Buletin Peternakan 42 (3): 244-249, August 2018

ISSTAP
Indonesian Society for Sustainable Tropical Animal Production

Bulletin of Animal Science

ISSN-0126-4400/E-ISSN-2407-876X Accredited: 36a/E/KPT/2016
http://buletinpeternakan.fapet.ugm.ac.id/

Doi: 10.21059/buletinpeternak.v42i3.28155

Direct Stimulation by Methanol Addition on the Cultured Medium for Methanol Dehydrogenase Protein Purification from *Bradyrhizobium japonicum USDA110*

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Article history
Submitted: 8 September 2017
Accepted: 13 July 2018

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ABSTRACT

Methanol dehydrogenase (MDH) enzyme was purified from *Bradyrhizobium japonicum* USDA110 cell-free extract. The bacteria were grown in a culture medium with direct 0.5% methanol addition aimed to stimulates the MDH catalytic enzyme activation. *Bradyrhizobium japonicum* USDA110 MDH enzyme was purified by using 25 mM 2-(N-morpholine) ethanesulfonic acid/MES pH 5.5 buffer and 1 M sodium chloride/NaCl which separated into three columns, the first column was PD-10 for buffer exchange; the second column was HiTrap Sepharose HP to obtain unbonded fraction in the column; and the third column was Mono S 5/50 GL integrated with two pumps HPLC (high-performance liquid chromatography) to obtain pure MDH enzyme for serial changing of 1 M NaCl-25mM MES pH 5.5 with the flow rate at 1 ml/min. The protein concentration and MDH catalytic enzyme activity were observed on each purification process starting from the cell-free extract to pure MDH enzyme. The pure MDH enzyme was obtained by Mono S 5/50 GL-HPLC purification which showed a single band on SDS PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The MDH enzyme purification from *Bradyrhizobium japonicum* USDA110 showed 90-fold purification, a sub-molecular weight of 63 kDa, specific activity at 2.69 U/mg, and optimum activity at a 35ºC temperature and pH 9.

Keywords: High-performance liquid chromatography, MDH, Sodium chloride, 2-(N-morpholine) ethanesulfonic acid

Introduction

Methanol dehydrogenase (MDH) enzyme from *Bradyrhizobium japonicum* USDA110 has been known to have a certain role in symbiosis and soybean nodule formation (Sudtachat *et al.,* 2009). This enzyme catalyzes methanol as an energy source for bacterial cell growth. Methanol is the smallest structure derivatives produced from lignin degradation and intermediate plant metabolism (Marshall *et al.,* 1995). On a small amount, methanol can be oxidized by soil bacteria through dehydrogenation mechanism, whether by methylotrophic or non-methylotrophic bacteria with the help of catalytic enzyme namely methanol dehydrogenase or MDH. Soil bacteria, especially nitrogen-fixing bacteria, which has a symbiotic relationship with plants required MDH enzyme for the initial symbiotic process, such as lignin and cellulose degradation on roots (Renier *et al.,* 2011). The availability of MDH enzyme would help germination and sprout formation on non-legumes (Abanda-Nkpwatt *et al.,* 2006) and has been one growth stimulus for legumes through root nodules formation (Renier *et al.,* 2011).

The catalytic activity of MDH enzyme could help bacteria to grow in methylotrophic condition by oxidizing methanol as the sole carbon and energy source. There are currently two known MDH enzymes which were originated from two different genes, which are mxaF or mxaF' and xoxF, with both, had different gene expression. The MDH enzyme originated from mxaF or mxaF' is expressed from methanol and other low energy sugars induction, while xoxF gene expression is based on the existence of lanthanide ions La^{3+} (Hibi *et al.,* 2011; Nakagawa *et al.,* 2012).

