

**PREPARATION OF SILK FIBROIN/CELLULOSE WHISKERS  
BIONANOCOMPOSITE SPONGES FOR YEAST CELL IMMOBILIZATION  
USED IN CONTINUOUS ETHANOL PRODUCTION BY PACKED BED  
BIOREACTOR**

Sureerat Jampa

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

2013

I 28372724

**Thesis Title:** Preparation of Silk Fibroin/Cellulose Whiskers  
Bionanocomposite Sponges for Yeast Cell Immobilization  
Used in Continuous Ethanol Production by Packed Bed  
Bioreactor

**By:** Sureerat Jampa

**Program:** Polymer Science

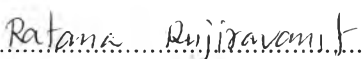
**Thesis Advisors:** Assoc. Prof. Ratana Rujiravanit  
Prof. Emeritus Seiichi Tokura

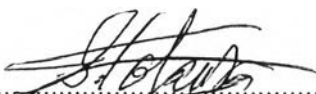
---


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:**

  
.....  
(Assoc. Prof. Ratana Rujiravanit)

  
.....  
(Prof. Emeritus Seiichi Tokura)

  
.....  
(Prof. Sumaeth Chavadej)

  
.....  
(Asst. Prof. Manisara Phiriyawirut)

## ABSTRACT

5472048063: Polymer Science Program  
Sureerat Jampa: Preparation of Silk Fibroin/Cellulose Whiskers  
Bionanocomposite Sponges for Yeast Cell Immobilization Used in  
Continuous Ethanol Production by Packed Bed Bioreactor  
Thesis Advisors: Assoc. Prof. Ratana Rujiravanit and Prof. Emeritus  
Seiichi Tokura 150 pp.  
Keywords: Silk fibroin/ Cellulose whiskers/ Yeast cell immobilization/  
Continuous ethanol fermentation

Silk fibroin (SF)/cellulose whiskers (CLWs) bionanocomposite sponges were prepared as a supporting material for yeast cell immobilization for further using in continuous ethanol production. The SF/CLWs bionanocomposite sponges with high porosity were fabricated by freeze-drying a silk fibroin solution containing cellulose whiskers at different SF/CLWs weight ratios. The freeze-dried SF/CLWs sponges were subjected to methanol treatment to increase water stability. Increasing the CLWs content resulted in higher water stability, less shrinkage, and better mechanical properties of SF/CLWs bionanocomposite sponges. In the continuous ethanol fermentation process, the bionanocomposite sponges with a SF/CLWs weight ratio of 50/50 were used to immobilize *Saccharomyces cerevisiae burgundy* KY11 yeast cells. The bionanocomposite sponges with the immobilized yeast cells were loaded into the glass column of a packed bed bioreactor. Continuous ethanol fermentation was operated by varying D-glucose concentrations (100, 150, 200 g/l), dilution rates (0.15, 0.20, 0.25 hr<sup>-1</sup>), and hydraulic retention times (6.87, 5.15, 4.12 hr). The continuous ethanol fermentation with a high concentration of D-glucose, low dilution rate, and high hydraulic retention time resulted in the production of a higher ethanol concentration. At 0.15 hr<sup>-1</sup> dilution rate and 200 g/l feed glucose concentration, the maximum ethanol production of 51.18 g/l with the volumetric ethanol productivity of 7.68 gl<sup>-1</sup>hr<sup>-1</sup> was obtained under continuous ethanol fermentation.

## บทคัดย่อ

สุวีรัตน์ จำปา : การเตรียมวัสดุคอมโพสิตที่มีรูพรุนจากไฟโบรอินจากรังไหมที่เสริมแรงด้วยเซลลูโลสวิสเกอร์เพื่อใช้ในการตรึงเซลล์ยีสต์สำหรับการหมักเอทานอลแบบต่อเนื่อง (Preparation of Silk Fibroin/Cellulose Whiskers Bionanocomposite Sponges for Yeast Cell Immobilization Used in Continuous Ethanol Production by Packed Bed Bioreactor)  
 อ. ที่ปรึกษา : รศ. ดร. รัตนา รุจิวานิช และ ศ. ดร. เซอิชิ โทกุระ 150 หน้า

