japonicum* USDA110 is measured without adding methanol content in the medium but by adding metal ions from different elemental groups. The MDH enzymatic activity on methanol measurement was done after the bacterial cell was extracted or *in vitro*. The extraction aimed to obtain the methanol-free bacteria which grew in metal ions added medium so that it can resemble actual soil environment with the anaerobic condition in plant root nodule such as in soybean.

Methanol is produced by plant leaves and roots from cellulose wall fermentation during the plant's growth (Abanda-Nkpwatt *et al.,* 2006; Balachandar *et al*., 2008; Irvine *et al*., 2012). Methanol is also found in soil, which originated from pectin and lignin decomposition from dead plants (Del Rocío Bustillos-Cristales *et al.,* 2017). Methanol is a carbon source without any other carbon chain or it has only one carbon element and belongs to alcohol primary group. Almost all bacteria are capable to produce MDH enzyme, especially bacteria which exist and undergo a symbiotic process in leaves surface (phyllospheric bacteria). Besides the leaves surface, methanol could also be produced on soil (rhizosphere) from the degradation of cellulose, and then became a simple energy source, easy and fast to be utilized

for the microorganism. It should be noted that the number of metal ions on soil and leaves surface are different, as metal ions on soil environment are much higher. However, the dust from soil or volcano which carried out by the air could bring metal ions to plant leaves (Tyler, 2004; Pol *et al.,* 2014).

One metal ion which easies to find and highly available in soil is calcium, with the amount around 0.92%-92.3% (Romero-Freire *et al.,* 2015). However, calcium as the sole metal ion source is not enough for the growth of agricultural microorganism like *Bradyhizobium japonicum* USDA110. The common metal ion available in plant fertilizer is potassium which associated with phosphate. The effect of various metal ions absorption and pollutant from hydrocarbon derivatives to the microbial growth could be detected with dehydrogenase enzyme like MDH enzyme through Nitro Blue Tetrazolium (NBT)/ Phenazine Ethosulfate (PES) PAGE staining method (Cassida, 1977; Skovran *et al*., 2011; Kaczynka *et al.*, 2015). This research observed the MDH enzymatic activity of *Bradyhizobium japonicum* USDA110, bacteria which is known as nitrogen-fixing and symbiotic bacteria in soybean roots, after the addition of metal ions which usually not available in plant fertilizer. The MDH enzymatic activity measurement is essential to further understand its methanol oxidation process which usually happened in the soil environment.

Materials and Methods

Media and bacterial growth condition

Bradyrhizobium japonicum USDA110 is obtained from NBRC with the registration number 14792. The microbial culture was done in Yeast extract-Mannitol medium/YM medium (Saito *et al.,* 1998). The bacteria were then enriched in 10ml of 10^{-1} diluted nutrient media for two days or 48 hours as the starter for 1-liter growth media. The nutrient medium consisted of 0.1% polypeptone, 0.05% NaCl, and 0.1% meat extract at the pH of 7.0-7.2. The growth media were then added with 30µM metal ions from 30mM metal ions stock. The used metal ions sources are KCl, $MgCl_3$, $CaCl_2{\cdot}2H_2O$, VCl_3, $Na_2MoO_4{\cdot}2H_2O$, $MnCl_2{\cdot}4H_2O$, $FeCl_3{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, $ZnCl_2$, and $AlCl_3{\cdot}6H_2O$. The bacteria were grown at the room temperature of 30ºC and the bacteria harvesting was done on the log phase or after 48 hours of cultivation. The bacterial growth was measured by spectrophotometer at 600 nm wavelength for every 24 hours. The bacteria harvesting was done by centrifugation at 10,000 rpm with the temperature of 4ºC for 10 minutes. The bacterial cell was washed out from attached debris for 3 times by using a 20mM Tris-HCl buffer at pH 8 and then kept at -20ºC until the extraction is performed.

Methanol dehydrogenase (MDH) enzymatic activity measurement

The measurement of MDH enzymatic activity was done on crude bacterial cell extract. The extraction was done with Tomy Disruptor UD-210 Ultrasonic on an ice base to keep the extraction process and cell extract cool. The extractions were done by 15 times for 20 seconds, with 30 seconds rest after each extraction. The produced bacterial crude cell extractions were separated by centrifugation at 16,000 rpm with the temperature of 4ºC for 30 minutes. The crude MDH enzyme extraction was then obtained in the supernatant.