Studies on MDH enzyme catalytic activity and purification have been intensively done on *Methylobacterium extorquense* AM1, a methylotrophic bacteria and initially known as *Pseudomonas extorquense*, which consisted of α sub-unit or large sub-unit and β sub-unit or small sub-unit (Anthony, 1982; Liu *et al.,* 2006). The first MDH enzyme purification from non-methylotrophic bacteria was done on *Bradyrhizobium* genus, which is *Bradyrhizobium sp*. MAFF211645, by

cerium ions and methanol induction on diluted growth media. Furthermore, the purified MDH enzyme from *Methylobacterium extorquense* AM1 showed high similarity with the MDH enzyme purified from *Bradyrhizobium* strain ORS278, BTAi1, and *B. japonicum* USDA110 based on the amino acids sequence and the α sub-unit (Fitriyanto *et al.,* 2011). The *mxaF'* gene which responsible for the MDH enzyme translation and transcription in *Bradyrhizobium japonicum* strain USDA110 is reported to be induced by organic compounds as well, like methanol and vanillate (a lignin derivate) (Ito *et al.,* 2006; Sudtachat *et al.,* 2009). The alternative MDH gene expression induction method thus encourages us to purify MDH enzyme from *Bradyrhizobium japonicum* USDA110 grown in 0.5% methanol addition media to stimulate the bacteria growth in the 10^{-1} nutrient medium.

Materials and Methods

Media and bacterial growth condition

Bradyrhizobium japonicum USDA110 registration number NBRC 14792 was grown in 10^{-1} diluted nutrient medium. The medium composition for bacterial growth is according to research by Fitriyanto *et al.* (2011), Hibi (2011) dan Nakagawa *et al*. (2012) consisted of 0.1% meat extract, 0.1% polypeptone and 0.05% sodium chloride. Methanol was added to the medium after sterilized and cooled down into room temperature. The methanol used in this research had 99% concentration and adjusted to 0.5% (v/v) from the nutrient medium volume. The bacteria preculture was isolated from bacteria growth resumption in the 1 L nutrient medium without methanol addition after reaching log phase (after 48 hours). The obtained bacteria preculture as much as 10 ml was then grown in the nutrient medium with methanol addition and used for MDH enzyme purification after reaching the log phase (48 hours). The bacterial growth was observed on Sakaguchi container consisted of 100 ml medium and 5 ml bacteria preculture and then grown for 2 days or after 48 hours. The observation was done by using spectrophotometer at 600 nm wavelength.

Purification and methanol dehydrogenase enzyme analysis

MDH enzyme purification from *Bradyrhizobium japonicum* USDA110 was done by using 3 commercial columns from GE Healthcare. Bacteria cell extraction was done before column purification. The bacteria cell extraction was done by growing the bacteria in 1 L nutrient medium for 48 hours and then washed and dissolved with 20 mM Tris-HCl pH 8.0 buffer, followed with Ultrasonic Tomy Disruptor UD-210 extraction which has ice flakes at the base to keep the extraction temperature cool. The extraction was done for 15 times in 20 seconds with 30 seconds rest after each extraction. The obtained bacterial cell-free extract as much as 4.5 ml was then filtered through PES filter, and then placed on a PD-10 column (first column) for buffer exchange (Tris-HCl ph 8.0 to 25 mM MES pH 5.5). The sample obtained from the first column (3 ml) was then purified in the 1 ml HiTrap Sepharose HP (second column) by dissolving the sample bound in the sepharose with 25 mM MES pH 5.5. The HiTrap Sepharose HP purification yielded around 1.5 ml sample with the first 0.5 ml yields were thrown away. At this point, the sample already contained the MDH enzyme. The process was then followed by cold centrifugation with Amicon tube to reduce the buffer volume. The result of the centrifugation (around 50 µl) was then purified further by using Mono S 5/50 GL column (third column) integrated with two pumps HPLC with the flow rate at 1ml/min. In the third column, MDH enzyme was obtained by collecting the 25 mM MES pH 5.5 and 25 mM MES -1 M NaCl pH 5.5 serial solutions in the first 10 minutes after the solution was placed in the column for 10 minutes. The solution ratio was 30:70 (25 mM MES -1 M NaCl: 25 mM MES pH 5.5), and fractions which contained MDH enzyme was obtained at 14 to 15 minutes. The MDH enzyme activity and protein concentration was observed in every purification process and followed by MDH enzyme purity measurement. The enzymatic activity was measured with the method adopted from Day and Anthony (1990), while the MDH concentration was measured according to the method by Bradford (1976) with Pierce® BCA Protein Assay Kit. The MDH enzyme purity on each process was visualized through dodecyl polyacrylamide gel electrophoresis or SDS PAGE with 20% concentration.

