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Short Communications

Revisiting the role of the electron transport chain in molybdate reduction by *Enterobacter cloacae* strain 48

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Molybdenum reduction to molybdenum blue by microbes is a potential bioremediation tool for molybdenum pollution. A previous work using cyanide as a respiratory inhibitor has shown that the site of molybdenum reduction in *Enterobacter cloacae* strain 48 is at the electron transport pathway. In the present work, several respiratory inhibitors together with cyanide were used to reinvestigate the site of molybdenum reduction in *E. cloacae* strain 48. All the respiratory inhibitors tested showed no inhibition to the molybdenum-reducing capability of the bacterium. It was also discovered that cyanide caused a large increase in the pH of the enzymatic reaction mixture and, therefore, the inhibition previously seen was likely due to unfavourable pH for the enzyme activity. Based on these results, the site of molybdenum reduction in this bacterium is suggested not to be the component of electron transport pathway.

Keywords: Azide, cyanide, *Enterobacter cloacae*, molybdenum-reducing bacterium, respiratory inhibitors, rotenone

Microbes can resist the toxic effects of heavy metals by employing a variety of mechanisms, such as, bioprecipitation, sequestration, bioreduction to an oxidation state that is less toxic and having pumps that can remove heavy metals from the cytosolic environment[1,2]. One of the heavy metals that has recently been emerged as a significant pollutant is molybdenum (Mo). In aqueous environment, Mo exists as molybdate ions[3] and microbes have long been known to be able to reduce molybdate to Mo-blue[4,5]. In 1985, Campbell *et al*[6] rejuvenated this phenomenon by reporting molybdate reduction in *Escherichia coli* K12. Sugio *et al*[7] reported the reduction of molybdate into Mo-blue by *Thiobacillus ferreoxidans* strain AP19-3. Later, Ghani *et al*[8] isolated and characterized *Enterobacter cloacae* strain 48 (EC 48) as Mo-reducing bacterium. For the past 25 years, studies on molybdate reduction to Mo-blue were carried out predominantly on Malaysian local bacterial isolates including EC 48[8-13].

Studies on cellular extracts of EC 48 have shown that Mo-reducing enzyme reduces molybdate to Mo-blue using NADH as the principal e^- donor. The optimum pH for enzyme activity is 7.0[8]. Since cyanide inhibits Mo-blue production but not cytochrome b reduction in the cell extract, the site of molybdate reduction to Mo-blue in EC 48 is proposed as the reduced component after cytochrome b. In the original Mo-reducing enzyme assay of the above study, the main buffering species was phosphate with a final concentration of 0.1 mM. This low concentration of buffering species was suspected to affect the pH of the reaction mixture when high concentration of inhibitors was used. The objective of this work is to determine the effect of cyanide on the pH of the reaction mixture to ascertain whether the inhibition seen with cyanide is due to the changes in pH or a true inhibition of the Mo-reducing enzyme activity.

EC 48 was maintained on low phosphate (2.9 mM phosphate) agar medium (pH 7.5) containing (g L^{-1}): glucose (10), $(NH_4)2SO_4$ (3), $MgSO_4 \cdot 7H_2O$ (0.5), NaCl (5), yeast extract (0.5), $NaMoO_4 \cdot 2H_2O$ (2.42) and Na_2HPO_4 (0.5). However, for the large scale growth, a high phosphate medium was used; the only difference from the low phosphate medium was the phosphate concentration (100 mM)[8]. Growth on low phosphate resulted in a blue sticky culture that complicated the preparation of crude enzyme and enzyme assay.

For preparation of crude enzyme, EC 48 was grown in 1 L of the high phosphate media at 30°C for 24 h on an orbital shaker (100 rpm). Although the high phosphate inhibits molybdate reduction to molybdenum blue, the cells contain active enzymes. The following procedure was carried out at 4°C unless stated otherwise. Cells were harvested through centrifugation at 10,000 g for 10 min. They were washed at least once with distilled water, resuspended and recentrifuged. The pellet was reconstituted with 10 mL of 50 mM Tris buffer pH 7.5 (Tris buffer

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prepared at 4°C) containing 0.5 mM dithiothreitol and 0.1 mM PMSF (phenylmethanesulfonyl fluoride). Cells were sonicated for 1 min on an ice bath with 4 min cooling until a total sonication time of at least 20 min was achieved. The sonicated fraction was centrifuged at 10,000 g for 20 min and the supernatant consisting of the crude enzyme fraction was taken[8].

