PREPARATION AND CHARACTERIZATION OF CELLULOSE WHISKERS - REINFORCED SILK FIBROIN SPONGE FOR YEAST IMMOBILIZATION FOR ETHANOL PRODUCTION

Vasithee Punyavardhana

A Thesis Submitted in Partial Fulfilment of the Requirements
for the Degree of Master of Science
The Petroleum and Petrochemical College, Chulalongkorn University
in Academic Partnership with
The University of Michigan, The University of Oklahoma,
and Case Western Reserve University
2012

Thesis Title:	Preparation and Characterization of Cellulose Whiskers-Reinforced Silk Fibroin Sponge for Yeast Immobilization for Ethanol Production
By:	Vasithee Punyavardhana
Program:	Polymer Science
Thesis Advisors:	Assoc. Prof. Ratana Rujiravanit
	Asst. Prof. Manit Nithitanakul

Accepted by the Petroleum and Petrochemical College, Chulalongkorn University, in partial fulfilment of the requirements for the Degree of Master of Science.

..... College Dean

(Asst. Prof. Pomthong Malakul)

Thesis Committee:

Ratana Rujiravanit

(Assoc. Prof. Ratana Rujiravanit)

.....

(Asst. Prof. Manit Nithitanakul)

Sumaeth Chavadej

(Prof. Sumaeth Chavadej)

.....

(Dr. Orathai Pornsunthorntawee)

ABSTRACT

5372032063: Polymer Science Program
Vasithee Punyavardhana: Preparation and Characterization of Cellulose Whiskers- Reinforced Silk Fibroin Sponge for Yeast Immobilization for Ethanol Production
Thesis Advisors: Assoc. Prof. Ratana Rujiravanit and Asst. Prof. Manit Nithitanakul 131 pp
Keywords: Silk fibroin/ Cellulose whisker/ Yeast immobilization/ Ethanol

Silk fibroin (SF) is a natural protein produced by the mulberry silkworm (*Bombyx mori*). It was chosen as a matrix in bionanocomposite sponges for yeast cell immobilization. Cellulose whiskers (CLWs), having an aspect ratio of 80, were used as reinforcements. The bionanocomposite sponges at SF/CLWs weight ratios of 100/0, 90/10, 80/20, 70/30, 60/40 and 50/50 were fabricated by using a freeze-drying technique before being treated with an aqueous methanol solution. At any studied SF/CLWs weight ratios, Fourier Transform Infrared Spectrophotometer spectra indicated beta-sheet conformation of SF after the methanol treatment. The presence of CLWs not only increased the compression modulus but also reduced the shrinkage of the bionanocomposite sponges as well as enhanced the conformation transition of SF. The formation of beta-sheet structure of SF significantly increased water stability of the bionanocomposite sponges. The Field Emission Scanning Electron micrographs showed that the bionanocomposite sponges exhibited an interconnected porous structure, providing high surface area for immobilizing *Saccharomyces cerevisiae burgundy* KY11 yeast cells. The sponge with the SF/CLWs weight ratio of 50/50 showed the highest average number of yeast cell attachment at 3.1×10^{10} cells/g sponge. The maximun ethanol production of immobilized yeast cell was 65% higher when compared with free yeast cell fermentation system.

บทคัดย่อ

วาสิฐี บุณยวรรธนะ : การเตรียม และการวิเคราะห์คุณสมบัติของวัสดุรูพรุนจากโปรตีนไฟโบรอินจากรังไหมที่เสริมแรงด้วยเซลลูโลสวิสเกอร์ เพื่อใช้ในการตรึงเซลล์ยีสต์สำหรับการผลิตเอทานอล (Preparation and Characterization of Cellulose Whiskers- Reinforced Silk Fibroin Sponge for Yeast Immobilization for Ethanol Production) อ. ที่ปรึกษา : รศ. ดร. รัตนา รุจิวานิช และ ผศ. ดร. มานิตย์ นิธิธนากุล 131 หน้า