The MDH enzymatic activity measurement was done by using MDH assay method (Day and Anthony, 1990). The solution for enzymatic activity measurement consisted 0.5 ml of 0.6 M buffer Tris-HCl at pH 9, 0.1 ml of 0.45 M ammonium chloride, 0.1 ml of 0.3 methanol, 0.1 ml of 2.6 mM 2,6-Dichlorophenolindophenol (DCPIP), 33 mM Phenazine Ethosulfate (PES), sample and distilled water until the volume reached 3 ml. The solutions will have yellowish green color and then placed on glass cuvette for spectrophotometer observation at 600 nm wavelength for 15 seconds. The epsilon DCPIP coefficient value for enzymatic activity measurement is $1.9x10^4$ $M^{-1}Cm^{-1}$ (Liu *et al*., 2006). One unit of MDH enzyme is stated as 1 *µ*mol of enzymatically reduced methanol with ammonium chloride as activator while DCPIP and PES as the electron donor and acceptor which measured by absorbance after 10 seconds until 30 seconds from the initial reaction. The MDH enzymatic activity is described in the unit for each milligram protein enzyme. The protein concentration measurement was done by linear regression from the commercial BCA Protein Assay Kit measurement.

MDH enzymatic activity staining measurement

The bacterial crude extract MDH enzymatic activity was also measured with Nitro Blue Tetrazolium (NBT)/ Phenazine Ethosulfate (PES) PAGE staining method (Skovran *et al.,* 2011). The 10% Polyacrylamide gel electrophoresis without the addition of sodium dodecyl sulfate (SDS) was done to measure the protein content without denaturation like in SDS PAGE. The solution for MDH enzymatic activity staining method consisted 5 ml of 0.6 M Tris-HCl at pH 9, 1 ml of 0.45 M ammonium chloride, 0.3 M Methanol, 33 mM Phenazine Ethosulfate (PES), Nitro Blue Tetrazolium 16 mg, dan distilled water until the total volume reached 30 ml. The PAGE for bacterial crude extract running was incubated on staining solution for 30 minutes at 30ºC. The existence of MDH enzyme can be seen in a form of a thick dark purple line.

Result and Discussion

Bacterial growth

The nitrogen-fixing symbiotic bacteria *Bradyrhizobium japonicum* USDA110 which

cultivated with the addition of Mg^{2+} metal ions addition showed a faster growth phase which can be seen from the increase of bacterial cells absorbance (Figure 1).

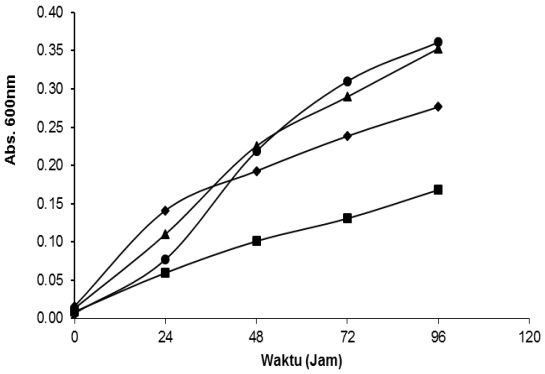

Figure 1. Metal ions biosorption to the growth of *Bradyrhizobium japonicum* USDA110. The added metal ions concentrations were 30µM: K^{1+}, KCl (square); Mg^{2+}, $MgCl_2$ (triangle); Ca^{2+}, $CaCl_2$ (circle) and 10^{-1} diluted nutrient medium without metal ions addition as control (diamond).

The bacterial growth on medium with other metal ions addition like Fe^{3+}, V^{3+}, Mo^{6+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, and Al^{3+} showed the absorbance value less than control. It is known that metal ions Mg^{2+} is needed for cell division and DNA transcription and translation (Deleebeeck *et al.,* 2009). While the effect of this metal ions on MDH enzymatic activity is not clearly known. Until today, the known metal ions which act as MDH enzyme cofactor is Ca^{2+}. The calcium ion is known as a cofactor to accept electrons from MDH enzyme on several bacteria, one of it is *Methylobacteriuam extorquens* AM1 (Del Rocío Bustillos-Cristales *et al.,* 2017). The observation of similar *Bradyrhizobium japonicum* USDA110 bacterial growth between the addition of Mg^{2+} and Ca^{2+} ions on media without methanol addition showed that these metal ions are involved in bacterial cell growth and furthermore the possibility in MDH enzyme protein formation. It can be seen from the MDH enzymatic activity from crude bacterial cell extraction after Mg^{2+} and Ca^{2+} absorption which discussed below.