Molecular weight estimation of MDH enzyme

The molecular weight of pure MDH enzyme was estimated by comparing the protein bands migration (cm) of the fourth purification column sample which has the highest MDH enzymatic activity with the standard proteins or markers. The visualization result from SDS-PAGE was then converted into JPEG to measure and compare the band's migration. The measurement was done through logarithmic formula with the bands migration of pure MDH enzyme and standard proteins used as variables. The protein standards or markers used in this research were benchmark protein ladder molecular weight marker adapted from research by Weber and Osborn (1969).

Result and Discussion

Methanol as a sole energy source

Bradyrhizobium japonicum USDA110 is a bacteria which the whole genome has already been studied and also known to has MDH enzyme-encoding gene (Ito *et al*., 2006). The MDH enzyme-encoding gene is located on the locus 10 with gene code blr6213 or *mxaF* (Ito *et al*., 2006; Sudtachat *et al*., 2009). *Bradyrhizobium japonicum* USDA110 which grown in oligotrophic

condition or a low nutrient availability medium could utilize methanol as the energy source. Furthermore, the bacteria also are known to be able to tolerate methanol in the medium up to 0.5%. The growth curve of *Bradyrhizobium japonicum* USDA110 in the nutrient medium with and without methanol addition could be seen in Figure 1.

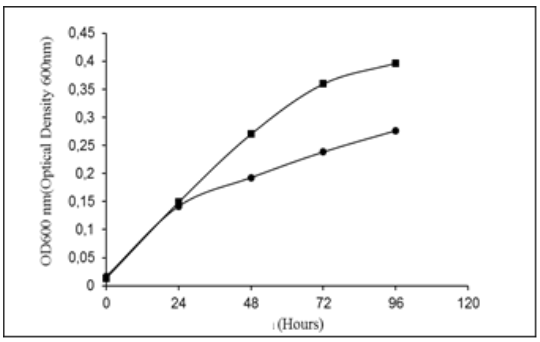

Figure 1. Growth curve of *Bradyrhizobium japonicum* USDA110 on the 10^{-1} diluted nutrient medium. Nutrient medium without methanol addition (circle) and 0.5% methanol addition (square).

In the medium, methanol will be oxidized by MDH enzyme to provide energy for the bacteria reproduction. In Figure 1, it can be seen that *Bradyrhizobium japonicum* USDA110 which was grown in medium with methanol addition has higher absorbances compared to the medium without methanol addition. The higher absorbances of *Bradyrhizobium japonicum* USDA110 indicates that higher bacterial cell reproduction occurred in the medium with methanol addition.

The methanol oxidation to produce energy yields formaldehyde, a toxic compound for the bacteria and thus should be converted into carbon dioxide quickly. *Bradyrhizobium japonicum* USDA110 has the ability to utilize methanol in the medium as an energy source by using MDH enzyme located in the periplasmic area between the inner and outer membrane of the bacterial cell. The *Bradyrhizobium japonicum* USDA110 ability to oxidize methanol was also supported by the cofactor enzyme synthesized from nutrient anabolism process originated from the meat extract and polypeptone during the bacterial growth.

