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SHORT COMMUNICATION

Purification of methanol dehydrogenase from mouth methylotrophic bacteria of tropical region

Diana Waturangi*, Nico Marko and Maggy Thenawidjaja Suhartono

Faculty of Biotechnology, Universitas Katolik Indonesia Atma Jaya, Jalan Jenderal Sudirman 51, Jakarta 12930, Indonesia.
E-mail: diana.waturangi@atmajaya.ac.id

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ABSTRACT

Aims: Purification of methanol dehydrogenase (MDH) from methylotrophic bacteria was conducted to obtain pure enzyme for further research and industrial applications due to the enzyme's unique activity that catalyzes oxidation of methanol as an important carbon source in methylotrophic bacteria.
Methodology and Results: The enzyme was screened from methylotrophic bacteria isolated from human mouth. Purification of this enzyme was conducted using ammonium sulphate precipitation followed by cation exchange chromatography. Two types of media were used to produce the enzymes: luria broth and standard mineral salts media (MSM). MSM produced MDH with higher specific activity than LB. Specific activity was also increased along with the purification steps. Application of ammonium sulphate increased the purity of enzyme and was more effective for the enzyme produced in LB. Using sepharose increased the enzyme activity 10 -57 folds.
Conclusion, significant and impact of this study: With this, ammonium sulphate precipitation coupled with single cation exchange chromatographic system has been proved to provide sufficient purified of methanol dehydrogenase from methylotrophic bacteria origin of human mouth with high specific activity for further application.

Keywords: methanol dehydrogenase, methylotrophs, enzyme purification

INTRODUCTION

Methylotrophic bacteria are aerobic bacteria that utilize single-carbon compounds as carbon and energy source (Kim *et al.*, 1991; Hanson and Hanson, 1996). This group of bacteria can be found in various habitats such as leaf, air, soil, hypersaline inland lakes, and even in drinking water (Gallego *et al.*, 2005; Sorokin *et al.*, 2007). A number of enzymes contribute in metabolizing these compounds; among them is methanol dehydrogenase that plays a key role in the oxidation of methanol (Anthony, 2000).

Methanol dehydrogenase (MDH, EC 1.1.99.8) is a quinoprotein contributes in metabolizing methanol to support methylotropic bacterial growth. In facultative methylotrophs, this enzyme is produced at high concentration, up to 10-15 % in bacteria cell. MDH exists in $\alpha_2\beta_2$ tetramer. The larger subunit is 66 kDa, whereas the smaller subunit is 8.5 kDa (Anthony and Williams, 2003). Pyrroloquinoline quinone (PQQ) and ion calcium are located in the active side of the enzyme. Both compounds contribute mainly in the mechanism of action of MDH (Liu *et al.*, 2005). To produce MDH, at least 26 genes are required, among them are genes involve in the coding of polypeptide structure, cytochrome cL, and the synthesis of PQQ molecules (Davagnino *et al*., 1998).

MDH has the ability to metabolize wide range of substrates, such as methanol, methane, formaldehyde, and methylamine (Whang and Kim, 1990; Liu *et al.*, 2005). In human body, methylotrophs are also found as a normal part of microflora and may utilize organosulfur compounds, such as dimethylsulfide, to maintain their growth (Anesti *et al.,* 2005). This organosulfur compounds are predicted responsible for human body odor formation, especially in mouth (van den Velde *et al.*, 2009).

Due to the action of mechanisms to utilize single carbon compounds, MDH can be applied to many industrial and environmental applications. Recent publications showed that MDH may contribute in reducing the human mouth bad smell and still in progress for other application, such as biocatalyst and bioremediation agent. However, the research about this microorganism and its enzyme isolated from tropical region was rarely investigated. Therefore, this research aimed to explore the diversity of rarely or even new microorganisms from tropical region with further applications performed with the purified enzyme.

MATERIALS AND METHODS

Sample collection

***Corresponding author**

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The microorganisms were isolated from mouth, screened on minimal media supplemented with 1 % methanol, and coded as M27G2, M27L1, M27L3, M41L3, dan M51G1. Isolates were frozen and stored at –70 °C in Atma Jaya Catholic University.

Cell growth

All isolates were grown aerobically in a rotary shaker at 28 °C, 110 rpm in Luria Broth and standard mineral salts media with methanol 25 mM served as the electron donor and carbon source. The bacteria were harvested at the end of an exponential growth period of 66 h.

Enzyme extraction

About 35 g of isolates were harvested and centrifuged (SorvallLegend^{TM} RT) at 9000 x *g* for 45 min, –4 °C. Cell paste was suspended with distilled water (w/v = 1:4). Intracellular enzyme and methanol dehydrogenase from periplasm space were obtained by breaking the membrane of bacteria. The cell suspension was treated for 2 min with an ultrasonic device (Biologics Inc., ultrasonic homogenizer, model 150 V/T), output 50, using 70 % pulses. The cell paste was then centrifuged at 9 000 × *g* (SorvallLegend^{TM} RT) for 60 min to separate the cell from other solute. The resulting supernatant was mixed with 100 mM 4-morpholine ethanesulfonic acid (Mes) buffer (Sigma-Aldrich, USA) (v/v= 3:1) to a final concentration of 25 mM (Liu *et al.*, 2005). Crude extract was stored in freezer for further analysis.