Methanol dehydrogenase (MDH) enzymatic activity

The bacterial cells were harvested during the log phase (48 hours) or on the phase where the bacteria double quickly and then extracted by ultrasonic for MDH enzymatic activity measurement and staining (Table 1, Figure 2).

The result of MDH enzymatic activity with the Day and Anthony (1990) solution showed that the addition of Mg^{2+} metal ions would give the highest MDH enzymatic activity and relative activity, which was 1.28 higher than the control medium or without metal ions addition. The Mg^{2+} metal ions addition would also yield higher bacterial cells compared to control medium and Ca^{2+} added media.

On the contrary, the staining MDH enzyme activity on native PAGE, a bright formazan dye was seen on crude cell extract grown on medium without metal ions addition, and with the metal ions Cu^{2+}, K^{1+}, and Al^{3+} addition. The formazan staining method is known to detect dehydrogenase enzymes like MDH on soil microorganism in which indicate the occurrence of pollutants (Cassida, 1977; Skovran *et al*., 2011; Kaczynka *et al*, 2015). Furthermore, MDH enzyme staining could be used to describe the significance of occurred soil pollutants in the form of metal ions Cu, K and Al. However, *Bradyrhizobium japonicum* USDA 110 showed a slow growth with low MDH enzymatic activity.

The effect of metal ions biosorption

Nitrogen-fixing symbiotic bacteria like *Bradyrhizobium japonicum* USDA 110 has been widely used as research standard in agriculture and environment area. The effect of biosorption from bio-accumulated metal ions to the bacteria can be used as soil fertility indicator. In Figure 3., it can be seen that metal ions addition like vanadium/V^{3+}, manganese/Mn^{2+}, iron/Fe^{3+}, copper/Cu^{2+}, zinc/Zn^{2+}, and aluminium/Al^{3+} resulted in lower MDH enzymatic activity compared on the control medium.

Table 1. Metal ions biosorption on MDH enzymatic activity during log phase (48 h) of *Bradyrhizobium japonicum* USDA110 grown in nutrient medium

crude cell extracts	MDH enzymatic activity (U/mg)	MDH relative activity	Bacterial biomass (log phase) each liter (gram)
Nutrient medium without metal ions addition/control	0.06	1	0.31
K^{1+}	0.05	0,81	0.17
Mg^{2+}	0.08	1,28	0.35
Ca^{2+}	0.06	1	0.29
V^{3+}	0.01	0,22	0.05
Mo^{6+}	0.06	0,89	0.24
Mn^{2+}	0.02	0,31	0.27
Fe^{3+}	0.02	0,29	0.24
Cu^{2+}	0.01	0,16	0.27
Zn^{2+}	0.02	0,27	0.16
Al^{3+}	0.02	0,25	0.27