The reaction mixture for Mo-reducing enzyme assay[8] contained cell extract (20 mg of protein), Na_2MoO_4 (5 mM), Na_2HPO_4 (0.1 mM), glucose (10 mM) (pH 7.0) and 8 mL of salts solution (pH 7.0) containing (g L^{-1}): $(NH_4)_2SO_4$ (3), $MgSO_4 \cdot 7H_2O$ (0.5) and NaCl (5); the total volume was maintained at 10 mL. The anaerobic condition of the assay was achieved by gassing the reaction vessel with nitrogen gas for 10 min (Fig. 1). The crude enzyme (50 µL) was injected through the silicone septa to start the reaction. The increase in absorbance at 710 nm was measured over a period of 20 min of incubation. The amount of Mo-blue produced was quantified using an ascorbate-reduced molybdate and one unit of enzyme activity was defined as the production of 1.0 µmol Mo-blue per min. The specific extinction coefficient[9] at 865 nm was 16.7 mM^{-1} cm^{-1}.

The buffering species for the enzymatic reaction mixture was phosphate at 0.1 mM final concentration. At this concentration, effect of metabolic inhibitors to the pH of reaction mixture was suspected. Thus to study this effect, sodium cyanide (1 to 10 mM) was dissolved in deionised water and added to the reaction mixture. For control, enzyme was substituted with deionized water. The increase or decrease of pH was monitored with a Mettler and Toledo pH meter.

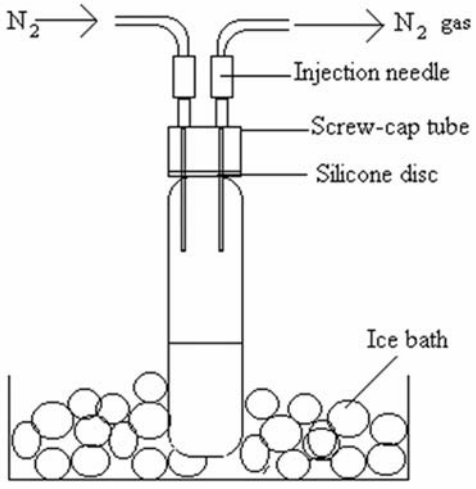

Fig. 1—Procedure for maintenance of anaerobic conditions for molybdenum reduction.

In order to study the effect of other metabolic inhibitors on molybdate reduction at exactly pH 7.0, a modification to the reaction mixture was carried out. Phosphate concentration was increased from 0.1 to 20 mM (pH 7.0), whilst cyanide and molybdate stock solutions were adjusted to pH 7.0 prior to addition to the reaction mixture. Other electron transport inhibitors, such as, HOQNO (hydroxy quinoline-N oxide), antimycin A and rotenone, were dissolved in acetone, while sodium azide was dissolved in deionized water[14]. Antimycin A, HOQNO, rotenone and azide were added into the reaction mixture to the final concentrations of 1.2, 0.02, 0.2 and 10 mM, respectively.

The addition of 10 mM cyanide into the reaction mixture showed that the pH of original reaction mixture rose from pH 7.0 to 10.17 (Fig. 2). Since the optimum pH for the Mo-reducing enzyme is 7.0 and a deviation of 1.0 pH unit causes more than 50% inhibition of the enzyme as reported by Ghani *et al*[8], this large deviation in pH in the present case is a major concern. The generally used concentration of cyanide for enzyme inhibition studies is between 0.1 to 10 mM[15] and the use of higher concentrations of cyanide was not attempted. When the concentration of the buffering species (phosphate) was increased to 20 mM and holding the cyanide concentration at 10 mM, no inhibitory effect of cyanide was observed. At 20 mM phosphate all of the other inhibitors tested did not show any inhibition to the Mo-reducing activity in the EC 48 bacterium ($F_{6,14}$= 0.1983, P>0.05) (Fig. 3).

A similar observation of cyanide effect to the pH of the enzymatic reaction mixture, in a study with peanut

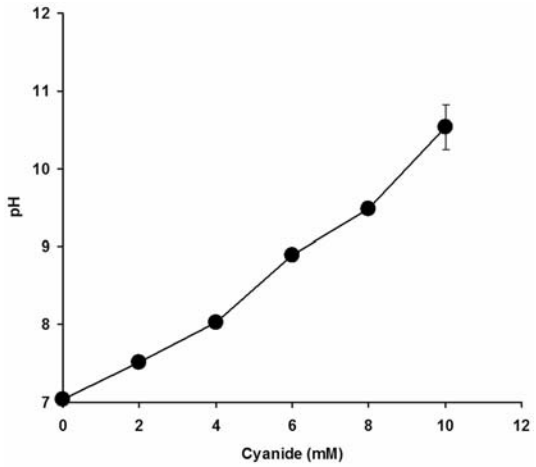

Fig. 2—Effect of cyanide on pH of reaction mixture. [Values represent mean±SE of 3 replicates]

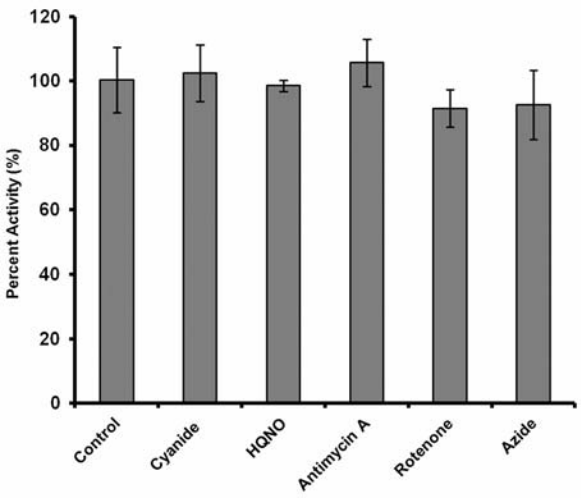

Fig. 3—Effect of various inhibitors on the enzymatic reduction of molybdate in *E. cloacae* strain EC 48. [Bars represent mean±SE of 3 replicates]

lipoxygenase, was reported by Angelo and Kuck[16]. Being aware about the conflicting reports on the effects of cyanide on lipoxygenase, they discovered that cyanide is very alkaline in nature and increases the pH of the reaction mixture. This increase of pH decreases the enzyme activity significantly[16].

It is known that sodium or potassium cyanide dissociates in water forming hydrogen cyanide and NaOH; the latter contribute to the high alkalinity of the reaction mixture. This increase in pH was not observed with other inhibitors[15]. Antimycin A and HQNO (hydroxyquinoline-N-oxide) are inhibitors to cytochrome b. Rotenone is an inhibitor to NADH dehydrogenase, whilst sodium azide and cyanide are inhibitors to the terminal cytochrome d oxidase[15]. All of these are components of the respiratory chain complex. Hence, the results suggest that the electron transport chain of this bacterium is not the site of molybdate reduction. However, the electron transport chain has previously been suggested as the site of molybdate reduction in EC 48 based on the inhibition of Mo-reducing enzyme by cyanide[8]. Respiratory inhibitors have been used to probe the location or identity of metal-reducing enzymes in various studies. Respiratory inhibitors, such as, rotenone, azide and cyanide failed to inhibit chromate reduction in *E. coli*[17] and in *Pseudomonas mendocina*[18], while cyanide and azide inhibit the reduction of chromate in *Bacillus subtilis*[19] and oxidation of arsenite in *Alcaligens* sp.[20].

The results obtained in the present study indicated that the effect of cyanide was probably not at the enzyme level but because of increased pH of reaction mixture caused by cyanide. Further, using more robust buffering conditions, it was shown that cyanide and other inhibitors did not inhibit the Mo-reducing enzyme activity in EC 48, leading to the conclusion that the electron transport chain of EC 48 is not the site of molybdate reduction.

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