Enzyme purification

The purification was conducted with AKTA FPLC Amersham® Biosciences according to the methods of Liu *et al.* (2005). The crude extract was first precipitated with 40 % ammonium sulfate and then filtered through 0.8 mm cellulose acetate membrane. The supernatant was applied onto a HiTrap SP Sepharose XL Model column (bed volume 1 mL), which was preconditioned with 25 mM Mes, pH 5.5. The extract was first washed with 25 mM Mes, pH 5.5, to remove unbound proteins and then eluted with a programmed gradient of 0 - 100% of 25 mM Mes, pH 5.5, containing 250 mM NaCl.

Enzyme assay

The assay mixture was composed as suggested by Liu *et al* (2005). Reagent 6-dichloroindophenolate (DCIP) and Phenazinemethosulfaten (PMes) (Sigma-Aldrich, Austria) were used in the reaction mixture. The reaction was initiated by the addition of PMes and methanol dehydrogenase activity was measured by monitoring the decrease of DCIP absorbance at 600 nm within 1 min. The assay mixture without enzyme served as the control. One unit of enzyme activity was defined as the amount that catalyzed reduction of 1 µmol DCIP per min. Protein concentrations were measured by the Bradford method with Bovine Serum albumin (BSA) as the standard (Bradford, 1976)**.**

RESULTS

The specific activity was increased as expected along with the purification steps conducted as shown in Table 1. In this research, we also tried to compare the activity of enzyme produce in Luria Broth versus the media with standard mineral salts media (MSM). M27G2 isolate showed highest specific activity after purifications. The purity of the enzyme also increased ranging from 2.88 to 57.18 folds.

Tabel 1: Specific activity of enzyme following salt precipitation and ion exchange.

Isolate	Purification step	Specific activity (U/mg)		Purification fold	
		Luria broth	MSM	Luria broth	MSM
M27G2	Crude extract	56.63	238.96	1.00	1.00
	Ammonium sulfate	188.89	376.61	3.34	1.68
	HiTrap Sepharose	3238.19	4544.69	57.18	10.84
M27L1	Crude extract	129.47	196.99	1.00	1.00
	Ammonium sulfate	372.43	390.76	2.88	1.98
	HiTrap Sepharose	466.20	2195.07	3.60	11.14
M27L3	Crude extract	100.19	186.90	1.00	1.00
	Ammonium sulfate	900.23	402.12	8.99	2.15
	HiTrap Sepharose	1564.93	2027.33	15.62	10.85
M41L3	Crude extract	138.18	333.09	1.00	1.00
	Ammonium sulfate	549.08	488.06	3.97	1.47
	HiTrap Sepharose	1575.61	2407.52	11.40	7.23
M51G1	Crude extract	39.09	141.12	1.00	1.00
	Ammonium sulfate	172.48	230.68	4.41	1.63
	HiTrap Sepharose	1119.65	1353.20	28.64	9.59

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DISCUSSION

There were five isolates used in this research. They were M27G2 which has coccus shape and appears as creamy white colonies, M27L1 which has coccus shaped and appears as orange colonies, M27L1 which has coccus shaped and appears as white colonies, M41L3 which has coccus shaped and appears as yellow colonies, and M51G1 which has bacil shaped and appears as creamy white colonies. All these cultures were maintained in Luria agar. Along with the research, the colonies tend to change its appearance to yellowish color, especially M27L3. The diversion in color occurred because the cultures were not maintained in MSM that provides methanol and other minerals which are usually has specific nutrition for methylotrophs.

The crude extract showed high concentration of total protein but low in specific activity. This is related to the protein come from debris cells and media. The first step of the purification was conducted using ammonium sulfate precipitation. Preliminary experiments were conducted to evaluate several possible concentrations of ammonium sulfate, ranging from 20 - 70 %, for initial purification of MDH. Among all of the concentration tested, 40 % $(NH_4)_2SO_4$ gave the best protein precipitation by giving highest specific activity. The next step of purification was conducted using single cation exchange chromatography because MDH from methylotrophs has isoelectric point (p*I*) of 8.8 and therefore will be positively charged at low pH solutions (Day and Anthony, 1990).

The resulting enzyme from the chromatographic system showed increasing value of specific activity. The increasing value of specific activity was showed by the amount of DCIP molecules that were reduced by MDH. In every step done, there was an increasing value of purity. In Table 1, we can see the increasing value of enzyme purity based on the specific activity. However, there was a significant loss in total enzyme concentration in every step of purification conducted.

To increase the specific activity, isolates were regrown on standard mineral salts media (MSM) supplemented with 1 % of methanol. This media contains various minerals that support the growth of methylotrophs. Ammonium compound plays important role as an essential activator for MDH (Anthony, 2004). The pH of this media was maintained at 6.0 as the best condition for MDH and the cofactor (PQQ).

The use of MSM increased the specific activity as shown in Table 1. Low concentration of methanol as the sole (single) carbon and energy source may contribute in increasing the enzyme activity since the enzyme production itself was actually triggered by existence of single carbon compounds. In fact, MDH in most methylotrophs synthesized in the media with multi-carbon substrates, but present at higher levels if the cells are grown in the media with methanol (Lidstrom and Stirling, 1990; Xu *et al*., 1993; Jakobsen *et al*., 2006). Media with no single carbon compounds, tend to suppress enzyme production.

CONCLUSION

Ammonium sulfate precipitation coupled with single cation exchange chromatographic system has been proved to provide sufficient purified MDH with high specific activity for further application. The use of MSM gave higher specific activity than the other media.

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