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Characterization of Chromate Reducing *Pseudomonas Aeruginosa* Strain Mie3 Isolated from Juru River Sludge and its Potential on Azo Dye Decolorization

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ABSTRACT

Chromate and azo dyes are common compounds used in the industrial applications and released into the environments. Therefore studies of bioremediation treatment methods should involve the removal of both pollutants. In this study, we report, a novel chromate reducing bacteria with the capability to decolorize 4 type of dye namely Amaranth Dye, Biebrich Scarlet, Direct Blue 71 and Methanil Yellow under aerobic conditions. The isolate identified as *Pseudomonas Aeruginosa* strain MIE3 and reduced 52% of 150 ppm potassium dichromate (Cr(VI)) in the nutrient broth after 24 hours incubation under shaking condition at 150 rpm. Optimization using one factor at a time (OFAT) showed the optimal conditions for chromate reduction include nutrient broth concentration was 8 g/L, temperature between 30 and 35^{o}C, and pH was 7.8. The ability of this bacterium to detoxify these toxicants make the bacterium a valuable tool for future wastewater and soil bioremediation.

KEY WORDS: Chromate, Azo dye, Reduction, Decolorization.

1. INTRODUCTION

Chromium (Cr) is one of the toxic heavy metal that is introduced into the environment by several industries such as textile dyeing, leather tanning, chromate plating and alloying formation (Dhal, 2013; Deshpande, 2005). Chromium is available in different oxidation states; nevertheless, hexavalent chromium and trivalent chromium are the most dominant and stable forms in the environment as compared to others redox states with hexavalent chromium is considered as the most toxic due to its solubility and mobility in the environment (Mishra and Bharagava, 2016). The exposure to hexavalent chromium contributes to several toxicity disorders to human being for instance; vomiting, skin allergy, etc. resulting from its strong oxidizing agent, mutagenic and teratogenic characteristics (Fernando, 2016). In the plant, hexavalent chromium is noticed to have an impact on several metabolic pathways in plants resulting in chlorosis, inhibition of root growth, the fatality of the plant, etc. Due to these toxic characteristics, there is a need to remove this contaminant from the environment (Lukina, 2016).

Conventional techniques for eliminating metals from industrial effluents might be ineffective or highly expensive especially when the metals available in a concentration range of 1-100 mg/L (Hackbarth, 2016). Due to this fact, it is necessary to develop an innovative, low-cost, and eco-friendly technique for of toxic heavy metal removal from the wastewater (Halmi, 2016). Recently, biotechnological methods for the restoration of the polluted environment have received global interest. Bioremediation is regarded as an economical and eco-friendly technology with promising to reduce toxic substances from a polluted environment or treatment solutions of wastewater (Halmi, 2016, Shukor, 2016). Biological reduction by microorganisms of toxic hexavalent chromium has been accepted as a tool to change the redox state to non-toxic form and highly soluble, trivalent chromium. Many microorganisms such as *Bacillus, Pseudomonas and Shewanella* have been reported able to carry out this function. In this paper, we describe the characterization of chromate reducing bacteria from sludge collecting from Prai Industrial Estate in Penang, Malaysia and examine its Cr(VI) reducing capability. The potential on dye decolorization by the chosen isolate has been assessed in this study.

2. MATERIALS AND METHODS

Chemicals: The chemicals used in this study were of analytical grade from several chemicals suppliers such as Fisher (Malaysia) and Merck (Darmstadt, Germany) and Sigma, Aldrich (USA).

Measurement of chromate reduction: Chromate reducing activity was measured according to calorimetric changes using 1,5-diphenyl carbazide (Sigma, USA). The calorimetric reagent was prepared by dissolving 0.025 g 1,5-diphenyl carbazide in 100 mL of analytical grade acetone to minimize deterioration. The hexavalent chromium in the culture was assayed by reacting 400 µL of culture supernatant with 400 µL of 1 N H_2SO_4 and 200 µL 1,5-diphenylcarbazide solution and further analyzed spectrophotometrically at 540 nm (Megharaj, 2003).

