UNIVERSITI PUTRA MALAYSIA

BIODEGRADATION OF CYANIDE BY CYANIDE DIHYDRATASE FROM LOCALLY ISOLATED Serratia marcescens ISOLATE AQ07

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By

KARAMBA KABIRU IBRAHIM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

I dedicated this thesis to Bauchi State University Gadau (BASUG) for giving me the opportunity to serve and obtain a Philosophy Doctorate Degree (PhD) under its umbrella. I hope the University will grow and be the No. 1 University in the Federal Republic of Nigeria.

Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfillment of the requirements for the Degree of Doctor of Philosophy

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Chairman : Siti Aqlima Ahmad,PhD
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Cyanide is a very toxic chemical and is one of the environmental pollutants found in sewage. *Serratia marcescens* isolated from soil sample around Universiti Putra Malaysia (3˚00'23.91"N, 101˚42'31.45"E) was found to have cyanide degrading capability. Spectrophotometric method was used to examine the biodegradation ability of the bacteria in free and immobilised forms using cyanide incorporated buffer medium. Factors affecting cyanide biodegradation such as carbon and nitrogen sources, pH of medium, inoculums size, cyanide concentration and temperature were optimised using one factor at time and response surface methods. Cyanide tolerance and effect of heavy metals (silver, arsenic, cadmium, cobalt, chromium, copper, mercury, nickel, lead and zinc) were investigated. The results illustrates that glucose at 5.5 g/L, yeast extract at 0.55 g/L, pH 6, 20% inoculums size, 200 mg/L cyanide concentration and 32.5ºC are the optimum biodegradation conditions required by the bacteria. Immobilised form of the bacteria showed better biodegradation in terms of duration as it degrades the cyanide in 24 hours compared to free cells that require 72 hours degradation process. The bacteria can tolerate 700 mg/L cyanide concentration in free cells and 900 mg/L in immobilised forms. Heavy metals tested at 1 ppm illustrates that the bacteria could stand their effect with the exception of mercury, which degraded only 24.7% in free cells and 61.6% in immobilised forms. Enzyme activity assay illustrates that the bacteria follow the hydrolytic pathway catalysed by cyanide dihydratase to degrade the cyanide. The purified enzyme was able to detoxify 82% of 2 mM potassium cyanide in 10 min of incubation and the rate of cyanide depletion improved linearly as the enzyme concentration is increased. Hydrolysis of cyanide by the purified enzyme fits Michaelis-Menten saturation kinetics when examined over cyanide concentration of 5 mM potassium cyanide. Lineweaver-Burk plot revealed a linear response at 5 mM KCN and less. Michaelis-Menten constant (K_m) for best-fit values of 26.52 and V_{max} value of 1.13 and R^2 value of 0.9 were determined. Total enzyme activity for crude extract stands at 79.9 and 49, 880 mg/L total protein. After final purification process, the total enzyme activity stands at 0.165 with a total protein of 52 mg/L demonstrating yield of 0.207% and purification fold of 65.78. Effect of pH and temperature revealed that enzyme activity was most active at pH of 8 and

temperature of 27ºC. The temperature stability test carried out on the enzyme illustrated that it was stable for 70 days at – 20ºC and when stored at 4ºC, the stability starts reducing after 4 days of incubation. Furthermore, SDS-PAGE electrophoresis post purification revealed the molecular weight of the enzyme to be ~38 kDa, which is a further affirmation. *Serratia marcescens* isolate AQ07 was observed to have the ability to degrade cyanide. Suitable growth and biodegradation conditions were obtained using the optimisation methods. It demonstrates that immobilised cells of the bacteria have a greater ability for cyanide biodegradation compared to free cells, which can be applied for cyanide treatment in sewage. It has been registered in the gene bank as isolate AQ07 with assigned accession number KP213291