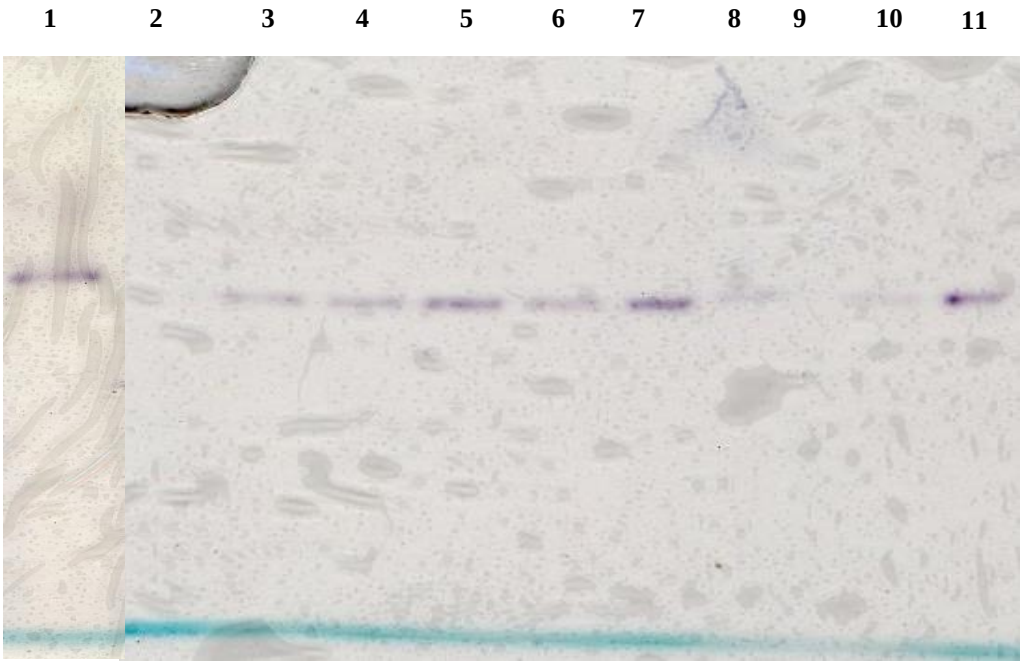

Figure 2. The result of methanol dehydrogenase (MDH) enzymatic activity staining by *Bradhyrhizobium japonicum* USDA110. Bacterial cell extract of *Bradyrhizobium japonicum* USDA110 which grown on nutrient medium (line 1), and with metal ions addition (Fe^{3+}, V^{3+}, Mo^{6+}, Cu^{2+}, Ca^{2+}, K^{1+}, Mg^{3+}, Mn^{2+}, Zn^{2+}, and Al^{3+}) presented on line 2 to 11, respectively, were measured with formazan dyes formation on the NBT/PES PAGE staining.

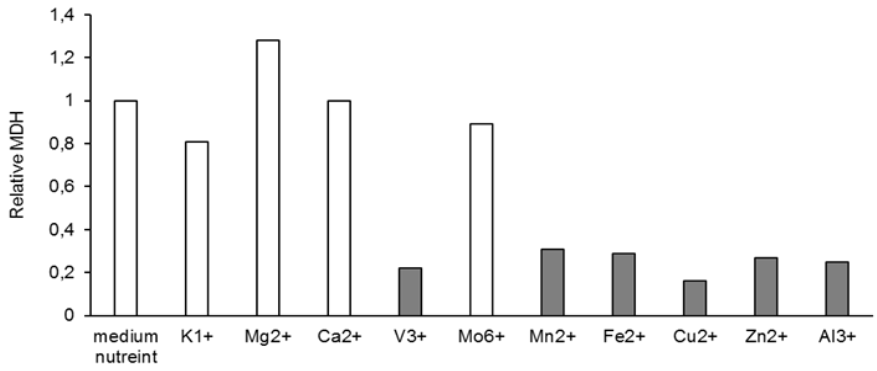

Figure 3. The methanol dehydrogenase (MDH) relative enzymatic activity in *Bradyrhizobium japonicum* USDA110 cell extract. The effect of added metal ions which inhibit MDH enzymatic activity presented on grey bars. The added metal ions were at 30 µM concentration.

The MDH enzymatic activity could be used as the basis to measure pollutant metal ions. The staining on MDH enzymatic activity after added with metal ions also showed a brighter color, which indicates that the metal ions were accumulated inside the bacterial cells. The accumulated metal ions inside cell provide more electron acceptors which resulted in brighter staining color (Skovran *et al*., 2011). Moreover, the bio-accumulation of metal ions will inhibit the reproduction of the cells, thus resulted in low bacterial biomass (Dourado *et al.,* 2015). Furthermore, the effect would also inhibit the anaerobic nitrogen fixation on plants and formation of nodules as the sign of symbiotic activity.

Conclusion

The *Bradyrhizobium japonicum* USDA110 bacteria showed the ability to absorb and accumulate metal ions inside its cells which affect overall cell metabolism. The MDH enzymatic activity of the bacteria is one of useful dehydrogenase enzyme for metal ions biosorption indicator in the environment. The *Bradyrhizobium japonicum* USDA110 which are commonly used in various agriculture and environment research showed different MDH enzymatic activity after absorbing the added metal ions in the growth medium compared to the control medium. Besides of the different MDH enzymatic activity, the

absorbed metal ions also affect the bacterial growth capability. A low MDH enzymatic activity and bacterial growth during log phase (48 h) also showed in several metal ions added medium. The condition thus describes that the respected metal ions are considered pollutant in the environment. However, magnesium and calcium added medium showed high MDH enzymatic activities which support bacterial growth, thus explained that these ion metals are essential for bacterial growth and nitrogen fixation for the symbiotic process.

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