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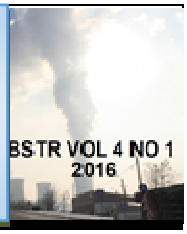

Short Communication

Partial Purification and Characterization of the Molybdenum-reducing Enzyme from the Glyphosate-degrading *Burkholderia vietnamiensis* strain AQ5-12

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ABSTRACT

In this study, a novel glyphosate-degrading shows the ability to reduce molybdenum to molybdenum blue. The enzyme from this bacterium was partially purified and partially characterized to ascertain whether the Mo-reducing enzyme from this bacterium shows better or lower efficiency in reducing molybdenum compared to other Mo-reducing bacterium that only exhibits a single biotransformation activity. The enzyme was partially purified using ammonium sulphate fractionation. The V_{max} for the electron donating substrate or NADH was at 1.905 nmole Mo blue/min while the K_m was 6.146 mM. The regression coefficient was 0.98. Comparative assessment with the previously characterized Mo-reducing enzyme from various bacteria showed that the Mo-reducing enzyme from *Burkholderia vietnamiensis* strain AQ5-12 showed a lower enzyme activity.

INTRODUCTION

Molybdenum is an essential heavy metal needed for a trace amount. However, it can be toxic when the concentration exceeds a certain level [1–4]. Molybdenum contamination in water bodies and soils may lead to heavy metal pollution and can disrupt the environment and ecosystem [5–7]. Molybdenum may exist in two forms, namely hexavalent molybdenum and molybdenum blue. Comparatively, hexavalent molybdenum is water soluble and possesses toxic properties while molybdenum blue is non-soluble in water and is non-toxic [8].

Molybdenum is an important heavy metal with plenty of industrial utilizations. For instance, it is used in the steel industry as an alloying agent to increase the hardness of tempered steels. Moreover, its alloying properties are utilized as an anti-corrosion agent in water-base hydraulic systems and automobile engine anti-freeze [9]. Besides that, molybdenum in the form of molybdenum disulphide is commonly used as a lubricant.

However, problems arise as wide usage of molybdenum prompted a few cases of water pollution all around the globe. So far, some notable cases have been reported in Japan, Austria and the Black Sea [10]. Presence of molybdenum in water at concentrations as small as several parts per million brings bad consequences to organisms such as catfish as it is capable of being a spermatogenesis inhibitor and thus stops embryogenesis from occurring [1,11]. Bioremediation of molybdenum is deemed as the best way to solve these problems as it is an environmentally safe way of removing toxic metals at a cheap cost [12]. Tyrol, Australia was the first location where molybdenum removal was accomplished successfully [10]. In this study, a novel glyphosate-degrading bacterium previously isolated has shown the ability to reduce molybdenum to molybdenum blue. One important study that needs to be ascertained is whether the Mo-reducing enzyme from this

bacterium shows better or lower efficiency in reducing molybdenum compared to previously isolated strains which show only molybdenum reduction capacity [13,14]. A higher molybdenum reduction capacity measured regarding the Michaelis-Menten constants would indicate a possibly better multiremediating capacity than single biotransformation activity.

MATERIALS AND METHODS

Growth and maintenance of the Mo-reducing bacterium *Burkholderia vietnamiensis* strain AQ5-12

The bacterium was originally isolated as a glyphosate-degrading bacterium [15], and the results of the molybdenum reduction characterization are published elsewhere.

Preparation of culture media

Low phosphate media (LPM)

The chemicals needed to make LPM in 1 litre of deionised water were as followed: 3 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract, 5 g of NaCl, 2.42 g of $Na_2MoO_4.2H_2O$, 0.73 g of $Na_2HPO_4$and 10 g of glucose. The pH of the media was fixed to 7.5. It was autoclaved at 121°C at 115 kPa for 15 minutes before usage. It is important to note that glucose was autoclaved separately, and only added to the autoclaved medium before inoculation.