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

BIOPENURUNAN SIANIDA OLEH SIANIDA DIHIDRATASE DARIPADA PEMENCILAN TEMPATAN *Serratia marcescens* PENCILAN AQ07

Oleh

KARAMBA KABIRU IBRAHIM

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Pengerusi : Siti Aqlima Ahmad, PhD
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Sianida adalah bahan kimia yang sangat toksik dan adalah salah satu daripada pencemaran alam sekitar yang dijumpai di dalam kumbahan. *Serratia marcescens* dipencilkan daripada sampel tanah di Universiti Putra Malaysia (3˚00’23.91"N, 101˚42’31.45"E) didapati mempunyai keupayaan menurunkan sianida. Kaedah spektrofotometri telah digunakan untuk mengkaji keupayaan biopenurunan oleh bakteria dalam bentuk yang bebas dan sekat gerak menggunakan sianida yang dicampar dengan media penimbal. Faktor yang mempengaruhi biopenurunan sianida seperti sumber karbon dan nitrogen, medium pH, saiz inokulum, kepekatan sianida dan suhu telah dioptimumkan menggunakan satu faktor pada masa dan kaedah gerak balas permukaan. Toleransi sianida dan kesan logam berat (perak, arsenik, kadmium, kobalt, kromium, tembaga, merkuri, nikel, plumbum dan zink) telah dikaji. Keputusan menunjukkan bahawa glukosa pada 5.5 g/L, ekstrak yis pada 0.55 g/L, pH 6, 20% saiz inokulum, 200 mg/L kepekatan sianida dan 32.5°C adalah diperlukan bagi biopenurunan optima oleh bakteria. Bentuk sekat gerak bakteria menunjukkan biopenurunan lebih baik dari segi jangka masa kerana ia menurunkan sianida dalam tempoh 24 jam berbanding dengan sel-sel bebas yang memerlukan 72 jam proses penurunan. Bakteria boleh bertoleransi dengan 700 mg/L kepekatan sianida dalam sel bebas dan 900 mg/L dalam bentuk sekat gerak. Logam berat diuji pada 1 ppm menggambarkan bahawa bakteria boleh tahan kesannya kecuali merkuri, yang menurunkan hanya 24.7% dalam sel bebas dan 61.6% dalam bentuk sekat gerak. Aktiviti enzim asai menunjukkan bahawa bakteria mengikuti laluan hidrolitik menjadi pemangkin oleh sianida hidratase untuk menurunkan sianida. Enzim tulen dapat menyahtoksik 82% daripada 2 mM kalium sianida dalam 10 min masa pengeraman dan kadar pengurangan sianida meningkat secara linear sebagai kepekatan enzim bertambah. Hidrolisis sianida oleh enzim tulen sepadan kinetik tepu Michaelis-Menten apabila diperiksa atas kepekatan sianida pada 5 mM kalium sianida. Plot Lineweaver-Burk mendedahkan tindak balas linear pada 5 mM KCN dan kurang. Pemalar Michaelis-Menten (Km) untuk nilai-nilai terbaik patut 26.52 da n nilai Vmax 1.13 dan nilai R2 0.9 telah ditentukan. Jumlah aktiviti enzim untuk ekstrak mentah berada pada 79.9 dan 49, 880 jumlah protein. Selepas proses penulenan berakhir, aktiviti enzim jumlah mencecah 0.165 dengan jumlah protein 52

mg/L menunjukkan hasil 0.207% dan pembersihan kali ganda 65.78. Kesan pH dan suhu menunjukkan bahawa aktiviti enzim adalah yang paling aktif pada pH 8 dan suhu 27ºC. Ujian kestabilan suhu dijalankan ke atas enzim menunjukkan bahawa ia adalah stabil selama 70 hari di -20ºC dan apabila disimpan pada 4ºC, kestabilan mula mengurangkan selepas 4 hari pengeraman. Tambahan pula, SDS-PAGE elektroforesis penulinan menunjukkan berat molekul enzim menjadi ~38 kDa, yang merupakan ikrar selanjutnya. *Serratia marcescens* pencilan AQ07 telah diperhatikan mempunyai keupayaan untuk menurunkan sianida. Keadaan pertumbuhan dan biopuraian yang sesuai telah diperolehi dengan menggunakan kaedah pengoptimuman. Ia menunjukkan bahawa sel sekat gerak daripada bakteria mempunyai kemampuan yang lebih besar untuk biopenurunan sianida berbanding sel bebas, yang boleh digunakan untuk rawatan sianida dalam kumbahan. Ia telah didaftarkan di GenBank sebagai pencilan AQ07 dengan nombor kesertaan diberikan KP213291.