วัสดุคอมโพสิตที่มีรูพรุนซึ่งเตรียมจากไฟโบรอินจากรังไหมผสมกับเซลลูโลสวิสเกอร์ ถูกใช้เป็นวัสดุตรึงเซลล์ยีสต์ สำหรับการผลิตเอทานอลแบบต่อเนื่อง วัสดุรูพรุนถูกเตรียมโดยการทำแห้งแบบเยือกแข็งของสารละลายไฟโบรอินจากรังไหมที่ผสมกับเซลลูโลสวิสเกอร์ในอัตราส่วนต่างๆ โดยน้ำหนัก วัสดุรูพรุนที่เตรียมได้ถูกจุ่มในสารละลายเมทานอลเพื่อเพิ่มความสามารถในการคงรูปอยู่ในน้ำของวัสดุ นอกจากนี้การเพิ่มปริมาณเซลลูโลสวิสเกอร์ส่งผลทำให้วัสดุสามารถคงรูปอยู่ในน้ำได้ดีขึ้น การหาคัดของวัสดุลดลงและยังช่วยเพิ่มสมบัติเชิงกลของวัสดุอีกด้วย วัสดุคอมโพสิตที่มีรูพรุนในอัตราส่วนของไฟโบรอินจากรังไหม/เซลลูโลสวิสเกอร์เท่ากับ 50/50 โดยน้ำหนัก ถูกใช้เพื่อตรึงเซลล์ยีสต์สำหรับกระบวนการหมักเอทานอลแบบต่อเนื่อง วัสดุรูพรุนที่ตรึงเซลล์ยีสต์ถูกบรรจุในคอลัมน์แก้วเพื่อใช้ในการหมักเอทานอลแบบต่อเนื่องซึ่งความเข้มข้นน้ำตาลกลูโคส (100, 150, 200 กรัมต่อลิตร) อัตราการเจือจาง (0.15, 0.20, 0.25 ต่อชั่วโมง) และระยะเวลาการกักน้ำหมักในระบบ (6.87, 5.15, 4.12 ชั่วโมง) ถูกใช้สำหรับกระบวนการหมักเอทานอล นอกจากนี้ยังพบว่ากระบวนการหมักเอทานอลแบบต่อเนื่องที่มีการใช้น้ำตาลกลูโคสความเข้มข้นสูง อัตราการเจือจางต่ำและมีระยะเวลาการกักน้ำหมักในระบบนานทำให้ปริมาณเอทานอลที่ผลิตได้มีความเข้มข้นสูง การหมักเอทานอลแบบต่อเนื่องที่อัตราการเจือจาง 0.15 ต่อชั่วโมงและความเข้มข้นน้ำตาลกลูโคส 200 กรัมต่อลิตรจะทำให้ได้ปริมาณเอทานอลสูงสุดเท่ากับ 51.18 กรัมต่อลิตรที่ความสามารถในการผลิตเอทานอลเท่ากับ 7.68 กรัมต่อลิตรต่อชั่วโมง

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisors Assoc. Prof. Ratana Rujiravanit and Prof. Emeritus Seiichi Tokura for their sincere assistances, invaluable knowledge, and beneficial techniques. Without their constructive suggestions and knowledgeable guidance in this study, this research would never have successfully been accomplished. I really appreciate in her kindness and would like to sincerely thank here.

Sincere thanks and deep appreciation are also extended to Prof. Sumaeth Chavadej and Asst. Prof. Manisara Phiriyawirut, my thesis examination committee, for their comments and useful suggestions. Moreover, I would like to show my heartfelt thanks to all teachers who have taught me all along.

I am grateful for the scholarship and financial support of Development and Promotion of Science and Technology Talents Project (DPST), the Petroleum and Petrochemical College, Chulalongkorn University, and the Center of Excellence on Petrochemical and Materials Technology, Thailand.

I greatly thank Dr. Chutima Vanichvattanadecha, Dr. Orathai Pornsunthorntawee, my Ratana's seniors, my friends, and all PPC staffs who gave me encouragement and heartfelt assist.

Finally, I would like to express my appreciation to my family for their love, understanding, help, encouragement and support throughout my life.