Research showed that formaldehyde is an intermediate metabolite produced during methanol oxidation process, while the compound is also produced during vanillin and vanillate metabolism by *Bradyrhizobium japonicum* USDA110 which then oxidized into carbon dioxide with NADH and glutathione as the cofactors (Ito *et al*., 2006; Sudtachat *et al*., 2009). The MDH enzyme produced by *Bradyrhizobium japonicum* USDA110 could also oxidize methanol as much as 0.5% in the 10^{-1} diluted nutrient medium consisted of meat extract and polypeptone (Fitriyanto *et al.,* 2011). The cell reproduction of *Bradyrhizobium japonicum* USDA110 is higher in the medium with methanol addition which indicates that direct methanol addition could stimulate the bacteria cell metabolism. The MDH enzyme activity from cell-free extract produced in the medium with and without methanol addition can be seen in Figure 2.

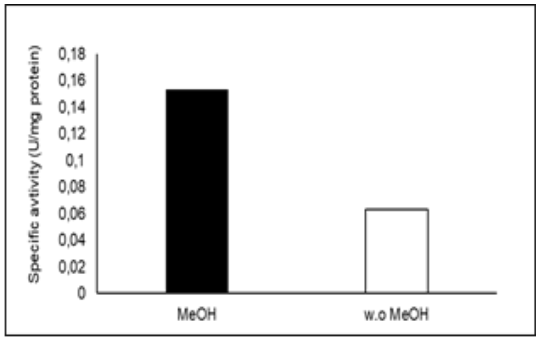

Figure 2. MDH enzyme specific activity (U/mg protein) from *Bradyrhizobium japonicum* USDA110 cell-free extract. Cell-free extract with methanol stimulation (black bar-MeOH) and without methanol stimulation (white bar-w.o MeOH).

The result indicates that methanol could stimulate *Bradyrhizobium japonicum* USDA110 cell to produce and activate MDH enzyme which encoded in the blr6213 *maxF*' gene with NADH and glutathione as the cofactor from the diluted nutrient medium. MDH enzyme is a unique enzyme regarding its specific role to oxidize methanol for energy production while also quickly produced carbon dioxide as the end metabolism product. The energy produced by the methanol oxidation will then be used for cell reproduction through other metabolism cycles, while in *Bradyrhizobium japonicum* USDA110, one of the cycles is serine metabolism which produced cell constituent compounds with formaldehyde as the main pathway from the methanol oxidation process to serine metabolism cycle (Sudtachat *et al*., 2009).

MDH enzyme purification

The inoculated *Bradyrhizobium japonicum* USDA110 cell during log phase (48 hours) was used for MDH enzyme purification. The MDH enzyme purification was done in 4 steps, and the SDS-PAGE visualization can be seen in Figure 3A.

The result of SDS-PAGE was then used to measure the molecular weight of the MDH enzyme by comparing the band's migration with a marker protein (Figure 3B). Furthermore, the enzymatic activity measurement on each purification process can be seen in Table 1.

The end of purification process in this research resulted in an MDH enzyme with 90-fold purity. The bacterial cell purification of *Bradyrhizobium japonicum* USDA110 which was grown with methanol stimulation showed similarity with the bacterial cell purification of the cerium ions and methanol-induced bacteria with the only

difference is on the specific catalytic activity of MDH enzyme. Furthermore, the characteristic observation of MDH enzyme produced by *Bradyrhizobium japonicium* USDA110 showed that the optimum enzyme activity was at 35°C with a pH value at 9.0. The result of temperature and pH observation can be seen in Figure 4 and 5.

The optimum temperature and pH of MDH enzyme produced by *Bradyrhizobium japonicium* USDA110 was not significantly different with the MDH enzyme produced from *Methylobacterium* bacteria. In accordance with its name, *Methylobacterium* bacteria specifically could live in a methylotrophic environment, or in other words, the bacteria are capable of utilizing methanol as the sole carbon and energy source. The methanol metabolism by *Metehylobacterium extorquense* used about 6 genes which encode methanol dehydrogenase enzyme (Vuilleumier *et al.,* 2009). The condition thus differentiates the pure methylotrophic with non-methylotrophic bacteria such as *Bradyhizobium* with the differences extend to the amount of methanol the bacteria able to oxidize in their respective metabolism process.