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Ma shaa Allah, Alhamdulillah

I certify that a Thesis Examination Committee has met on 03 June 2016 to conduct the final examination of Karamba Kabiru Ibrahim on his thesis entitled "Biodegradation of Cyanide by Cyanide Dihydratase from Locally Isolated *Serratia marcescens* Isolate AQ07" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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$(NH4)_2SO_4$	Ammonium sulphate
>	Greater than
%	Percent
<	Less than
µL	Microliter
^oC	Degrees Celsius
µM	Micro molar
As	Arsenic
Ag	Argentum
ATP	Adenosine triphosphate
CFU	Colony Forming Unit
Cd	Cadmium
cm	Centimetre
Cr	Chromium
Co	Cobalt
Cu	Copper
dH_2O	Distilled water
DEAE	Diethylaminoethylamine
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
Fe	Iron
et al	and friends
G	gram
Hg	Mercury

HCL	Hydrogen chloride
HPLC	High Performance Liquid Chromatography
hrs	Hours
Kb	Kilobase
kDa	Kilodaltons
KCN	Potassium Cyanide
Kg	Kilogram
L	Litre
Km	Michaelis-Menten constant
M	Meter
mA	Milliampere
M	Molar
mg	Milligram
mAu	Milli Absorbance Unit
$MgCl_2$	Magnesium Chloride
Min	Minutes
$MgSO_4$	Magnesium Sulphate
mM	Millimolar
MSM	Mineral Salt Medium
MW	Molecular Weight
K_2HPO_4	di-Potassium Hydrogen Phosphate
NA	Nutrient Agar
KH_2PO_4	Potassium dihydrogen Phosphate
NaCl	Sodium Chloride
NAD^+	Nicotinamide Adenine-dinucleotide oxidized form

NADH	Nicotinamide Adenine-dinucleotide reduced form
Ni	Nickel
OD	Optical Density
Nm	Nanometer
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
Pb	Lead
pH	Log concentration of H^+ ion (*Puissance hydrogen*)
PO_4^{3-}	Phosphate
ppm	Parts Per Million
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
TBE	Tri-borate EDTA
Taq	Thermus Aquaticus
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
v/v	Volume/Volume
UV	Ultraviolet
V_{max}	Maximum Velocity
Zn	Zinc
w/v	Weight/Volume
ɤ	Gamma
β	Beta
α	Alpha

CHAPTER 1

INTRODUCTION

Free cyanide like Hydrogen Cyanide and CN^- are reflected as the most toxic forms of cyanide because of their great metabolic inhibition capacity (Gurbuz *et al.,* 2009). They are universally found in the environment. Amygdalin found in apricots, fruits, vegetables, seeds, cashew nuts, cherries, bean sprouts, is a normal source of HCN (DOH, 2004). Cyanide ensues as normal metabolite in a wide-range of animals, plants and fungi inspite of its noxiousness. It is reflected as one of the poisonous chemicals worldwide. It virtually affects all living organisms as it disables the respiratory functions of a living cell by resolutely binding to the terminal oxidase (Chen *et al.,* 2008). Contact with deadly dosage of cyanide via ingestion, inhalation or skin adsorption devoid of quick first aid action can be tremendously lethal to human being in just a few minutes (Badugu *et al.,* 2005).

Additionally, cyanide also arises anthropogenically. Utilisation of huge amounts of cyanide in several manufacturing practices like metal plating, polymer synthesis, electroplating, steel tempering, and mining signify the significant sources of possible cyanide effluence in the environment (Ebbs et al., 2010). Moreover, cyanide is utilised in gold extraction and jewellery manufacturing that produce wastes highly contaminated with cyanide. The cyanidation method utilised for gold mining has generated over 20% of worldwide cyanide production (Luque-Almagro *et al.,* 2011). Through the cyanidation course, cyanide leaching produces various complexes of metal cyanide.

Due to industrial actions, cyanide compounds and complexes are released as industrial waste into the environment, whereby a predicted 14 million kg/year of entire cyanide discharge from these industries have been reported (Dash *et al.,* 2009). The wastes from these manufacturing industries commonly contain between 0.01 and 10 ppm of total cyanide. Conversely, these figures can increase up to 10,000 to 30,000 ppm as certain cyanide effluents from discrete processes at metal plating and electroplating finishing plants can be stowed for years. Actually, greater levels of cyanide amounting to 100,000 ppm can be obtained in some industrial wastes in which it surpasses the acceptable standards for release to the environment.

