UNIVERSITI PUTRA MALAYSIA

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NENI GUSMANIZAR

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By

NENI GUSMANIZAR

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

November 2006

DEDICATION

Dedicated to my parents, especially to my husband and son who are always giving me encouragement and support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PURIFICATION AND CHARACTERIZATION OF ACRYLAMIDE-DEGRADING ENZYME FROM *Burkholderia* sp. DR.Y27

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Chairman : Mohd. Yunus bin Abd. Shukor, PhD

Faculty : Biotechnology and Biomolecular Sciences

Acrylamide is a toxic and carcinogenic compound. There are many sources of acrylamide pollution in soil. Three major documented sources are polyacrylamide used liberally as a flocculating agent in water treatment, acrylamide waste from acrylic industries, and the other is from the formulation in the herbicide glyphosate. It has been documented that approximately 0.1% of polyacrylamide is degraded yearly to the carcinogenic acrylamide in soil by soil bacteria. Some of the acrylamide is used as carbon and nitrogen sources by soil bacteria whilst it is suggested that the remaining becomes a source of contamination in vegetables and potatoes. Understanding acrylamide degradation in soil is vital not only to the microbiological point of view, but the prospect of lowering acrylamide

concentrations via bioremediation would lower the potential of acrylamide as a pollutant and contaminant. Several local bacteria have been isolated from glyphosate-contaminated soils at various locations throughout Malaysia. Out of these isolates we have singled out a potent acrylamide-degrading bacterium, which could be potentially used in the bioremediation of acrylamide. Quantitative degradation of acrylamide was performed using High Performance Liquid Chromatography (HPLC), whilst bacterial growth was carried out by plate counting. Isolate 2.7 could degrade 99.84% of 100 mg/L acrylamide as the sole nitrogen source after 48 hours of incubation. Isolate 2.7 was identified as *Burkholderia* sp. Strain DR.Y27 using 16S rRNA and Biolog™ microbial identification system. *Burkholderia* sp. Strain DR.Y27 showed an optimum temperature for growth at 30°C, and optimum initial pH medium for bacterial growth at pH 7.5. *Burkholderia* sp. strain DR.Y27 showed maximum growth in medium containing 1 % glucose and when 500 mg/L acrylamide was provided. The acrylamide-degrading enzyme, amidase, from this bacterium was stable at pH 8 when stored at 4 and -20 °C. Amidase activity was not affected by 1 mM of all metal ions tested, such as WO_4^{2-}, $L1^{2+}$, Fe^{2+}, As^{4+}, Ni^{2+}, Se^{2+}, Zn^{2+}, Cs^{2+}, Cr^{2+}, Al^{3+}, Mn^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, Ag^{2+}, Hg^{2+}, the enzyme activity also was not affected by EDTA, β-Merchaptoethanol and DTT. The maximum velocity in the order of decreasing rates using various substrates were 1.99 ± 0.11 Units/mg protein, 1.50 ± 0.09 Units/mg protein, 1.5 ± 0.02 Units/mg protein, 0.6 ±

0.04 Units/mg protein, 0.48± 0.01 Units/mg protein and 0.34 ± 0.02 Units/mg protein for propionamide, acrylamide, urea, acetamide and 2-cloroacetamide, respectively. The apparent K*m* for these substrates in the order of decreasing affinity are 0.27 ± 0.19 mM, 1.21 ± 0.13 mM, 1.88 ± 0.28 mM, 2.39 ± 1.84 mM and 4.29 ± 0.87 mM for acetamide, 2-cloroacetamide, urea, acrylamide and propionamide, respectively. The amidase from *Burkholderia* sp. strain DR.Y27 could not use metachrylamide and nicotinamide as substrate. The amidase exhibited maximal activity at 40°C and at pH 8.0 of phosphate buffer. The apparent K_m and V_{max} values for amidase were 2.39 ± 1.84 mM mM acrylamide and, 1.50 ± 0.09 µmol min^{-1} mg^{-1} protein, respectively using acrylamide as a substrate. The amidase was purified to homogeneity by a combination of anion exchange and gel filtration chromatography. The purification strategy achieved 11.15 of purification fold and a yield of 1.55%. Pure amidase showed a homogenous protein band with approximate MW of 186 kDa using gel filtration Zorbax^R GF-250 column chromatography. The purified enzyme migrated as a single band in SDS-PAGE in the presence of β-mercaptoethanol with a molecular mass of 47 kDa. It indicates that the native enzyme was a homotetramer.