The result of bacterial growth and enzyme purification showed that *Bradyhizobium japonicum* USDA110 is able to directly oxidize methanol whether *in vivo* or *in vitro*. The bacteria capability to oxidize methanol is caused by the existence of 1 MDH encoding gene blr6213 *mxaF'*, which can be seen in *Bradyrhizobium japonicum* USDA110 whole genome sequencing done by researchers in Kazusa DNA Research Institute (Kaneko *et al.,* 2002a,b). Furthermore, *Bradyrhizobium japonicum* USDA110 showed the capability to live and grow in an oligotrophic environment with methanol as the sole energy source. The methanol in the environment is produced by leaves stomata and also from cellulose and hemicellulose degradation (Peyraud *et al.,* 2012). The existence of MDH enzyme in symbiotic nitrogen-fixing bacteria such as *Bradyrhizobium japonicum* USDA110 is allegedly needed to initiate the symbiotic process which realized in the form of roots nodules, especially on legumes.

Table 1. The *B. japonicum* USDA110 MDH enzyme purification in 0.5% methanol addition medium

Purification columns	Specific activity (U/mg)	Purification level	Yield (%)	Total activity (U)	Total protein (mg)
Crude cell extract	0.03	1	100	4.36	147.44
PD-10 (Sephdex G-25)	0.03	1.05	63.33	2.76	88.64
HiTrap Sepharose HP	0.21	6.94	28.13	1.23	5.97
MONO S 5/50 GL	2.69	90.75	5	0.22	0.08