ในปัจจุบัน พลังงานทดแทนจากไบโอเอทานอลได้มีบทบาทมากขึ้นเพื่อบรรเทาปัญหาการขาดแคลนพลังงานฟอสซิล การผลิตไบโอเอทานอลสามารถทำได้โดยใช้จุลินทรีย์ คือ ยีสต์ ในกระบวนการหมักทางชีวภาพ งานวิจัยนี้มีจุดประสงค์ที่จะพัฒนากระบวนการหมักโดยใช้เทคนิคการตรึงเซลล์ยีสต์เพื่อเพิ่มอัตราผลิตเอทานอล โดยสังเคราะห์วัสดุรูพรุนจากโปรตีนไฟโบรอินซึ่งสกัดจากรังไหมของตัวไหม *บอมบิกซ์ โมริ* ซึ่งโครงสร้างมีความเป็นระเบียบ มีโครงสร้างทุติยภูมิเป็นแผ่นพับเบต้าแบบสวนขนาน ส่งผลให้สามารถคงรูปได้ในน้ำ อีกทั้งยังมีความเข้ากันได้กับเซลล์และถูกนำมาใช้เป็นวัสดุทางชีวการแพทย์อย่างหลากหลาย เสริมแรงวัสดุรูพรุนด้วยเซลลูโลสวิสเกอร์ซึ่งสกัดจากเครือกล้วยน้ำว้า เพื่อเพิ่มความแข็งแรงของวัสดุ ทำการขึ้นรูปวัสดุโดยการทำให้แห้งภายใต้สภาวะสูญญากาศ ซึ่งพบว่าสัณฐานของวัสดุมีความเป็นรูพรุนที่ต่อเนื่อง และปริมาณของเซลลูโลสวิสเกอร์ที่เหมาะสมโดยน้ำหนักของวัสดุรูพรุน คือ 50% วัสดุที่ได้มีความสามารถในการคงรูปได้ภายใต้สภาวะการหมัก และมีขนาดของรูที่เหมาะสม ทำให้ตรึงเซลล์ยีสต์ได้ 3.1×10^{10} เซลล์ ต่อกรัมของวัสดุรูพรุน ซึ่งแสดงให้เห็นถึงประสิทธิภาพในการใช้เป็นวัสดุตรึงเซลล์ ผลผลิตเอทานอลที่ได้จากการหมักโดยใช้การตรึงเซลล์ในวัสดุรูพรุนเพิ่มขึ้น 65% โดยน้ำหนักเมื่อเทียบกับการหมักโดยใช้เซลล์ยีสต์อิสระ นอกจากนี้การตรึงเซลล์ยีสต์ในวัสดุรูพรุนยังช่วยลดผลของการยับยั้งการผลิตเอทานอลเนื่องจากสารตั้งต้นหรือผลิตภัณฑ์ที่มีความเข้มข้นสูง ซึ่งทำให้เซลล์ยีสต์ตายได้ และยังสามารถนำวัสดุรูพรุนตรึงเซลล์ยีสต์กลับมาใช้ซ้ำในกระบวนการหมัก โดยมีปริมาณผลผลิตที่คงที่

ACKNOWLEDGEMENTS

First of all, the author wishes to express her gratefulness to her advisors— Assoc. Prof. Ratana Rujiravanit and Asst. Prof. Manit Nithitanakul — for providing useful recommendations, creative comment, and great encouragement thoughout this thesis. The author would like to thank Prof. Sumaeth Chavadej and Dr. Orathai Pornsunthorntawee for being her thesis committees, and for giving valuable comments and suggestions.

This thesis work is funded by the Petroleum and Petrochemical College, and by the Center of Excellence on Petrochemicals and Materials Technology, Thailand.

The author would like to thank the PPC where the author have gained the precious knowledge in the Polymer Science program and the author would like to thank all faculty and staff members at the PPC for their kindnesses and useful suggestions. The author would like to thank PPC Ph.D. students, RR group, and all her PPC friends for their friendly assistance, cheerfulness, and great encouragement.

Finally, the author would like to thank her family for their love, understanding and supports during her study at the PPC.

TABLE OF CONTENTS

		PAGE
Title Page		i
Abstract (in English)		iii
Abstract (in Thai)		iv
Acknowledgements		v
Table of Contents		vi
List of Tables		x
List of Figures		xi
CHAPTER		
I	**INTRODUCTION**	1
II	**LITERATURE REVIEW**	3
	2.1 Ethanol	3
	2.1.1 Production of Ethanol	4
	2.2 Fermentation	5
	2.2.1 Ethanol Fermentation	5
	2.3 Cell immobilization	7
	2.3.1 Adsorption to a Pre-formed Carrier	8
	2.3.2 Cell Entrapment	8
	2.3.3 Self-aggregation	9
	2.3.4 Containment of Cells Behind Carrier	10
	2.4 Silk Fibroin (SF)	13
	2.4.1 SF as a Biomaterial	16
	2.4.2 Structure of SF	18
	2.4.2.1 Primary Structure	18
	2.4.2.2 Secondary Structure	19