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

PEMENCILAN, PEMURNIAN DAN SIFAT *Burkholderia* sp. DR.Y27 YANG BOLEH MENGURAIKAN AKRILAMIDA

Oleh

NENI GUSMANIZAR

November 2006

Pengerusi : Mohd. Yunus bin Abd. Shukor, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Akrilamida merupakan satu bahan toksik yabg bersifat karsinogen. Terdapat pelbagai sumber pencemaran akrilamida dalam tanah. Tiga sumber yang telah direkodkan ialah poliakrilamida yang digunakan sebagai agen pengenapan di dalam perawatan air, pembuangan akrilamida dari sisa industri akrilik dan daripada formulasi di dalam herbisid glifosit. Diketahui 0.1% poliakrilamida boleh didegradasikan kepada akrilamida oleh bakteria tanah dalam tempoh satu tahun. Sesetengah akrilamida digunakan sebagai sumber karbon dan nitrogen oleh bakteria tanah, manakala terdapat pendapat yang mengatakan bahawa lebihannya telah menjadi sumber pencemar di dalam sayur dan kentang. Pemahaman degradasi akrilamida dalam tanah bukan sahaja penting dari segi mikrobiologi malah prospek meminimumkan kepekatan

akrilamida sebagai pencemar melalui bioremediasi dapat dijalankan. Beberapa bakteri tempatan yang telah dipencilkan dari pada tanah yang tercemar oleh glifosit di pelbagai lokasi di seluruh Malaysia. Daripada pemencilan ini kita telah mendapati satu pendegradasi akrilamida yang berpotensi untuk digunakan dalam biodegradasi akrilamida. Pemerhatian kuantitatif degradasi akrilamida telah dijalankan dengan dengan menggunakan Kromatografi cecari berprestasi tinggi (HPLC) manakala pemerhatian kuantitatif pertumbuhan bakteria dijalankan dengan pengiraan plat. Isolat 2.7 boleh mendegradasikan 99.4% daripada 100 mg/L akrilamida sebagai satu-satunya sumber nitrogen selepas 48 jam inkubasi. Isolat 2.7 telah dikenal pasti sebagai *Burkholderia* sp. Strain Dr.Y27 dengan menggunakan 16s rRNA molekul filogenetik dan sistem Biolog™. *Burkholderia* sp. Strain Dr.Y27 menunjukan suhu optimum pertumbuhan pada 30˚C dan nilai pH optimum untuk pertumbuhan adalah pH 7.5. *Burkholderia* sp. Strain Dr.Y27 menunjukan pertumbuhan maksimum dalam media yang mengandungi 1% glukosa dan 500 mg/L akrilamida. Enzim pengurai akrilamida iaitu amidase adalah stabil pada pH 8 apabila disimpan pada suhu 4 dan -20˚C. Aktiviti amidase tidak dipengaruhi oleh 1 mM ion logam yang diuji seperti WO_4^{2-}, $L1^{2+}$, Fe^{2+}, As^{4+}, Ni^{2+}, Se^{2+}, Zn^{2+}, Cs^{2+}, Cr^{2+}, Al^{3+}, Mn^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, Ag^{2+}, Hg^{2+}. Enzim aktiviti juga tidak dipengaruhi oleh EDTA, ß-merkaptoetanol dan DTT. Pada kepekatan 5 mM tiada logam berat yang mengakibatkan 50% perencatan oleh amidase berbanding dengan