High phosphate media (LPM)

The chemicals needed for HPM were the same as LPM, but HPM had the significantly higher amount of phosphate compared to LPM. The chemicals needed to make HPM in 1 litre of deionised water were as followed: 3 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract, 5 g of NaCl, 5 g of $Na_2MoO_4.2H_2O$, 14.6 g of $Na_2HPO_4$and 10 g of glucose. The pH of the media was fixed to 7.5. It was autoclaved at 121°C at 115 kPa for 15 minutes before usage. It is important to note that glucose was autoclaved separately, and only added to the autoclaved medium before inoculation [16].

Low phosphate agar

The chemicals needed to make low phosphate agar in 1 litre of deionised water were as followed: 3 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract, 5 g of NaCl, 2.42 g of $Na_2MoO_4.2H_2O$, 0.73 g of Na_2HPO_4, 10 g of glucose and 18 g of agar. The pH of the media was fixed to 7.5. It was autoclaved at 121°C at 115 kPa for 15 minutes before usage. I It is important to note that glucose was autoclaved separately, and only added to the autoclaved medium before inoculation.

After autoclaving, the molten agar was allowed to cool down at around 50 °C before it was poured into sterile disposable Petri dishes. In order to store the agar for a longer period, the plate was sealed with parafilm tape to avoid the agar from drying out, before the plates were stored in the cold room.

Characterisation of Mo-reducing enzyme

The crude Mo-reducing enzyme from *Burkholderia vietnamiensis* strain AQ5-12 was obtained by growing the strain in high phosphate media at a large scale. Next, the enzyme was purified via ammonium sulphate fractionation. After that enzyme kinetic studies were carried out to determine its V_m and $K_{max.}$

Preparation of crude enzyme

Burkholderia vietnamiensis strain AQ5-12 was grown in 1 L of high phosphate media (HPM). The preparation steps were explained in 3.2.1. The bacterium would not produce Mo-blue in HPM [16]. This was because the high concentration of phosphate (100 mM) will inhibit the production of Mo-blue. Even so, the enzyme would stay active [17].

After the strain was grown in HPM for 48 hours, it was centrifuged at 10,000 x g at 4°C for 10 minutes. Next, the cells were resuspended in 200 ml of LPM.After that, the cells were centrifuged at 15,000 x g at 4°C for 10 minutes. The next step is to take the pellet and reconstitute it with 20 ml of 50 mM Tris-HCl buffer. The buffer was at pH 7.5. The buffer also contained 0.5 mM DTT and 0.1 mM PMSF. Then, the cells were sonicated for 1 minute in an ice bath and 4 minutes cooling until a total sonication time of 20 minutes. Subsequently, the cells were centrifuged at 15,000 x g at 4°C for 1 hour. Later, the supernatant was collected and ultracentrifuged at 36,500 rpm at 4°C for 1 hour [18].

Purification via ammonium sulphate fractionation

The crude enzyme obtained underwent ammonium sulphate fractionation in order to obtain purified enzyme. Ammonium sulphate would remove water molecules from the crude enzyme, thus causing the enzyme to precipitate into its pure form. First of all, grounded ammonium sulphate was kept at 4°C before use. The crude enzyme was stirred slowly in a beaker. Then, ammonium sulphate was added slowly into the crude enzyme until the desired concentration was achieved. The percentages of saturation used in this experiment were between 0-20%, 20-30%, 30-40%, 40-50%, 50-60% and 60-70%. Previous studies showed that ammonium sulphate saturation of 70-100% did not give Mo-reducing enzyme activity.

Mo-reducing enzyme kinetic studies

Enzyme kinetic is characterised by the values of K_m and V_{max}. K_m is used to study the enzyme's ability to bind to the substrate. Therefore, it is also known as the affinity of the enzyme to bind to the substrate. On the other hand, V_{max} is used to measure the rate of enzyme reaction where V_{max} is the maximum initial velocity. The values of K_m and V_{max} were analysed by using a software called Graphpad Prism™ version 5.0. The software calculated the values via non-linear regression analysis.