## TABLE OF CONTENTS

	<b>PAGE</b>
Title Page	i
Abstract (in English)	iii
Abstract (in Thai)	iv
Acknowledgements	v
Table of Contents	vi
List of Tables	xi
List of Figures	xii
<b>CHAPTER</b>	
<b>I INTRODUCTION</b>	<b>1</b>
<b>II THEORETICAL BACKGROUND AND LITERATURE REVIEW</b>	<b>3</b>
2.1 Ethanol	3
2.1.1 Ethanol Production	4
2.2 Fermentation	4
2.2.1 Ethanol Fermentation	5
2.2.2 Continuous Ethanol Fermentation	8
2.3 Cell Immobilization System	10
2.3.1 Adsorption to a Pre-formed Carrier	11
2.3.2 Cell Entrapment	12
2.3.3 Self-aggregation	12
2.3.4 Containment of Cells behind a Barrier	13
2.4 Silk Fibroin (SF)	16
2.4.1 Structure of SF	19
2.4.1.1 Primary Structure	19
2.4.1.2 Secondary Structure	19
2.4.2 SF as a Biomaterial	22

<b>CHAPTER</b>	<b>PAGE</b>
2.5 Cellulose (CL)	25
2.5.1 Cellulose Whiskers (CLWs)	27
<b>III METHODOLOGY</b>	<b>32</b>
3.1 Materials	32
3.1.1 Silk Cocoon and Banana Rachis	32
3.1.2 Yeast Cells	32
3.1.3 Other Chemicals	32
3.2 Experimental Methods	33
3.2.1 Preparation of SF Solution	33
3.2.2 Preparation of CLWs	33
3.2.3 Preparation of SF/CLWs Bionanocomposite Sponge	33
3.2.4 Methanol Treatment of SF/CLWs Bionanocomposite Sponge	34
3.2.5 Inoculums Preparation	34
3.2.6 Yeast Cells Immobilization	34
3.2.7 Continuous Ethanol Fermentation	34
3.3 Equipments and Characterizations	35
3.3.1 Fourier Transformed Infrared (FTIR) Spectroscopy	35
3.3.2 Transmission Electron Microscopy (TEM)	35
3.3.3 Field Emission Scanning Electron Microscopy (FE-SEM)	36
3.3.4 Lloyd Instrumental	36
3.3.5 Optical Microscopy (OM)	36
3.3.6 Ultraviolet-visible (UV-vis) Spectroscopy	36
3.3.7 Gas Chromatography (GC)	36
3.4 Analytical measurements	37
3.4.1 Weight Loss of SF/CLWs Bionanocomposite Sponge	37
3.4.2 Shrinkage of SF/CLWs Bionanocomposite Sponge	37
3.4.3 Immobilization and Leakage of Yeast Cells	37

<b>CHAPTER</b>	<b>PAGE</b>
3.4.4 Sugar Consumption during Fermentation	38
3.4.5 Ethanol Production during Fermentation	39
3.4.5.1 Ethanol Production	39
3.4.5.2 Volumetric Ethanol Productivity ( $Q_p$ )	39
3.4.5.3 Ethanol Yield ( $Y_{P/S}$ )	39
<b>IV PREPARATION OF SILK FIBROIN/CELLULOSE WHISKERS BIONANOCOMPOSITE SPONGES FOR YEAST CELL IMMOBILIZATION USED IN CONTINUOUS ETHANOL PRODUCTION BY PACKED BED BIOREACTOR</b>	<b>40</b>
4.1 Abstract	40
4.2 Introduction	41
4.3 Experimental	43
4.4 Results and Discussion	49
4.5 Conclusions	78
4.6 Acknowledgements	79
4.7 References	79
<b>V CONCLUSIONS AND RECOMMENDATIONS</b>	<b>83</b>
<b>REFERENCES</b>	<b>84</b>
<b>APPENDICES</b>	<b>88</b>
<b>Appendix A</b> The Bionanocomposite Sponge Codes at Various Weight Ratios of SF to CLWs	88
<b>Appendix B</b> Weight Loss of Silk Fibroin/Cellulose Whiskers Bionanocomposite Sponges at Various Weight Ratios of SF/CLWs	89