Ozonation, alkaline chlorination, sulphur-based technologies and wet air oxidation are some of the presently obtainable chemical approaches for cyanide containing effluent treatments. The utilisation of expensive and unsafe compounds as depolluting agents attested to be unfavourable (Luque-Almagro *et al.,* 2011). Moreover, total removal of cyanide complexes is not attainable by these methods. The employment of commercially prevailing physical and chemical approaches of cyanide removal inclines to produce noxious spinoffs, which also need appropriate treatment and consequently increase the total cost of wastes treatment. Furthermore, exceptional equipment and upkeep are also needed to employ these techniques. Owing to these

demerits, cyanide bearing sewages from several manufacturing plants are incompletely detoxified to the cyanate stage or in vilest scenarios straight release devoid of treatment so as to reduce operational cost (Dash *et al.*, 2009). Hence, the exploration of a substitute treatment method capable of attaining high detoxification efficacy at subsidised cost is very significant.

On the other hand, biological detoxification can be used to treat cyanide bearing effluents. Biological methods of cyanide detoxification that are less expensive and free of risk have consequently been accepted, predominantly in mining industries where cyanide can be converted to inoffensive end products (Ebbs, 2004). Nevertheless, the detoxification of cyanide harbouring effluents by biological methods has not been broadly investigated, which could be attributable to the noxiousness of cyanide and deficiency of the essential knowledge on bacteriological processes (Khor, 2009). Though most bacteria are very subtle to cyanide, others not only endure it, but even utilise it in selected instances as a source of sustenance. Cyanide forbearance can be instructed in two ways, which are by the production of cyanide resilient cytochrome oxidases and by the metabolism of cyanide to harmless end-products (Kunz, 2004).

With fast proliferations of many manufacturing plants that regularly use cyanide, cost-effective and effective degradation methodologies are necessary. Constant researches for assessing the degrading capacities of the new microbes for different cyanide compounds from effluents have to be mutually conducted, in the laboratory and in real scale. As a result, the assortment of microbes has to be based on their capacity to degrade cyanide and also to endure the supplementary pressures, like the thrilling environmental conditions such as higher or lower pH and toxicity effect of other contaminants. The microbes must be capable of competing with native microbial inhabitants efficiently in the environment wherein they will be operative (Dash *et al.*, 2009). Primarily, biological detoxification method has to be established and improvised so as to surpass present technologies with added value benefits as well as filling particular essentials related to the treatment of industrial waste waters.

A broad variety of microbes has been identified to break down the extremely noxious cyanide and therefore established the cyanide metabolic degradation pathways, which have been applied in manufacturing plants for the previous 40 years (Hong, 2006). Several previous efforts in designing a biological method for the degradation of cyanide have focused on cyanide-degrading moulds such as *Trichoderma* and *Fusarium* and quite a few reports have been also reported on the utilisation of bacterial strains like *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus* and *Alcalegens*.

The utilisation of bacteria inter alia appeared to be the best acknowledged and best viable method of biological cyanide treatment. Strains of bacteria are capable of adapting and growing in the cyanide bearing medium either by inducing degradation enzymes or by the inducing of cyanide resisting enzymes (Naveen *et al.*, 2011). Meanwhile, the use of bacterial strains demonstrates to be achievable for practical application in degrading cyanide containing effluent, thus the necessity to explore

extra cyanide detoxification microbes is of great importance. Nevertheless, no additional study on the bacteria was conducted in relation to the removal capacity of its immobilised form or other environmental conditions that could affect the degradation or the degradation pathways that bacteria utilise in the removal process. The dearth of thorough valuation on these bacteria encouraged the present study to deliver a better all-inclusive account on the series of actions that could result to the cyanide degradation. Furthermore, both one factor at a time and response method methodology were employed as the optimisation methods to bring out more detailed options required by the bacteria in the removal of cyanide.

This study aims to bring out a biological methodology for degradation of cyanide by means of bacterial isolate. Bacteria were anticipated to induce cyanide detoxification enzymes or to develop cyanide-resistant enzymes as well as to allow them to adapt and proliferate in the cyanide bearing medium.

The specific objectives of this study are:

1. To isolate, screen and identify cyanide degrading bacteria from soil.
2. To optimise cyanide degradation condition using one factor at a time (OFAT) approach and response surface methodology (RSM) by free and immobilised cells.
3. To determine the effect of cyanide concentration and heavy metals on biodegradation of cyanide by free and immobilised cells.
4. To purify the enzyme responsible for the degradation of cyanide
5. To examine the capacity and kinetic properties of cyanide degradation enzymes

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