Referring to a previous study, 12-phosphomolybdate and labaratory-prepared phosphomolybdate (LPPM) acted as electron acceptors while NADH acted as the electron donor. [18]. In this work only NADH is considered and LPPM was under saturating level at all values of NADH.

K_m and V_{max} of NADH as the electron donor

400 mM of NADH stock solution and 100 µl of 50 mM LPPM with pH 5 were prepared. The concentrations of NADH were made varied by adding 50 mM of Tris buffer with pH 7 in the stock solution. After the desired concentration was obtained, 150 µl of purified enzyme fraction was added. The total volume of NADH, Tris and enzyme mixture was 1 ml. This experiment was done at room temperature. The increase in absorbance at 865 nm was measured by using UV-spectrophotometer after 5 minutes incubation time. The activity of the enzyme is expressed as nanomole Mo-blue produced per min [18].

RESULTS AND DISCUSSION

Characterisation of *Burkholderia vietnamiensis* strain AQ5-12's Mo-reducing enzyme

Based on the results, the V_{max} for NADH was at 1.905 nmole Mo blue/min. The best regression coefficient was 0.98. The K_m was 6.146 mM (**Fig. 1**). Comparisons to previous studies showed that it has lower V_{max} than *Enterobacter cloacae* 48 [13] and *Serratia* sp. strain Dr.Y5 [14] which recorded V_{max} values of 6.28 nmole Mo blue/min and 12 nmole Mo blue/min respectively. On the other hand, the mo-reducing enzyme of *Burkholderia vietnamiensis* strain AQ5-12 has a K_m higher than those two strains. *Enterobacter cloacae* 48 [13] had a K_m of 1.65 mM while *Serratia sp.* strain Dr.Y5 [14] had a K_m of 0.79.Therefore, it can be said that the mo-reducing enzyme of *Burkholderia vietnamiensis* strain AQ5-12 had a lower affinity to bind to the substrate, NADH.

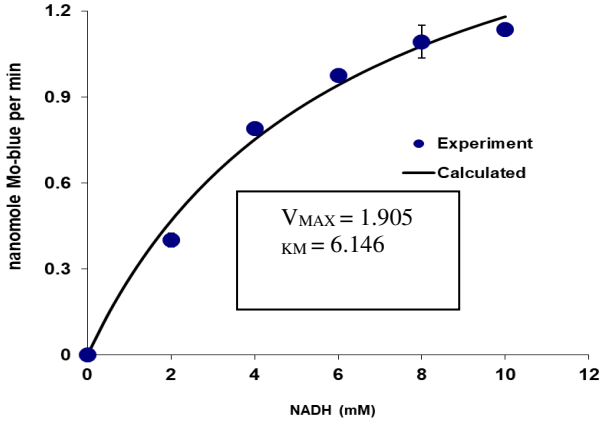

Fig. 1. Michaelis-Menten plot of the amount of Molybdenum-blue formed (nmole/min) versus the electron donor substrate (NADH). The concentration of the electron acceptor 10:4-phosphomolybdate was at saturation level at all points. Each reading is a mean of 3 replicates, and the bars represent the standard error means (SEM).

CONCLUSION

This study was conducted to ascertain the kinetics parameters of enzymatic reduction of molybdenum to molybdenum blue from the glyphosate-degrading *Burkholderia vietnamiensis* strain AQ5-12. Based on the Michaelis-Menten kinetics constants obtained, this bacterium was found to have a lower enzymatic conversion process that other single transforming capacity bacterium. Despite this fact, the ability of this bacterium to be able to biotransform more than one toxicants is an important step for bioremediation regarding finding multibiotransforming microorganisms as more and more polluted sites in the world have a combination of heavy metals and organic pollutants.

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