kawalan. Halaju maksimum dalam susunan menurun menggunakan pelbagai substrat adalah 1.99± 0.11 Units/mg protein, 1.50± 0.09 Units/mg protein, 1.5± 0.02 Units/mg protein, 0.6± 0.04 Units/mg protein, 0.48± 0.01 Units/mg protein and 0.34 ± 0.02 Units/mg protein bagi propionamida, akrilamida, urea, asetamida, dan 2-kloroasetamida. Amidase dari bakterium ini tidak boleh menggunakan metakrilamida dan nikotinamida sebagai substrat. Amidase ini menunjukan aktiviti maksimum pada 40˚C dan pada pH 8 menggunakan penimbal fosfat. Nilai K_m dan V_{max} ketara untuk amidase adalah 2.39± 1.84 mM mM akrilamida and , 1.50± 0.09 µmol min^{-1} mg^{-1} protein. Nilai K_m ketara dalam susunan keafinan merendah adalah 0.27± 0.19 mM, 1.21± 0.13 mM, 1.88± 0.28 mM, 2.39± 1.84 mM and 4.29± 0.87 mM masing-masing untuk asetamida, 2-kloroasetamida, urea, akrilamida dan propionamida. Amidase itu ditulenkan dengan kombinasi kromatogafi penukaran ion dan penurasan gel. Penulenan itu memberikan 11.15 kali penulenan dan hasil sebanyak 1.55%. Berat molekul natif amidase adalah 186 kDa selepas ditentukan menggunakan kromatografi penurasan gel pada turus Zorbax^R GF-250. Enzim ini didapati berada dalam jalur tunggal pada berat molekul 47 kDa pada gel elektroforesis poliakrilamida SDS dengan kehadiran β-merkaptoetanol. Berdasarkan maklumat ini, amidase dari bakterium ini adalah berada dalam bentuk homotetramer.

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I certify that an Examination Committee met on 13th of November 2006 to conduct the final examination of Neni Gusmanizar on her Doctor of Philosophy thesis entitled "Purification and Characterization of Acrylamide-Degrading Enzyme From *Burkholderia* sp. DR.Y27" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination committee are as follows:

Raja Noor Zaliha Raja Abd. Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Foo Hooi Ling, PhD
Associate Professor,
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Maziah Mahmood, PhD
Professor,
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Dato' Mohammed Noor Embi, PhD
Professor,
Chairman of National Biotechnology Division,
Ministry of Science, technology and innovation, Malaysia
Level 3, Block C4, Parcel C
Federal Government Administrative Centre,
62662 Putrajaya
(External Examiner)

HASANAH MOHD GHAZALI, PhD
Professor/ Deputy Dean
School of Graduate studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follow:

Mohd. Yunus bin Abdul Shukor, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd. Arif Syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Johari Ramli, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/ Dean
School of Graduate studies
Universiti Putra Malaysia

Date: 17 July 2007

DECLARATION

I hereby declare that the thesis in based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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NENI GUSMANIZAR

Date : 19 June 2007

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
NA	Nutrient Agar
bp	Base pair
CFU	Colony forming unit
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DTT	Ditiothreithol
EC	European Commission
EC	Enzyme Commission
EDTA	Ethylene diamine tetraacetic acid
HPLC	High Performance Liquid Chromatography
Kb	kilobase
KCl	Potassium Chloride
kDa	kilodalton
K_m	Michaelis-Menten Constant
µl	microlitre
µM	micromolar
M	Molar
mA	milliampere
mAu	mili absorbance unit
mg	miligram

min	minute
mM	milimolar
mL	milliliter
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEEK	Polyether ether ketone
PMSF	Phenylmethylsulfonylfluoride
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sp	species
TCA cycle	Tricarboxylic acid cycle
PAGE	Polyacrylamide gel electrophoresis
UV	Ultraviolet
w/v	weight/ volume
v/v	volume/ volume
V_{max}	Maximum velocity
WHO	World Heath Organization
NMR	Nuclear magnetic resonance

CHAPTER 1

INTRODUCTION

Acrylamide is the building block for the polymer, polyacrylamide (PAM). It is known that commercial polyacrylamide preparations may be contaminated with its toxic monomer, acrylamide. Thus, regulations have been set on the amount of acrylamide that is present in polyacrylamide. For example, a limit of 500 ppm in polyacrylamide preparations is used in agriculture or water treatment. Other uses of acrylamide include as sewage-flocculating agent (Myagchenkov and Proskurina, 2000), stabilising tunnels and dams, and in the industry as adhesives (IPCS, 2003). Thus it is not surprising that one of the known cases of acrylamide toxicity is due to the application of acrylamide for the stabilising of tunnel in Sweden. Acrylamide pumped to surrounding soil had resulted in acrylamide polluting the Hallandas village in Sweden causing death to cows and fish (Franzen *et al*., 2001).

In Malaysia, large amount of polyacrylamide are used yearly for drinking water treatment. For example, in the Sarawak state alone, almost 800 kgs of polyacrylamide additive (Superfloc and Praestol 2530) are used yearly by the Kuching Water Board (Auditor-General's Report, 2005). A direct introduction of acrylamide into agriculture soil comes from glyphosate application in