

การผลิตน้ำตาลแอมิโนจากไคทินของแกนหมึกโดยตัวเร่งปฏิกิริยาชีวภาพจากรา

นางสาว กฤษณา อ้วนนิรันดรกุล

ศูนย์วิทยทรัพยากร

จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์


คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2546

ISBN 974-17-3724-6

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

PRODUCTION OF AMINOSUGAR
FROM SQUID PEN CHITIN BY FUNGAL BIOCATALYST



Miss Krissana Auynirundronkul

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Petrochemistry and Polymer Science

Faculty of Science
Chulalongkorn University

Academic Year 2003


ISBN 974-17-3724-6

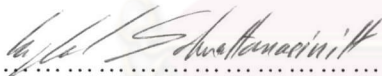
Thesis Title : Production of aminosugar from squid pen chitin by fungal biocatalyst
By : Miss Krissana Auynirundronkul
Field of Study : Petrochemistry and Polymer Science
Thesis Advisor : Assistant Professor Mongkol Sukwattanasinitt, Ph.D.
Thesis Co-advisor : Assistant Professor Hunsa Punapayuk, Ph.D.

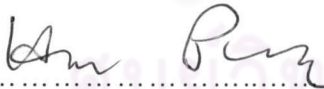
Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

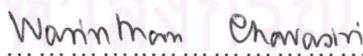

.....Dean, Faculty of Science
(Associate Professor Wanchai Phothiphichitr, Ph.D.)


Thesis Committee


.....Chairman
(Associate Professor Supawan Tantayanon, Ph.D.)


.....Thesis Advisor
(Assistant Professor Mongkol Sukwattanasinitt, Ph.D.)


.....Thesis Co-advisor
(Assistant Professor Hunsa Punapayuk, Ph.D.)


.....Member
(Assistant Professor Warinthorn Chavasiri, Ph.D.)


.....Member
(Rath Pichyangkura, Ph.D.)

กฤษฎณา อุ้ยนิรันดรกุล: การผลิตน้ำตาลเอมิโนจากไคตินของแกนหมึกโดยตัวเร่งปฏิกิริยาชีวภาพจากรา (PRODUCTION OF AMINOSUGAR FROM SQUID PEN CHITIN BY FUNGAL BIOCATALYST) อ.ที่ปรึกษา: ผศ.ดร. มงคล สุขวัฒนาสินิทธิ; อ.ที่ปรึกษาร่วม: ผศ.ดร. หารรษา ปุณณะพยัคฆ์ ; 68 หน้า ISBN 974-17-3724-6

ได้ทดสอบรา 5 สปีชีส์คือ *Aspergillus fumigatus*, *Trichoderma viride*, *Trichoderma aureoviride*, *Trichoderma reesei* และ *Mucor sp.* สำหรับการผลิตเอนไซม์ย่อยไคติน รา *Aspergillus fumigatus* สามารถชักนำให้เกิดเอนไซม์ได้สูงที่สุดโดยให้แอกติวิตีสูงถึง 438 mU/mL เมื่อเลี้ยงที่อุณหภูมิ 40 °C ในคอลถลอยคอลไคติน และมีปริมาณโปรตีนเป็น 1.70 mg/mL ปฏิกริยาไฮโดรไลซิสของเบต้าไคตินโดยเอนไซม์ย่อยไคตินที่ผลิตจากรา *Aspergillus fumigatus* ให้ผลิตภัณฑ์เป็นเอ็น-แอซิทิล-ดี-กลูโคซามีนในเปอร์เซ็นต์สูง (มากกว่า 70 % ใน 1 วัน) อัตราส่วนที่เหมาะสมของเอนไซม์ต่อไคตินคือ 1-4 mU/mg ที่ความเข้มข้นของซับสเตรทเป็น 20 mg/mL ช่วง pH ที่เหมาะสมคือ 3-5 และอุณหภูมิที่เหมาะสมคือ 45 °C ปฏิกริยาไฮโดรไลซิสสามารถทำได้โดยไม่ต้องมีบัฟเฟอร์ซึ่งง่ายต่อการทำให้ผลิตภัณฑ์บริสุทธิ์ในระดับการผลิต

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

หลักสูตร วิทยาศาสตรบัณฑิต สาขาวิชาวิทยาศาสตร์สุขภาพ วิทยาลัยวิทยาศาสตร์และเทคโนโลยีสุขภาพ 2546

ปีการศึกษา 2546

ลายมือชื่ออธิการบดี.....

ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4473411823: MAJOR PETROCHEMISTRY AND POLYMER SCIENCE

KEY WORD: β -CHITIN / CHITINOLYTIC ENZYME / ENZYMATIC HYDROLYSIS

KRISSANA AUYNIRUNDRONKUL: PRODUCTION OF AMINOSUGAR FROM SQUID PEN CHITIN BY FUNGAL BIOCATALYST. THESIS ADVISOR: ASST.PROF.MONGKOL SUKWATTASINITT, Ph.D.; THESIS CO-ADVISOR: ASST.PROF. HUNSA PUNAPAYUK, Ph.D., 68 pp. ISBN 974-17-3724-6.

Five species of fungi, *Aspergillus fumigatus*, *Trichoderma viride*, *Trichoderma aureoviride*, *Trichoderma reesei* and *Mucor sp.* were tested for production of chitinolytic enzymes. *Aspergillus fumigatus* was the most active fungus to be induced for production of chitinolytic enzymes. The ensemble of chitinolytic enzymes were produced from *Aspergillus fumigatus* with the maximum chitinolytic activity of 438 mU/mL at 40 °C in colloidal chitin minimum medium after 9 days of incubation. The enzyme preparation contained 1.70 mg protein per milliliter. The hydrolysis of β - chitin with the chitinolytic enzymes from *Aspergillus fumigatus* gave *N*-acetyl-D-glucosamine (GlcNAc) with high yields (over 70 % in 1 days). The optimum ratio of enzyme to chitin was found to be 1-4 mU/mg with optimum substrate concentration at 20 mg/mL. The optimum pH range was 3-5 and the optimum temperature was 45 °C. The hydrolysis could be carried out without any buffer that would simplify the product purification in a preparative scale.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Field of study Petrochemistry and polymer science Student's signature.....

Academic year 2003.....

Advisor's signature.....

Co-advisor's signature.....

ACKNOWLEDGEMENT

The author wishes to sincerely thank to her advisors, Assistant Professor Mongkol Sukwattanasinitt, and Assistant Professor Hunsa Punapayuk for their advice, help and suggestions the way to be good in study, research and chemistry. Their kind suggestions motivated the author with strength and happiness to do this thesis. Although, this thesis had obstacles, finally it could be completed by their advice.

In addition, the author is grateful to Professor Supawan Tantayanon chairman, and to Assistant Professor Warinthorn Chavasirias, thesis examiners, for all valuable comments regarding this thesis. And Dr. Rath Pichyangura, thesis examiners the author would like to thank for advice, help and kindly suggestion.

Moreover, the author would like to thank M.L. Siripastra Jayanta for her kindness, advice, help, and suggestions.

Special thanks are expanded to Mr. Akamol Klaikherd, Miss. Wasinee Prakobkij and Mr. Khanchai Danmek to succor my thesis. The author also greatest thanks to Scientific and Technological Research Equipment Center of Chulalongkorn University, Chulalongkorn University for giving the opportunity to study, laboratory facilities, chemicals and equipment. Finally, the author would like to thank my parents, my friends, MS group and Plant Biomass Utilization Research Unit members for strong moral support.

This work is part of department of chemistry OECF-JBIT project and financially support by National Metal and Materials Technology Center (MTEC), project code MT-B-46-POL-09-206-G.

CONTENTS

	Page
Abstract in Thai	iv
Abstract in English	v
Acknowledgement	vi
Contents	vii
List of Figures.....	xi
List of Tables	xiii
List of Abbreviations	xiv
CHAPTER I: INTRODUCTION.....	1
1.1 Chitin	1
1.2 The applications of chitin and their derivatives.....	4
1.3 Preparation of <i>N</i> -acetyl-D-glucosamine and chitooligosaccharide	7
1.3.1 Chemical hydrolysis	7
1.3.2 Enzymatic hydrolysis	8
1.4 Enzyme from fungi.....	13
1.5 Aims of thesis	13
CHAPTER II: METERIALS AND METHODS	14
2.1 Instruments and Apparatus	14
2.2 Chemicals	15
2.3 Fugal strains.....	16
2.4 General Procedure	16
2.4.1 Sauborand dextrose agar.....	16
2.4.2 Preparation of Lactose broth.....	16
2.4.3 Squid pen chitin 50 μ m.....	16
2.4.4 Swollen chitin	17
2.4.5 Preparation of colloidal chitin	17
2.4.6 Preparation of colloidal chitin minimum medium.....	17
2.4.7 Crude enzyme preparation	18

CONTENTS (Continued)