CHAPTER	PAGE
<b>Appendix C</b> Shrinkage of Silk Fibroin/Cellulose Whiskers Bionanocomposite Sponges after Methanol Treatment at Various Weight Ratios of SF/CLWs	101
<b>Appendix D</b> Compression Modulus of Methanol-treated Silk Fibroin/Cellulose Whiskers Bionanocomposite Sponges at Various Weight Ratios of SF/CLWs	104
<b>Appendix E</b> Growth Curve of <i>Saccharomyces Cerevisiae</i> <i>Burgundy</i> KY 11	105
<b>Appendix F</b> Yeast Cells in Methanol-treated Silk Fibroin/Cellulose Whiskers Bionanocomposite Sponges at Various Weight Ratios of SF/CLWs	108
<b>Appendix G</b> Leaked Yeast Cells from Methanol-treated Silk Fibroin/Cellulose Whiskers Bionanocomposite Sponges at Various Weight Ratios of SF/CLWs after Immersed in Distilled Water with 150 rpm Shaking at 30°C for 24 Hours	111
<b>Appendix H</b> % Yeast Cell Immobilization Efficiency and % Yeast Cell Leakage of Methanol-treated Silk Fibroin/Cellulose Whiskers Bionanocomposite Sponges at Various Weight Ratios of SF/CLWs after Immersed in Distilled Water with 150 rpm Shaking at 30°C for 24 Hours	114
<b>Appendix I</b> Determination of Ethanol Concentration in Continuous Ethanol Fermentation by Immobilized Yeast Cell	116
<b>Appendix J</b> Determination of Volumetric Ethanol Productivity ( $Q_p$ ) in Continuous Ethanol Fermentation by Immobilized Yeast Cell	124

<b>CHAPTER</b>	<b>PAGE</b>
<b>Appendix K</b> Determination of Sugar Concentration in Continuous Ethanol Fermentation by Immobilized Yeast Cell	127
<b>Appendix L</b> Determination of % Sugar Consumption in Continuous Ethanol Fermentation by Immobilized Yeast Cell	141
<b>Appendix M</b> Determination of Ethanol Yield ( $Y_{P/S}$ ) in Continuous Ethanol Fermentation by Immobilized Yeast Cell	144
<b>Appendix N</b> Plot Graph between Ethanol Production (g/l) and Time (hr) in Continuous Ethanol Fermentation by Immobilized Yeast Cell	147
<b>Appendix O</b> Photograph of MSF50 Bionanocomposite Sponge after Used in Continuous Ethanol Fermentation	149
<b>CURRICULUM VITAE</b>	150

**LIST OF TABLES**

<b>TABLE</b>		<b>PAGE</b>
2.1	Composition of silk produced by <i>Bombyx mori</i> silkworm	17
2.2	Amino acid composition of <i>Bombyx mori</i> fibroin	18
4.1	Production yield of silk fibroin solution	49
4.2	Production yield of CLWs suspension	52
4.3	The main functional groups of CLWs	56
4.4	The non-methanol treated bionanocomposite sponge codes at various weight ratios of SF to CLWs	57
4.5	The methanol-treated bionanocomposite sponge codes at various weight ratios of SF to CLWs	57
4.6	Average pore width of bionanocomposite sponge	58
4.7	A comparison of the fermentation condition and ethanol production	77

## LIST OF FIGURES

FIGURE	PAGE
2.1 Metabolic pathway of ethanol fermentation in <i>Saccharomyces cerevisiae</i> .	6
2.2 Sources of sugars for ethanol production, 1 Crops; 2 lignocellulose. Arrows represent hydrolysis (only monomers generated from hydrolysis, represented by dark arrows, can be fermented). G Glucose, Gal galactose, F fructose, Man mannose, X xylose, Ara arabinose, Other L-rhamnose, L-fucose, uronic acids.	7
2.3 Model of continuous fermentation system.	8
2.4 Effect of sugar concentration on ethanol production in a continuous immobilized cells reactor with different HRT.	9
2.5 Adsorption to a pre-formed carrier.	11
2.6 Cell entrapment within the carrier.	12
2.7 Self-aggregation of cells.	13
2.8 Containment of cells behind carrier.	13
2.9 Scanning electron micrograph of immobilized cells on BC after the immobilization culture.	14
2.10 A cross-section of a thin-shell silk cocoon (TSC) at the initial time (0 h), (A) and after the five-cycle repeated batch fermentation (240 h), (B).	15
2.11 Scanning electron microphotograph (1000 $\times$ ) of the (a) hydrated DSBP; <i>Saccharomyces cerevisiae</i> immobilized (b) on the surface and (c) inside the cavities of hydrated DSBP.	16
2.12 Physical structure of raw silk fiber.	17
2.13 Primary structure of SF.	19
2.14 (a) Structure of SF composed of amorphous and crystalline region and (b) $\beta$ -sheet conformation of the crystalline region of SF.	20