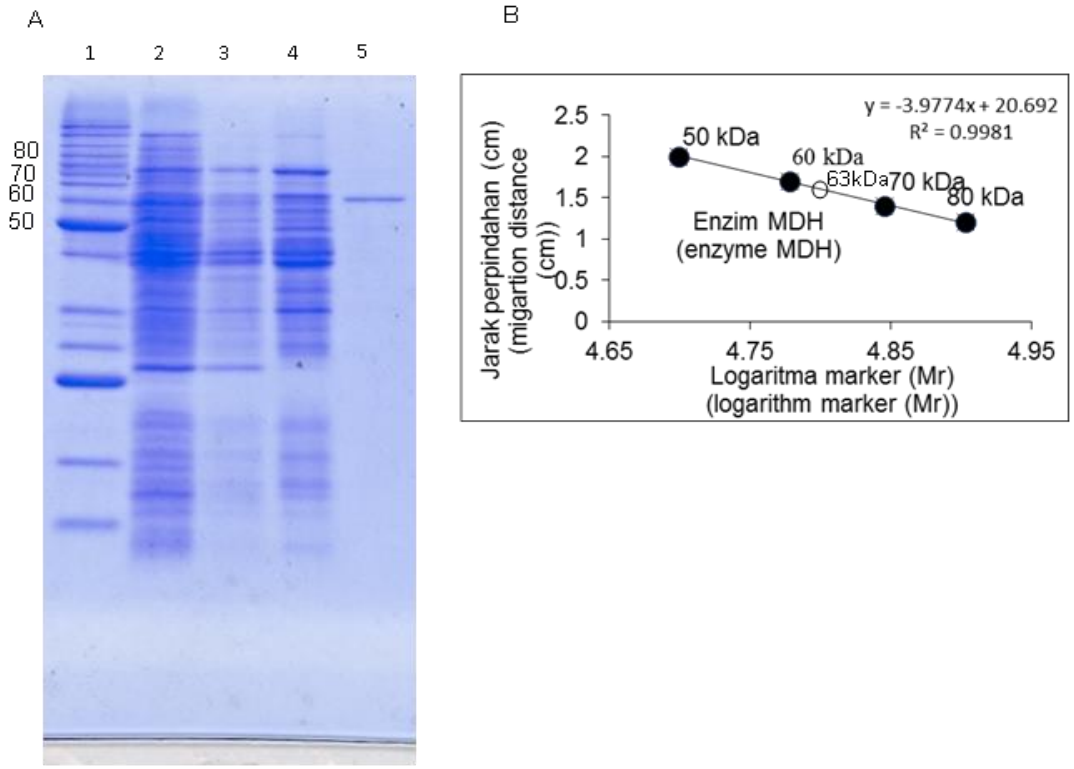

Figure 3. A: SDS-PAGE visualization of purified *B. japonicum* MDH protein. From left to right are (1) marker; (2) bacterial cell extract; (3) PD-10 (Sephadex G-25) column fractions; (4) HiTrap Sepharose HP column fractions; (5) Mono S 5/50 GL column fractions. B: Molecular weight estimation in one *B. japonicum* MDH protein unit. The open circle is MDH enzyme migration estimation at 1.65 cm on SDS PAGE. One unit of MDH enzyme is estimated to has 63 kDa molecular weight.

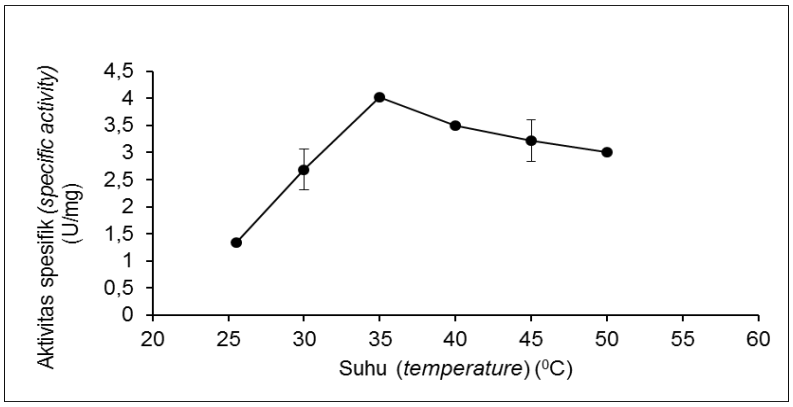

Figure 4. The optimum temperature for *B. japonicum* USDA110 MDH specific enzyme activity.

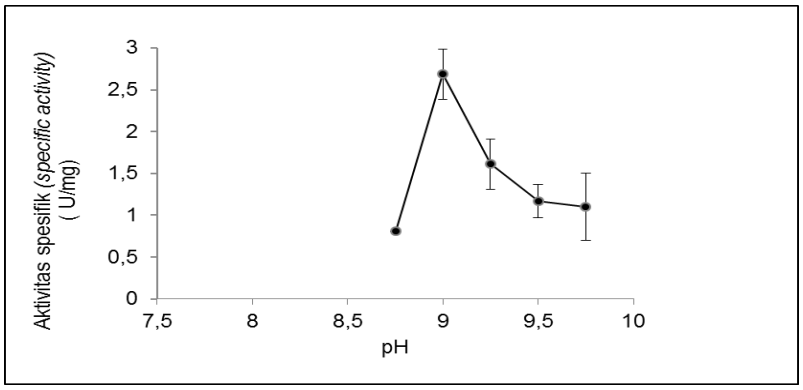

Figure 5. The optimum pH for *B. japonicum* USDA110 MDH enzyme specific activity.

Conclusions

Methanol as a sole energy source in the oligotrophic environment could stimulate the *Bradyrhizobium japonicum* USDA110 growth. The optimum oxidized methanol concentration was 0.5%. The *Bradyrhizobium japonicum* USDA110 MDH enzyme showed optimum methanol catalytic activity *in vitro* at a 35^0C temperature and pH 9.0 and had a molecular weight of 63 kDa which was measured through SDS-PAGE.

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