CHAPTER PAGE

2.5 Cellulose (CL) 21

2.5.1 Cellulose Whiskers (CLWs) 22

III METHODOLOGY 28

3.1 Materials 28

3.1.1 Silk Cocoons and Banana Rachises 28

3.1.2 Yeast Cells 28

3.1.3 Other Chemicals 28

3.2 Experimental Methods 28

3.2.1 Preparation of CLWs 28

3.2.2 Preparation of SF Solution 29

3.2.3 Preparation of CLWs-reinforced SF Sponges 29

3.2.4 Methanol Treatment of CLWs-reinforced SF Sponges 29

3.2.5 Inoculums Preparation 30

3.2.6 Cell Immobilization 30

3.2.7 Vitalization of Yeast Cell Immobilized in Bionanocomposite Sponges 30

3.2.8 Fermentation 30

3.3 Characterizations and Testing 31

3.3.1 Fourier Transformed Infrared (FTIR) Spectroscopy 31

3.3.2 Field Emission Scanning Electron Microscopy (FE-SEM) 31

3.3.3 Transmission Electron Microscopy (TEM) 31

3.3.4 Shrinkage of Bionanocomposite Sponges 31

3.3.5 Lloyd Instrumental 31

3.3.6 Weight Loss 32

3.3.7 Ultraviolet-visible (UV-vis) Spectroscopy 32

3.3.8 Gas Chromatography (GC) 32

3.3.9 Optical Microscopy (OM) 33

CHAPTER PAGE

IV PREPARATION AND CHARACTERIZATION OF CELLULOSE WHISKER- REINFORCED SILK FIBROIN SPONGE FOR YEAST IMMOBILIZATION FOR ETHANOL PRODUCTION 34

4.1 Abstract 34

4.2 Introduction 35

4.3 Experimental 36

4.4 Results and Discussion 43

5.5 Conclusions 70

5.6 Acknowledgements 71

5.7 References 71

V CONCLUSIONS 76

REFERENCES 77

APPENDICES

APPENDIX A Weight loss behaviour of bionanocomposite sponges 82

APPENDIX B Shrinkage of bionanocomposite sponges After treated with methanol 88

APPENDIX C Yeast cell in bionanocomposite sponges And yeast cell leakage 92

APPENDIX D Cell leakage of methanol-treated bionanocomposite sponges after immersed in distilled water with 150 rpm shaking at 30°C for 48 hours 95

CHAPTER **PAGE**

APPENDIX E	Growth curve of *S. cerevisiae burgundy* KY11	99
APPENDIX F	Compression modulus of methanol- treated bionanocomposite sponges at different SF/CLWs weight ratio	101
APPENDIX G	Determination of ethanol concentration in fermentation by using free cell, immobilized cell and crosslinked cell by using glucose concentration of 10-40% (w/v)	102
APPENDIX H	Determination of reducing sugar by dinitrosalicylic (DNS) colorimetric method	116
APPENDIX I	Immobilization efficiency between immobilized cell and glyoxal crosslink- immobilized cell	122
APPENDIX J	Ethanol production in 5 cycle repeated batch	127

CURRICULUM VITAE 131

LIST OF TABLES

TABLE		PAGE
2.1	Composition of silk produced by *Bombyx mori* silkworm	14
4.1	Production yield of silk fibroin solution	44
4.2	Production yield of CLWs suspension	45
4.3	The main functional group of CLWs and BC	47
4.4	Average pore size of bionanocomposite sponges	48
4.5	Macromolecules of cell wall of *S. cerevisiae*	62
4.6	The immobilized efficiency, glucose consumption (Y_s), ethanol production (Y_p), ethanol yield ($Y_{p/s}$) in ethanol production used 10% (w/v) glucose as substrate	63
4.7	The immobilized efficiency, glucose consumption (Y_s), ethanol production (Y_p), ethanol yield ($Y_{p/s}$) in ethanol production used 20% (w/v) glucose as substrate	63
4.8	The immobilized efficiency, glucose consumption (Y_s), ethanol production (Y_p), ethanol yield ($Y_{p/s}$) in ethanol production used 30% (w/v) glucose as substrate	64
4.9	The immobilized efficiency, glucose consumption (Y_s), ethanol production (Y_p), ethanol yield ($Y_{p/s}$) in ethanol production used 40% (w/v) glucose as substrate	64
4.10	The conversion of glucose into ethanol (%)	67
4.11	The sugar consumption (Y_s), ethanol production (P), ethanol yield ($Y_{p/s}$) and conversion of glucose into ethanol of five repeated batches fermentation by using free cell and immobilized cell.	69