Isolation of chromium-resistant bacteria: Chromate reducing bacteria were isolated from sludge obtained from an industrial area that was recognized as a heavy metal polluted site in Prai, Penang. Sludge was serially diluted and plated on nutrient agar plates amended with 100 ppm potassium dichromate (K_2CrO_4) and incubated at 30°C for 24 hours. Chromate-reducing strains that represent different colony morphologies were purified on the same agar medium, maintained on Cr(VI) amended nutrient agar slants for storage (Miller, 2016). Reduction of chromate was determined by inoculating the isolates in nutrient broth supplemented with 100 ppm of Cr(VI) and incubated at 30°C under continuous shaking (150 rpm) for 24 hours. The reduction was estimated by measuring the decrease in chromate in the culture filtrate following 1, 5-diphenylcarbazide method. The isolate with highest chromate reduction was chosen for further studies and identified by Biolog Identification System.

Chromium reduction experiments: The nutrient broth was used to investigate the effect of 3 parameters such as nutrient broth concentration, pH, and temperature on chromate reduction by chromate reducing bacteria. The determination of the optimum concentration of these parameters is necessary to maximize the reduction capacities and bacterial growth. A hundred milliliters of nutrient broth was used throughout the studies. Two milliliters of fresh bacteria cells in nutrient broth was pipetted and transferred into nutrient broth amended with 50 ppm chromate when the optimal density at 600 nm reached between of 0.9 to 1.0. All inoculated nutrient broths were incubated at 30°C for 24 hours except during the optimization of temperature. One milliliters sample from nutrient broth was pipetted for every two hours, and the bacterial growth was measured at 600 nm. Then, the collected sample was centrifuged at 10,000 xg for 20 minutes in an Eppendorf™ tube, and chromate reduction was measured according to calorimetric changes using 1,5-diphenylcarbazide (Swapna, 2016; Halmi, 2016). All data were analyzed using GraphPad Prism 5.0. Statistical analyses were performed using one–way analysis of variance (ANOVA) with post hoc analysis by Tukey's test with $p<0.05$ were considered statistically significant.

Decolorization of azo-dye: The potential of the chromate bacterium to decolorize various dyes was investigated in a minimal salt medium added with 100 ppm dye. The ingredients of the minimal salt media (% w/v) were as follows: glucose (1%), sodium lactate (1%), $NaNO_3$ (0.2%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), and Na_2HPO_4 (0.705% or 50 mM). The decolorization percentage was calculated through the difference between the absorbance values from the initial reading was subtracted from the final reading after incubated for 24 hours. All dyes were bought from Sigma-Aldrich (St. Loius, U.S.A.). The dyes were: Crocein Orange G (C.I. 15970) (482 nm), Evans Blue (C.I. 23860) (594 nm), Cresol Red (C.I. 1733-12-6) (570 nm), Congo Red (C.I. 22120) (498 nm), Fast Green FCF (C.I. 42053) (620 nm), Fuchsin Basic (C.I. 42510) (625 nm), Methyl Green (C.I. 42590) (635 nm), Metanil Yellow (C.I. 13065) (414 nm), Methyl Orange (C.I. 13025) (505 nm), Toluidine Blue (C.I. 52040) (626 nm), Methylene Blue (C.I. 52015) (590 nm), Nigrosin (C.I. 50415) (570 nm), Sudan Black B (C.I. 26150) (600 nm), Safranin O (C.I. 50240) (530 nm), Amaranth dye (C.I. 16185) (520 nm), Biebrich Scarlet (C.I. 26905) (506 nm) and Direct Blue 71 (C.I.34140) (587 nm) (Shukor, 2016).

3. RESULTS AND DISCUSSIONS

Isolation, bacterial identification and screening Cr(VI) reducing media: From 30 isolates, only isolate no 25, showed the highest reduction percentage after 24 hours incubation. Thus, isolate no 25 was chosen in this study. The isolate was an aerobic gram-negative bacterium identified as *P. Aeruginosa* according to Biolog Identification System with 0.603 similarity index. The similarity index greater than 0.500 indicating a strong similarity index for genus or species identification. The isolate demonstrated the most significant reduction in nutrient broth containing 50 ppm ppm chromate in comparison with the Luria bertani broth, minimal salt media, and acetate minimal media. Thus, the nutrient broth was selected as the best medium and used throughout this study.