<b>FIGURE</b>	<b>PAGE</b>
2.15 FT-IR spectra of the as-prepared silk fibroin sponge (A), methanol-treated sponges at various C/S ratios (B to E), and chitin whisker film (F). Arrows indicate the absorption shoulder.	21
2.16 Typical cell growth curves on silk fibroin from (a) <i>Bombyx Mori</i> domestic silkworm, (b) <i>Antheraea pernyi</i> wild silkworm.	22
2.17 The growth of cells on non-woven silk fibroin nets. Cell name and (type) shown are: (a) 1321N1 (glial); (b) A549 (epithelial); (c) CACO-2 (epithelial); (d) HaCaT (keratinocyte); (e) HPMEC ST1.6R (endothelial); (f) MRC-5 (fibroblast); (g) MG63 (osteoblast); (h) STML-12 (epithelial); and (i) U373 MG (glial).	23
2.18 SEM micrographs of attached cells on methanol-treated silk fibroin sponges for (a) 6 and (b) 24 h of cultivation and SEM micrographs of attached cells on methanol-treated chitin whisker/silk fibroin sponges at C/S ratio of 4/8 for (c) 6 and (d) 24 h of cultivation.	24
2.19 Chemical repeating unit of cellulose.	25
2.20 Cellulose fiber (A), Microfibrillated cellulose (B), Cellulose whiskers (C).	26
2.21 Inter and intra hydrogen bonding in CL.	27
2.22 Esterification of cellulose hydroxyl groups during sulfuric acid hydrolysis.	28
2.23 Suspensions of sugarcane bagasse whiskers.	28
2.24 TEM micrographs of negatively stained preparations of cellulose microfibrils isolated after different treatments: (a) peroxide alkaline (PA), (b) peroxide alkaline–hydrochloric acid (PA–HCl), (c) potassium hydroxide 5 wt% (KOH-5) and (d) potassium hydroxide 18 wt% (KOH-18).	30
2.25 Transmission electron micrograph from diluted suspension of CLWs extracted from rice husk fibers.	31

<b>FIGURE</b>	<b>PAGE</b>
4.1 Appearance of silk fibroin solution.	49
4.2 FTIR spectrum of SF sponge.	50
4.3 FTIR spectrum of methanol-treated SF sponge.	51
4.4 Chemical structure of silk fibroin.	51
4.5 Appearance of cellulose whiskers suspension.	52
4.6 TEM image of cellulose whiskers suspension.	53
4.7 (a) The diameter distribution and (b) length distribution of CLWs derived from banana rachis.	54
4.8 FTIR spectra of native cellulose (CL) and CLWs.	56
4.9 SEM images of surface of (a) methanol-treated SF sponge and (b), (c), (d), (e), (f) methanol-treated bionanocomposite sponges at SF/CLWs ratio of 90/10, 80/20, 70/30, 60/40 and 50/50, respectively.	59
4.10 SEM images of cross sectional part of (a) methanol-treated SF sponge and (b), (c), (d), (e) and (f) methanol-treated bionanocomposite sponges at SF/CLWs ratio of 90/10, 80/20, 70/30, 60/40 and 50/50, respectively.	60
4.11 FTIR spectra of SF, CLWs and SF/CLWs bionanocomposite sponge at various ratios after methanol treatment.	62
4.12 Weight loss of non-methanol treated bionanocomposite sponges.	64
4.13 Weight loss of methanol-treated bionanocomposite sponges.	64
4.14 Shrinkage (%) of bionanocomposite sponges at various ratios after methanol treatment.	65
4.15 Compression modulus of methanol- treated bionanocomposite sponges.	67
4.16 Possible intermolecular hydrogen bond between SF and CLWs.	67
4.17 (a) A number of immobilized yeast cells in the methanol-treated SF/CLWs bionanocomposite sponge at various weight ratios and (b) The % immobilization efficiency in the methanol-treated SF/CLWs bionanocomposite sponge at various weight ratios.	68

<b>FIGURE</b>		<b>PAGE</b>
4.18	SEM images of a large number of yeast cells attached inside MSF50 bionanocomposite sponge.	70
4.19	(a) A number of leaked yeast cells and (b) the % cell leakage from the methanol-treated SF/CLWs bionanocomposite sponge at various weight ratios.	71
4.20	The effect of feed glucose concentration and dilution rate on (a) the ethanol production, (b) volumetric ethanol productivity ( $Q_p$ ) and (c) ethanol yield ( $Y_{P/S}$ ).	74
4.21	The effect of feed glucose concentration and dilution rate on (a) % sugar consumption and (b) residual sugar concentration.	76