LIST OF FIGURES

FIGURE		PAGE
2.1	Growth rate of ethanol (million liters) between 1975 and 2010, according to an industry	4
2.2	Ethanol fermentation pathway	6
2.3	Various processes for the production of bioethanol from lignocellulosic biomass	7
2.4	Adsorption to a performed carrier	8
2.5	Cell entrapment within the carrier	9
2.6	Self aggregation of microbial cells	10
2.7	Containment of cells behind carrier	10
2.8	Electron micrographs of the surface of orange peel, before and after yeast immobilization at a magnification of 1200	11
2.9	Kinetics of glucose fermentation by free cell and immobilized cell	12
2.10	Viable cell counts of free and immobilized cells during repeated batch fermentations of glucose (as log10 colony forming unit per gram (cfu/g) of orange peel or log10 cfu/ml of fermentation broth)	13
2.11	Physical structure of raw silk fiber	15
2.12	Scanning electron micrograph (SEM) of virgin silk produced by *B. mori* (a) before and (b) after extraction of sericin protein coating	15
2.13	SEM micrographs of mouse connective tissue cells cultures attached on methanol-treated SF sponges for (a) 6 and (b) 24 h of cultivation	17

LIST OF FIGURES

FIGURE		PAGE
2.14	SEM micrographs of porous SF sheet (a) before degradation and after degradation by protease XIV for (b) 1 day, (c) 6 days, (d) 9 days, (e) 12 days, and (f) 15 days	18
2.15	Primary structure of SF	18
2.16	(a) Structure of single SF composed of amorphous and crystalline region and (b) β-sheet conformation of the crystalline region of SF	19
2.17	(a) FTIR spectra of SF–MCC composite films at SF/MCC ratio of (a)100/0, (b) 80/20, (c) 60/40, (d) 40/60, (e) 30/70, (f) 20/80, (g) 10/90, and (h)0/100. (b) Young's modulus of SF/MCC composite films	20
2.18	Chemical repeating units of CL	21
2.19	Inter- and intra- hydrogen bonding in CL	22
2.20	Transmission electron micrographs (TEM) of a dilute suspension of hydrolyzed (a) cotton (b) tunicin CLWs entrapment within the carrier	23
2.21	The distinguish between MFC and CLWs Containment of cells behind carrier	25
2.22	Banana rachis	26
2.23	SEM micrographs of the vascular bundles in banana rachis showing (a) microfibril (mf) and (b) cell wall (cw) as well as middle lamella (ml)	26
4.1	FTIR spectra of SF solution	44
4.2	Characteristic peaks of silk fibroin	45
4.3	TEM image of CLWs obtained from acid hydrolysis of banana rachis	46

LIST OF FIGURES

FIGURE		PAGE
4.4	FTIR spectra of native cellulose (banana rachis) and CLWs	47
4.5	SEM images of top surface of (a) SF sponge, (c) and (e) bionanocomposite sponges at SF/CLWs ratio of 90/10 and 80/20 respectively. (b), (c) and (f) represented cross sectional part of SF sponge, bionanocomposite sponges at SF/CLWs ratio of 90/10 and 80/20 respectively	49
4.6	SEM images of top surface of (a), (c) and (e) bionanocomposite sponges at SF/CLWs ratio of 70/30, 60/40 and 50/50 respectively. (b), (c) and (f) represented cross sectional part of bionanocomposite sponges at SF/CLWs ratio of 70/30, 60/40 and 50/50 respectively	50
4.7	FTIR spectra of SF, CLWs, and SF/CLWs bionanocomposite sponges (a) before and (b) after methanol treatment	52
4.8	Weight loss of (a) non-methanol treated and (b) methanol treated bionanocomposite sponges	53
4.9	Shrinkage (%) of bionanocomposite sponges after methanol treatment	56
4.10	Compression modulus of methanol- treated bionanocompostie sponges	56

LIST OF FIGURES

FIGURE		PAGE
4.11	Schematic model proposed for hydrogen bonding in SF/CLWs bionanocomposite sponge (a), (b), (c), (d), (e), and (f) represented bionanocomposite sponge at weight ratio of SF/CLWs 100/0, 90/10, 80/20, 70/30, 60/40 and 50/50 respectively (SF, CLWs, • hydrogen bond)	57
4.12	Field Emission Scanning Electron micrographs of bionanocomposite sponge having SF/CLWs weight ratio of 50/50 after immobilization of yeast cells	59
4.13	Yeast cell leakage in distilled water after immersed in distilled water for 48 hour under fermentation condition	60
4.14	Immobilization efficiency (%) of immobilized cell ( )and immobilized cell with glyoxal crosslinked ( ) at various glucose concentration	61
4.15	Ethanol production at varied glucose concentration –●– free yeast cell, –▼– immobilized yeast cell and –■– glyoxal crosslinked yeast cell system	66
4.16	Viable yeast cells at the end of fermentation in varied glucose concentration ■ free yeast cell, immobilized yeast cell and immobilized yeast cell with crosslinking	66
4.17	Ethanol production (g/l) and conversion of glucose into ethanol (%) during five- cycle repeated batches	70