Optimization of chromate reduction by chromate reducing bacteria: The optimum condition of *P. Aeruginosa* was determined using classical method, One-Factor-at-a-Time (OFAT) approach. The parameters such as nutrient broth concentration, pH, and temperature were characterized and optimized in this study. These parameters are vital factors that affect biological chromate reduction process due to their influence on enzymatic reduction activity and bacterial growth. For the effect of nutrient broth concentration, chromate reducing bacterium shows the highest reduction at nutrient broth concentration 8.0 g/L (Fig.1a). The reduction started to decrease after the bacterium was grown at a concentration above 8.0 g/L. In the presence of carbon sources in the nutrient broth, the bacteria would produce NADH and NADPH, an electron donating substrates through several metabolic pathways such as Kreb's cycle, glycolysis, and electron transport chain (Rahman, 2009; Suzuki, 1992). Both electrons donating substrates are needed by chromate reducing enzyme during the reduction process. Effect of temperature on chromate reduction was observed over a broad range of temperature (23 to 40°C) with the optimum temperature occurred at 30 to 35°C with no significant different ($p>0.05$) (Fig.1b). The broad optimum condition for temperature would be an advantage for bioremediation of hexavalent chromium in the tropical region that have an average temperature from 33 to 35°C (Shukor, 2008). The effect of pH was on chromate reduction examined at pH range from 5.5 to 9. The result demonstrated that the optimum initial pH is 7.8 with inhibition of reduction process at pH 7 and 9 (Fig.1c). Bacteria require a physiological pH to survive, similar to other living organisms. Their capability to thrive in high or low pH

relies upon their ability to control the pH difference between the external and internal of the cell. Furthermore, pH plays significant roles on the turning of ionic states of enzyme and proteins on the surface of the bacterial cells (Ilias, 2011). These could involve carbon source transporters and chromium transport protein and all of which are affected by a strong adjustment in pH of the environment leading to a decrease in the reduction process. Due to this optimization result, further experiments were performed at nutrient broth concentration of 8 g/L, pH 7.8 and a temperature of 30°C.

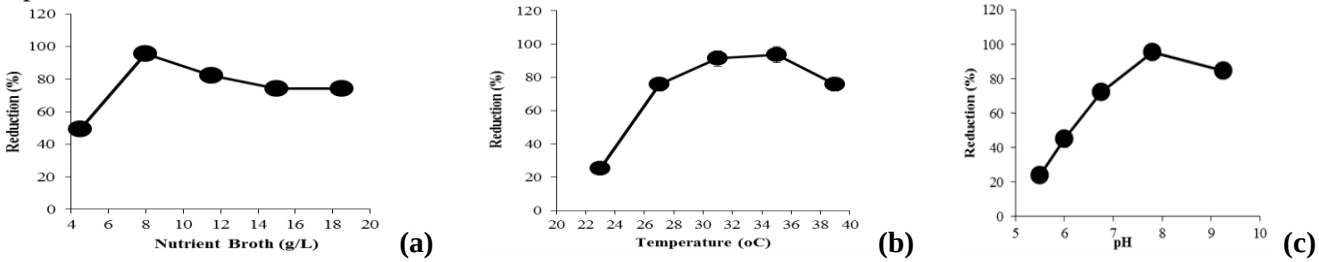

Figure.1. Optimization of chromate reducing capability of *P. Aeruginosa* strain MIE3

Time course chromate reduction Studies: Figure.2, shows the effect of varying chromate concentrations ranging from 20-200 ppm on chromate reduction and bacterial growth of *P. Aeruginos*a under batch culture. *P. Aeruginosa* demonstrated the reduction ability until 150 ppm with inhibitory of bacteria growth as the chromate concentration increases indicating that the reduction by *P. Aeruginos*a was found to be growth-associated. During the reduction of chromate in culture, there was a gradual decrease in growth rate and bioreduction rates as the chromate concentration increases over time. The inhibitory effect during the reduction process could due to the toxicity of chromate to the bacteria cells and the decrease in cells number as reported by Dey & Paul. (2012).