	Page
2.5 Protein assaying.....	18
2.5.1 Preparation of calibration curve.....	18
2.5.2 Biuret reagent	19
2.5.3 Preparation of standard calibration curve.....	19
2.5.4 Determination of the protein amount in the enzyme	19
2.6 Reducing sugar	19
2.6.1 Preparation of calibration curve	19
2.6.2 Preparation of standard calibration curve.....	20
2.6.3 Determination of the chitinolytic activity of the enzyme	20
2.7 Factors effecting production of chitinolytic enzyme.....	21
2.7.1 Carbon source	21
2.7.2 Nitrogen source.....	21
2.7.3 Incubation time effect.....	21
2.7.4 Inoculums of fungi.....	22
2.7.5 Trace elements (FeSO ₄ and MnSO ₄).....	22
2.7.6 Quantity of carbon source.....	22
2.7.7 Quantity of carbon source and inoculums of fungi	23
2.7.8 Optimum temperature.....	23
2.8 Characterization of chitinolytic enzyme.....	23
2.8.1 Optimum pH for hydrolysis chitin.....	23
2.8.2 Optimum temperature for hydrolysis chitin	24
2.8.3 The chitinolytic enzyme on different substrates.....	24
2.8.4 Optimum enzyme/substrate ratio for hydrolysis chitin	25
2.9 Characterization of chitinolytic enzyme by HPLC	25
2.9.1 Optimum concentration of McIlvaine buffer for chitin hydrolysis.....	25
2.9.2 Optimum substrate concentration for hydrolysis chitin	26
2.9.3 The fed-batch technique	27
2.9.4 Changing preservative from NaN ₃ to toluene.....	27
2.9.5 Comparison of fibrous chitin with swollen chitin	28
2.10 Product analysis by HPLC.....	29
2.10.1 Preparation of calibration curve	29

CONTENTS (Continued)

	Page
• <i>N</i> -acetyl-D-glucosamine.....	29
• <i>N,N'</i> -diacetylchitobiose.....	30
• <i>N,N',N''</i> -triacetylchitotriose.....	30
2.10.2 Analysis of products in hydrolysates.....	31
CHAPTER III: RESULTS AND DISCUSSION	32
3.1 Factors effecting the production of chitinolytic enzyme.	32
3.1.1 Carbon source	32
3.1.2 Nitrogen source.....	34
3.1.3 Incubation time	35
3.1.4 Inoculums of <i>Aspergillus fumigatus</i>	36
3.1.5 Trace elements (FeSO ₄ and MnSO ₄)	37
3.1.6 Amounts of colloidal chitin	38
3.1.7 Initial amounts of colloidal chitin and fungal inoculums.	39
3.1.8 Temperature.....	40
3.2 Study hydrolytic activity of the ensemble of chitinolytic enzymes from <i>Aspergillus fumigatus</i> by enzyme assaying with modified Schale's method.....	41
3.2.1 pH dependence	41
3.2.2 Temperature dependence.....	42
3.2.3 The chitinolytic susceptibility of different substrates.....	43
3.2.4 Enzyme/substrate ratio	44
3.3 Study for optimum condition toward a preparative of GlcNAc using HPLC	45
3.2.5 Effect of buffer concentration.....	45
3.2.6 Effect of substrate concentration	46
3.2.7 Fed-batch technique of fibrous chitin.....	47
3.2.8 Preservatives.....	48
3.2.9 Comparison of fibrous chitin versus swollen chitin	49
CHAPTER IV: CONCLUSION	51
Conclusion.....	51

CONTENTS (Continued)