Figure.2. Time course chromate reduction (A) and bacterial growth (B) of *P. Aeruginos*a strain MIE3 at difference chromate concentration.

Azo-dye Decolorization: Decolorization of 17 dyes by chromate reducing *P. Aeruginos*a strain MIE3 was examined and shown in Figure 3. The result shows the strain has decolorized four types of dyes such as Amaranth Dye, Biebrich Scarlet, Direct Blue 71 and Methanil Yellow (95-100% decolorization) after 24 hours incubation as compared to other dyes. The potential of this bacterium to detoxify multiple toxicants makes the bacterium an important tool for bioremediation. However, the capability of the bacterium to decolorize these azo dyes should be explored in details. Many microorganisms have been reported for effective remediation of dye and chromium alone, and a few studies are reported on simultaneous removal both compounds (Ng, 2010; Shukor, 2016). Therefore, the capability of chromate reducing bacteria to simultaneous decolorize dyes and reduce chromate should be investigated for future works. Many researchers have isolated several chromate reducing bacteria from various sources such as wastewater, soil, etc. These microorganisms have been reported to decolorize and reduce both dyes and chromate efficiently in the culture medium (Chaudhari, 2013; Huang, 2015; Mistry, 2010).

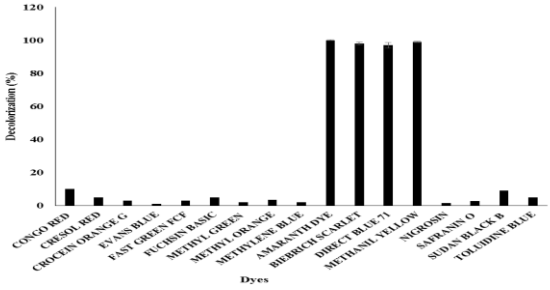

Figure.3. Decolorization of dyes by *P. Aeruginosa* strain MIE3. The bacterium was incubated for 24 hours. Error bars represent mean ± standard deviation (n = 3)

4. CONCLUSION

A chromate reducing bacterium, *P. Aeruginos*a with the novel ability to decolorize Amaranth Dye, Biebrich Scarlet, Direct Blue 71 and Methanil Yellow has been isolated. The bacterium reduces hexavalent chromium to trivalent chromium optimally at pH 7.8, temperatures of between 30 and 35ºC and nutrient broth concentration at 8.0 g/L. Time course chromate reduction by this strain was found to be growth-associated. The result suggests *P. Aeruginos*a strain MIE3 would be useful in detoxification of chromate and makes the bacterium an important tool for future bioremediation in a contaminated environment. Recently, attempts are ongoing to immobilize this bacterium in alginate beads and to fully characterize and optimize the dye-decolorizing ability using response surface methodology and artificial neural network.

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REFERENCES

Chaudhari A.U, Tapase S.R, Markad V.L & Kodam K.M, Simultaneous decolorization of reactive Orange M2R dye and reduction of chromate by Lysinibacillus sp. KMK-A, Journal of hazardous materials, 262**,** 2013, 580-588.

Deshpande K, Cheung S, Rao M.S & Dave B.C, Efficient sequestration and reduction of hexavalent chromium with organosilica sol–gels, Journal of Materials Chemistry, 15**,** 2005, 2997-3004.

Dey S & Paul A, Optimization of cultural conditions for growth associated chromate reduction by *Arthrobacter* sp. SUK 1201 isolated from chromite mine overburden, Journal of hazardous materials, 213**,** 2012, 200-206.

Dhal B, Thatoi H, Das N & Pandey B, Chemical and microbial remediation of hexavalent chromium from contaminated soil and mining/metallurgical solid waste, a review, Journal of hazardous materials, 250**,** 2013, 272-291.

Fernando V.A, Weerasena J, Lakraj G.P, Perera I.C, Dangalle C.D, Handunnetti S, Premawansa S & Wijesinghe M.R, Lethal and sub-lethal effects on the Asian common toad Duttaphrynus melanostictus from exposure to hexavalent chromium, Aquatic Toxicology, 177**,** 2016, 98-105.

Hackbarth F.V, Maass D, De Souza A.A.U, Vilar V.J & De Souza S.M.G.U, Removal of hexavalent chromium from electroplating wastewaters using marine macroalga Pelvetia canaliculata as natural electron donor, Chemical Engineering Journal, 290**,** 2016, 477-489.

Halmi M.I.E, Abdullah S.R.S, Wasoh H, Johari W.L.W, Bin Mohd Ali M.S, Shaharuddin N.A & Shukor M.Y, Optimization and maximization of hexavalent molybdenum reduction to Mo-blue by Serratia sp. strain MIE2 using response surface methodology, Rendiconti Lincei**,** 2016, 1-13.

Huang G, Wang W & Liu G, Simultaneous chromate reduction and azo dye decolourization by Lactobacillus paracase CL1107 isolated from deep sea sediment, Journal of environmental management, 157**,** 2015, 297-302.

Ilias M, Rafiqullah I.M, Debnath B.C, Mannan K.S.B, & Hoq M.M, Isolation and characterization of chromium (VI)-reducing bacteria from tannery effluents, Indian journal of microbiology, 51**,** 2011, 76-81.

Lukina A, Boutin C, Rowland O & Carpenter D, Evaluating trivalent chromium toxicity on wild terrestrial and wetland plants, Chemosphere, 162**,** 2016, 355-364.

Megharaj M, Avudainayagam S & Naidu R, Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste, Current microbiology, 47**,** 2003, 0051-0054.

Miller R.B, Giai C, Iannuzzi M, Monty C.N & Senko J.M, Microbial reduction of Cr (VI) in the presence of chromate conversion coating constituents, Bioremediation Journal**,** 2016.

Mishra S & Bharagava R.N, Toxic and genotoxic effects of hexavalent chromium in environment and its bioremediation strategies, Journal of Environmental Science and Health, Part C, 34**,** 2016, 1-32.

Mistry K, Desai C & Patel K, Chromate reduction by Vogococcus sp. isolated from Cr (VI) contaminated industrial effluent, Electronic Journal of Biology, 6**,** 2010, 6-12.

Ng T.W, Cai Q, Wong C.K, Chow A.T & Wong P.K, Simultaneous chromate reduction and azo dye decolourization by Brevibacterium casei, azo dye as electron donor for chromate reduction, Journal of hazardous materials, 182**,** 2010, 792-800.

Rahman M, Shukor M, Suhaili Z, Mustafa S, Shamaan N.A & Syed M.A, Reduction of Mo (VI) by the bacterium *Serratia* sp. strain DRY5, Journal of Environment Biology, 30 (1), 2009, 65-72.

Shukor M.S, Aftab K, Norazlina M, Effendi H.M.I, Sheikh A.S.R & Yunus S.M, Isolation of a Novel Molybdenum reducing and Azo Dye Decolorizing Enterobacter sp. Strain Aft-3 from Pakistan, Biodiversitas, 16 (2), 2015, 238-246.

Shukor M.Y, Habib S.H.M, Rahman M.F.A, Jirangon H, Abdullah M.P.A, Shamaan N.A & Syed M.A, Hexavalent molybdenum reduction to molybdenum blue by S. marcescens strain Dr. Y6, Applied Biochemistry and Biotechnology, 149 (1), 2008, 33-43.

Suzuki T, Miyata N, Horitsu H, Kawai K, Takamizawa K, Tai Y & Okazaki M, NAD (P) H-dependent chromium (VI) reductase of Pseudomonas ambigua G-1, a Cr (V) intermediate is formed during the reduction of Cr (VI) to Cr (III), Journal of bacteriology, 174**,** 1992, 5340-5345.

Swapna T, Papathoti N, Khan M, Reddy G & Hameeda B, Bioreduction of Cr (VI) by Biosurfactant Producing Marine Bacterium Bacillus subtilis, SHB, 75 (7), 2016, 432-438.