	Page
REFERENCES	52
APPENDICES	57
APPENDIX A.....	58
APPENDIX B.....	64
VITAE.....	68



ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figures	Page
1.1 Structure of (a) cellulose, (b) chitin, and (c) chitosan	1
1.2 Diagrammatic illustration with arrangement as antipararell and X-ray crystal structure of hydrogen bond linkage between to C=O...NH group of α -chitin.	3
1.3 Diagrammatic illustration with arrangement as antipararell and X-ray crystal structure of hydrogen bond linkage between to C=O...NH group of β -chitin.	3
1.4 simplified flowsheet for preparation of chitin and chitosan, their monomer and oligomers from invertebrate marine's waste	4
1.5 Acid hydrolysis of chitin	7
1.6 Pathway for conversion of chitin and chitosan into their oligomer by enzyme means	8
1.7 Preparation the (GlcNAc) ₂ by enzymatic transglycosylation.....	9
1.8 The action of chitinase on chitin and it product	11
1.9 The action of chitobiase, on chitin and product	12
1.10 The action of chitosanase, on chitin and product	12
2.1 The squid pen chitin (a) flake chitin, (b) fibrous β -chitin (25 × 50 μ m).....	17
3.1 The effect of carbon source on chitinolytic enzyme production from <i>Asprgillus fumigatus</i> , <i>Trichoderma viride</i> and <i>Trichoderma aureoviride</i>	33
3.2 The effect of the nitrogen sources on chitinolytic enzyme production from <i>Asprgillus fumigatus</i> , <i>Trichoderma viride</i> , <i>Trichoderma aureoviride</i> , <i>Trichoderma reesei</i> and <i>Mucor sp.</i>	34
3.3 The time course of the chitinolytic activity obtained from cultivation of <i>Aspergillus fumigatus</i>	35
3.4 The effect of inoculums pieces of <i>Aspergillus fumigatus</i> on chitinolytic enzyme production.....	36
3.5 The effect of trace elements (FeSO ₄ and MnSO ₄) on the chitinolytic enzyme production from <i>Aspergillusfumigatus</i>	37

CONTENTS (Continued)

Figures	Page
3.6 Amount of colloidal chitin on chitinolytic enzyme from <i>Aspergillus fumigatus</i>	38
3.7 Amount of colloidal chitin and amount of <i>Aspergillus fumigatus</i> on chitinolytic enzyme production.	39
3.8 Temperature for culturing <i>Aspergillus fumigatus</i> on chitinolytic enzyme production.	40
3.9 pH dependence of the ensemble of the chitinolytic enzyme from <i>Aspergillus fumigatus</i>	41
3.10 Temperature dependence of the activity of the ensemble of chitinolytic enzymes from <i>Aspergillus fumigatus</i>	42
3.11 The substrate dependence of the hydrolytic activity of the ensemble of chitinolytic enzymes from <i>Aspergillus fumigatus</i>	43
3.12 The effect of enzyme/chitin ratio on the chitinolysis.	44
3.13 The effect of concentration of buffer on the chitinolysis activity of the ensembles of enzyme from <i>Aspergillus fumigatus</i>	45
3.14 Effect of chitin concentration on the chitinolytic activity of enzyme from <i>Aspergillus fumigatus</i>	46
3.15 Yield of GlcNAc from fed -batch technique of fibrous chitin a single batch experiment.....	47
3.16 Hydrolytic profiles different preservative.....	48
3.17 Hydrolytic profiles for swollen and fibrous chitin.....	50

LIST OF TABLES

Tables	Page
1.1 Application of chitin, chitosan, their monomers and oligomers.....	6
2.1 Preparation of BSA standard solution.....	18
2.2 GlcNAc used and concentration of standard solution for enzyme assaying...20	20
2.3 Amount of colloidal chitin (g) added during incubation in shake flask.....	22
2.4 The amounts of reagents used in reaction in the study for optimum pH.....	23
2.5 The amounts of reagents used in reaction for optimum temperature.....	24
2.6 The amounts of reagents used in reaction in the study for type of substrates...24	24
2.7 The amounts of reagents used in reaction in the study enzyme/substrates.....	25
2.8 The concentration of buffer used in the reaction for finding the optimum concentration of McIlvaine buffer suitability.....	26
2.9 The amounts of reagents used in the reaction substrate suitability.....	26
2.10 The amount of reagents used in the reaction for fed-batch technique.....	27
2.11 Comparative preservation of NaN_3 and toluene.....	27
2.12 The amounts of reagents used in reaction.....	28
2.13 Preparation of GlcNAc standard solution.....	29
2.14 Preparation of $(\text{GlcNAc})_2$ standard solution.....	30
2.15 Preparation of $(\text{GlcNAc})_3$ standard solution.....	31

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

List of Abbreviations

α	alpha	GlcN	D-glucosamine
β	beta	HPLC	High Performance
$^{\circ}\text{C}$	degree celsius		Liquid Chromatography
cm	centimeter	mg	milligram
DI-water	deionized water	M	Molar
γ	gramma	min	minute
g	gram (s)	mL	milliliter
GlcNAc	<i>N</i> -acetyl-D-glucosamine	mM	millimolar
(GlcNAc) ₂	<i>N,N'</i> -diacetylchitobiose	mU	milliunit
(GlcNAc) ₃	<i>N,N',N''</i> -triacetyl- chitotriose	ppm	part per million
(GlcNAc) ₄	<i>N,N',N'',N'''</i> -tetraacetyl- chitotetrose	U	unit
		μL	microliter
		%	percent

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย