รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาวัสดุไบโอพอลิเมอร์เพื่อประยุกต์ใช้ในการแพทย์

Development of Biopolymer Material for Medical Application

โดย

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สัญญาเลขที่ RMU 5380024

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ผู้วิจัย สังกัด

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

สำนักงานคณะกรรมการการอุดมศึกษา

และจุฬาลงกรณ์มหาวิทยาลัย

กิตติกรรมประกาศ

โครงการวิจัยนี้ ได้รับทุนอุดหนุนการวิจัยจากทุนพัฒนานักวิจัย จากฝ่ายวิชาการ สำนักงานกองทุนสนับสนุนการวิจัย (สกว) ร่วมกับ สำนักงานคณะกรรมการการอุดมศึกษา (สกอ) และจุฬาลงกรณ์มหาวิทยาลัย (สัญญาเลขที่ RMU 5380024) นอกจากนี้ยังได้รับการสนับสนุนและความร่วมมือจากบุคคลต่างๆ ทำให้งานวิจัยสำเร็จลุล่วงไปด้วยดี ดังต่อไปนี้

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2. ดร. ผ่องพรรณ ศิริพงษ์ หัวหน้ากลุ่มงานวิจัยสมุนไพร กลุ่มงานวิจัย สถาบันมะเร็งแห่งชาติ

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ผู้วิจัยขอแสดงความขอบคุณอย่างยิ่งมา ณ ที่นี้

Abstract

Project Code: RMU 5380024

Project Title: Development of Biopolymer Material for Medical Application

Investigator: Associate Prof. Dr. Muenduen Phisalaphong, Chulalongkorn University

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Novel composites of bacterial cellulose (BC) have been developed in order to expand the scope of applications. The modifying effects of added biopolymers (alginate, chitosan, Aloe vera gel and gelatin) on physical, chemical and biological properties of the composites were investigated. It was found that at a certain ratio of the supplement, the modified BC composites displayed homogeneous structures, which exhibited a certain level of miscibility. The FTIR results demonstrated some specified interaction between the hydroxyl group of BC and the functional groups of the supplemented polymers. The supplements of these polymers significantly affected characteristics of porous structures, mechanical properties, water absorption capacity and biocompatibility. These modified composites have potential applications for a wide range of fields, including biomedical materials, tissue engineering, drug delivery, immobilization matrices for cells and enzymes, food packaging and membrane separation. For further work, the study of effects of modification of BC scaffold in order to giving rise to various types of differentiated cells is recommended. Based on this research, we can produce 6 international research articles, 1 book chapter, 1 Thai patent, 5 international conference proceedings and partially support research activities for 2 master degree students and 4 doctoral degree students.

Keywords: Bacterial Cellulose; Biopolymer; Medical; Cell Immobilization

บทคัดย่อ

รหัสโครงการ: RMU 5380024

ชื่อโครงการ:การพัฒนาวัสดุไบโอพอลิเมอร์เพื่อประยุกต์ใช้ในการแพทย์

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ระยะเวลาโครงการ: 15 มิถุนายน พ.ศ. 2553-2556

สารประกอบแบคทีเรียเซลลูโลสแบบใหม่ได้ถูกพัฒนาขึ้นเพื่อขยายขอบเขตของการนำไปประยุกต์ใช้ จากการตรวจสอบผลของการดัดแปลงโดยการเติมไบโอพอลิเมอร์ (อัลจิเนท ไคโตรซาน เจลว่านหางจระเข้ และ เจลาติน) ต่อสมบัติทางกายภาพ เคมี และชีวภาพ ของสารประกอบ พบว่าการเติมสารผสมที่สัดส่วนปริมาณหนึ่งจะได้สารประกอบมีลักษณะโครงสร้างที่เป็นเนื้อเดียวกัน ซึ่งแสดงถึงการผสมเข้ากันได้ดีของสารประกอบ จากการวิเคราะห์โดยเครื่อง FTIR แสดงให้เห็นถึงการปฏิสัมพันธ์โดยเฉพาะระหว่างหมู่ไฮดรอกซิล(hydroxyl group) ของแบคทีเรียเซลลลูโลสและ หมู่ฟังก์ชัน (functional groups) ของสารพอลิเมอร์ต่างๆที่เติมเข้าไป การเติมสารพอลิเมอร์เหล่านี้ส่งผลอย่างเด่นชัดต่อลักษณะโครงสร้างรูพรุน สมบัติเชิงกล ความสามารถในการอุ้มน้ำ และลักษณะความเข้ากันได้กับเนื้อเยื่อของสิ่งมีชีวิต สารประกอบที่ถูกดัดแปลงเหล่านี้มีศักยภาพในการนำไปประยุกต์ใช้ในหลายๆด้านรวมถึงวัสดุชีวภาพทางการแพทย์ วิศวกรรมเนื้อเยื่อ วัสดุนำส่งยา วัสดุสำหรับตรึงเซลล์และเอนไซม์ บรรจุภัณฑ์อาหาร และแผ่นเยื่อใช้ในกระบวนการแยก งานที่แนะนำสำหรับการศึกษาต่อไปคือการศึกษาผลการดัดแปลงโครงเลี้ยงเซลล์ทำจากแบคทีเรียเซลลูโลสต่อการเจริญของเซลล์หลากหลายรูปแบบ ทั้งนี้งานวิจัยนี้สร้างผลผลิตคือ บทความในวารวารวิจัยระดับนานาชาติ 6 เรื่อง บทความในหนังสือระดับนานาชาติ 1บท (book chapter) ยื่นจดสิทธิบัตร 1 ผลงาน นำเสนอในการประชุมระดับนานาชาติ 5 เรื่อง และมีส่วนช่วยสนับสนุนกิจกรรมการวิจัยของนิสิตในระดับปริญญาโท 2 คน ปริญญาเอก 4 คน

คำหลัก: แบคทีเรียเซลลูโลส ไบโอพอลิเมอร์ การแพทย์ การตรึงเซลล์

สารบัญ

เนื้อหางานวิจัย

บทนำ

ปัจจุบันนี้พอลิเมอร์จากธรรมชาติได้รับความสนใจเป็นอย่างมากในการนำมาประยุกต์ใช้ในงานทางชีวเวชในหลายๆด้าน อาทิเช่นนำมาประยุกต์ใช้เป็นแผ่นปิดแผล หลอดเลือดเทียม โครงเลี้ยงเซลล์ผิวหนัง เซลล์กล้ามเนื้อ เซลล์กระดูกอ่อน และ เซลล์ต้นกำเนิดเป็นต้น ทั้งนี้เพื่อนำเซลล์เหล่านั้นไปใช้ในการบำบัดรักษาหรือใช้ประโยชน์อื่นๆในทางการแพทย์ต่อไป โดยไบโอพอลีเมอร์ (Biopolymer) ต่างๆ ได้ถูกนำมาพัฒนาให้เป็นลักษณะแบบแผ่นบาง แบบท่อกลวง หรือ แบบโครง 3 มิติ ที่มีความพรุน ที่มีลักษณะโครงสร้างที่เหมาะสมตลอดจนทำการพัฒนาคุณสมบัติ เช่นความแข็งแรงเชิงกล ความสามารถในการอุ้มน้ำ การแพร่ผ่านของไอน้ำหรืออากาศ และชีวสมบัติเช่น ความเป็นมิตรต่อเซลล์ ความสมารถในการยับยั้งจุลินทรีย์ เป็นต้น เพื่อให้มีความเหมาะสมสำหรับการนำไปใช้ตามวัตถุประสงค์

เซลลูโลสเป็นสารพอลิเมอร์ทางธรรมชาติที่มีมากเป็นอันดับหนึ่งโดยส่วนใหญ่ได้จากพืช ปัจจุบันได้มีการนำเซลลูโลสมาใช้เป็นประกอบเป็นวัสดุอุปกรณ์หลากหลายรวมทั้งการนำมาพัฒนาคุณสมบัติและขึ้นรูปใช้เป็นแผ่นเยื่อกรอง หรือใช้เป็นวัสดุทางการแพทย์ อย่างไรก็ตามเซลลูโลสจากพืชมีองค์ประกอบของสารอื่นๆ เช่น ลิกนิน (Lignin) และ เฮมิเซลลูโลส (Hemicellulose) ปนอยู่ซึ่งทำให้คุณสมบัติโดยรวมด้อยลง จึงจำเป็นต้องมีขั้นตอนการทำให้บริสุทธิ์ซึ่งมีความยุ่งยาก ทำให้มีการปนเปื้อนของสารเคมี และทำให้โครงสร้างของเซลลูโลสเสียไป ในขณะที่แผ่นเซลลูโลสที่สังเคราะห์ได้จากจุลินทรีย์มีความบริสุทธิ์สูงไม่มีสารปนเปื้อน เช่น ลิกนิน (Lignin) หรือ เฮมิเซลลูโลส (Hemicellulose) มีเส้นใยนาโนขนาดเล็กประมาณ 1 ใน 100 ของเส้นใยจากพืช มีลักษณะเป็นโครงสร้างตาข่ายที่มีความพรุน มีขนาดรูพรุนขนาด 20-300 นาโนเมตร คุณสมบัติพิเศษหลายอย่างที่เหมาะสมในการนำมาประยุกต์ใช้เป็นวัสดุทางการแพทย์

ได้มีการนำแบคทีเรียเซลลูโลสไปประยุกต์ใช้ในทางการแพทย์ด้านต่างๆเช่น แผ่นเยื่อกรองสำหรับฟอกเลือด, แผ่นปิดแผล, หลอดเลือดเทียม เนื่องคุณสมบัติที่เหมาะสมหลายๆด้านที่สำคัญเช่น สมบัติที่สามารถย่อยสลายได้โดยกระบวนการชีวภาพ มีความสามารถในการอุ้มน้ำสูง ไม่มีความเป็นพิษ เข้ากับเซลล์มีชีวิตได้ดี โดยอาจมีการดัดแปลงโครงสร้างพื้นผิว เช่นการเติมพันธะต่างๆตามความต้องการเป็นต้น จากการศึกษาที่ผ่านมามีรายงานว่าแผ่นแบคทีเรียเซลลูโลสไม่มีพิษต่อเซลล์ผิวหนัง สนับสนุนการเจริญเติบโตของเซลล์ผิวหนังได้ดี นอกจากนี้การผลิตแผ่นแบคทีเรียเซลลูโลยังสามารถใช้วัตถุดิบซึ่งเป็นผลพลอยได้ของอุตสาหกรรมในประเทศ เช่นน้ำมะพร้าว หรือน้ำสัปรด เป็นวัตถุดิบหลัก ใช้จุลลินทรีย์ที่คัดเลือกได้จากการวิจัยในประเทศ ตลอดจนกรรมวิธีที่ไม่ยุ่งยาก ส่งผลให้ต้นทุนการผลิตผลิตภัณฑ์มีราคาถูก ทำให้สามารถนำไปประยุกต์ใช้ได้อย่างกว้างขวาง โดยได้มีรายงานการใช้แผ่นแบคทีเรียเซลลูโลสเป็นวัสดุปิดแผลที่ทำให้แผลหายเร็วขึ้น ใช้รักษาแผลไฟไหม้ หรือการนำมาดัดแปลงและทดสอบเพื่อใช้เป็นส่วนทดแทนเช่นหลอดเลือดเทียมเป็นต้น อย่างไรก็ตาม

สำหรับการนำมาประยุกต์ใช้เป็นโครงเลี้ยงเซลล์แบบสามมิติ โครงสร้างและสมบัติพื้นฐานของแผ่นแบคทีเรียเซลลูโลสยังจำเป็นต้องการพัฒนาเพื่อปรับปรุงคุณสมบัติให้เหมาะสมต่อไป

การปรับปรุงสมบัติของพอลิเมอร์อาจทำได้หลายอย่าง อย่างไรก็ตามพบว่าวิธีหนึ่งที่มีประสิทธิภาพมากทำโดยการนำพอลิเมอร์ที่มีสมบัติที่ต้องการมาผสมกันแล้วเชื่อมต่อกันโดยใช้เทคนิคทางเคมีหรือทางกายภาพ เพื่อให้ได้พอลิเมอร์ผสมแบบใหม่ที่มีสมบัติตามต้องการ โดยขั้นตอนต่างๆในขณะการขึ้นรูปและองค์ประกอบจะเป็นปัจจัยสำคัญในการกำหนดลักษณะโครงสร้างหรือลักษณะรูพรุนของโครงสามมิติ ตลอดจนสมบัติต่างๆ เป้าหมายของงานวิจัยนี้จะทำศึกษาพัฒนาโครงเลี้ยงเนื้อเยื่อสำหรับเซลล์ อาทิเช่น เซลล์ผิวหนังคน เซลล์ในช่องปาก เป็นต้น โดยใช้พอลิเมอร์ผสมจากพอลิเมอร์ธรรมชาติ เช่นแบคทีเรียเซลลูโลส อัลจิเนท เจลาติน ไคโตซาน และใยไหม เป็นต้น โดยทำการศึกษาขั้นตอนและปัจจัยต่างๆในการขึ้นรูป และทำการศึกษาลักษณะต่างๆที่สำคัญของโครงเลี้ยงเซลล์ เช่นโครงสร้าง รูพรุน สมบัติทางเชิงกล พันธะเคมีที่เปลี่ยนไป การอุ้มน้ำ ความสามารถในการย่อยสลายเป็นต้น จากนั้นศึกษาถึงปัจจัยสำคัญที่มีผลต่อการเจริญของเซลล์เป้าหมาย เช่น โครงเลี้ยงเซลล์จะต้องไม่มีความเป็นพิษต่อเซลล์ ส่งเสริมการเจริญเพิ่มปริมาณของเซลล์ และเซลล์มีลักษณะในรูปแบบที่เหมาะสม โดยงานวิจัยนี้มีจุดมุ่งหมายเพื่อพัฒนาองค์ความรู้ของการสร้างโครงเลี้ยงเซลล์แบบใหม่เพื่อการนำมาประยุกต์ใช้ในการบำบัดรักษา เช่นการรักษาบาดแผลที่ผิวหนัง การรักษาสภาพความพิการหรือบาดแผลในช่องปาก ตลอดจนศึกษาถึงความเหมาะสมในการนำไปประยุกต์ใช้ในด้านอื่นๆตามลักษณะของผลิตภัณฑ์ โดยพบว่ายังไม่มีรายงานการวิจัยพัฒนาโครงเลี้ยงเซลล์ในลักษณะที่จะศึกษาในงานวิจัยนี้ ทั้งนี้จะเน้นพัฒนากระบวนการที่ทำโดยใช้วัตถุดิบภายในประเทศเป็นหลักและมีราคาการผลิตที่ไม่แพง เพื่อให้สามารถนำไปผลิตใช้ได้อย่างกว้างขวาง มีจุดมุ่งหมายให้ผลิตภัณฑ์ที่พัฒนาขึ้นสามารถแข่งขันหรือทดแทนผลิตภัณฑ์ที่ต้องนำเข้าจากต่างประเทศที่มีราคาแพง

วิธีการทดลอง

- ศึกษาหลักการพื้นฐาน และรวบรวมงานวิจัยที่เกี่ยวข้อง
- เตรียมแผ่นแบคทีเรียเซลลูโลส จากการสังเคราะห์ของ *Acetobacter xylinum* และทำการดัดแปลงโดยใช้พอลิเมอร์ผสมจากพอลิเมอร์ธรรมชาติ เช่นแบคทีเรียเซลลูโลส อัลจิเนท เจลาติน ไคโตซาน และใยไหม เป็นต้น เพื่อให้มีสมบัติที่เหมาะสมต่อการนำไปใช้งาน
- ดัดแปลงโครงสร้างโดยใช้กระบวนการอบแห้งในรูปแบบต่างๆ
- ทดสอบคุณสมบัติของแผ่นหรือโครงสามมิติที่พัฒนาขึ้น
    1) ความหนาของแผ่นฟิล์มด้วยเครื่อง dial thickness gauge
    2) โครงสร้างพื้นผิวและตัดขวางของแผ่นฟิล์มโดยการถ่ายภาพด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (scanning electron microscope, SEM)
    3) รูพรุนของแผ่นฟิล์มด้วยเครื่อง Nitrogen Adsorption BET

4) ความแข็งแรงเชิงกล วัดความต้านทานแรงดึง และความยืดหยุนของโครงร่างโดย โดยเครื่องวัดความต้านแรงดึง (tensile stress) LLOYD, L2000R
5) คุณสมบัติความเป็นผลึกโดยใช้เครื่อง X-ray Diffraction (XRD)
6) พันธะทางเคมีโดยเครื่อง FTIR
7) สมบัติการย่อยสลายได้ทางชีวภาพ (Biodegradable)
8) สมบัติในการดูดซับน้ำ
9) สมบัติในการแพร่ผ่านไอน้ำ
10) สมบัติในการแพร่ผ่านออกซิเจน
11) สมบัติในการเป็นสารต้านแบคทีเรีย หรือ เชื้อรา
12) ความเป็นพิษ

- ศึกษาแนวโน้มการนำไปใช้เป็นวัสดุทางการแพทย์
    1) ทดสอบการเพาะเลี้ยงเซลล์ Keratinocyte และ Fibroblast

เซลล์ไลน์ human transformed skin keratinocyte (CRL-2309) และ human normal skin fibroblast (CCL-110) ซื้อจาก ATCC (American Type Culture Collection) โดย เซลล์ keratinocyte จะเลี้ยงในอาหารเลี้ยงเซลล์ชนิด Keratinocyte-Serum Free medium ที่เติม 0.05 มิลลิกรัม/มิลลิลิตร bovine pituitary extract (BPE) และ 35 ng/ml epidermal growth factor (EGF) เซลล์จะถูกถ่ายลงจานเลี้ยงเซลล์ใหม่สัปดาห์ละ 1 ครั้ง ในอัตราส่วน 1:2 สำหรับเซลล์ fibroblast จะเลี้ยงในอาหารเลี้ยงเซลล์ชนิด DMEM (Dulbecco Modified Eagle's Medium) ที่เติมสารประกอบดังต่อไปนี้ คือ ซีรัมจากฟีตัสของวัวร้อยละ10 (10% Fetal Bovine Serum), กลูตามีน (L-Glutamine) 2 มิลลิโมลาร์, เพนนิซิลิน (Penicillin) 100 ยูนิต/มิลลิลิตร, สเตร็ปโตมัยซินซัลเฟต (Streptomycin sulfate) 100 มิลลิกรัม/มิลลิลิตร และ แอมโฟเทอริซิน บี (Amphotericin B) 0.25 มิลลิกรัม/มิลลิลิตร เซลล์จะถูกถ่ายลงจานเลี้ยงเซลล์ใหม่สัปดาห์ละ 1 ครั้ง ในอัตราส่วน 1:4 เซลล์ทั้งสองชนิดเลี้ยงอยู่ในตู้อุณหภูมิ 37 องศาเซลเซียส ที่มีปริมาณก๊าซคาร์บอนไดออกไซด์ร้อยละ 5

2) ศึกษาความสามารถในการแบ่งตัวของเซลล์ โดยเซลล์ถูกถ่ายลงในจานเลี้ยงเซลล์แบบ 24 หลุม ที่ความหนาแน่น 25,000 เซลล์/หลุม เป็นเวลา 16 ชั่วโมง จากนั้นจึงเปลี่ยนอาหารเลี้ยงเซลล์เป็นชนิดที่ไม่มีซีรัมเลี้ยงต่อไปอีก 24 ชั่วโมงแล้วจึงเริ่มเข้าสู่การทดลอง ในการทดลองจะเป็นการเปรียบเทียบระหว่างเซลล์ที่เลี้ยงในหลุมเลี้ยงเซลล์พลาสติกธรรมดา (Polystylene) และเซลล์ที่เลี้ยงอยู่บนแผ่นเซลลูโลส โดยภายหลังจากที่เลี้ยงเซลล์ไปในระยะเวลาที่กำหนดคือ 0 , 24 และ 48 ชั่วโมง อาหารเลี้ยงเซลล์ในแต่ละหลุมจะถูกดูดออกแล้วล้างด้วยฟอสเฟสบัฟเฟอร์เซลายด์ 2 ครั้ง จากนั้นเติมสารละลาย MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) ลงในอาหารเลี้ยงเซลล์ที่ไม่มีซีรัมและฟีนอลเรด ให้ได้ความเข้มข้นสุดท้ายเท่ากับ 0.5 มิลลิกรัมต่อมิลลิลิตร ใน 4 ชั่วโมงสุดท้ายก่อนการเก็บผล ทำการดูดสารละลาย MTT ออก แล้วทำละลายผลึกสีม่วงเข้มของ formazan ด้วยสารละลายผสมระหว่างไกลซีนบัฟเฟอร์ และ DMSO (Dimethylsulfoxide) ในอัตราส่วน 1 ต่อ 9 ในปริมาตร 1 มิลลิลิตร จะได้สารละลายใสสีม่วง แล้วจึงนำไปวัดค่าการดูดกลืนแสงด้วยสเปค

โตรโฟโตมิเตอร์ที่ความยาวคลื่น 570 นาโนเมตร แล้วนำค่าที่ได้ไปเปรียบเทียบหาจำนวนเซลล์จากกราฟมาตรฐาน ทำการทดลองซ้ำอย่างน้อย 3 ครั้ง และในแต่ละการทดลองจะทำซ้ำอย่างน้อย 3 หลุม

3) ศึกษาการจัดเรียงตัวของเซลล์ Keratinocyte และ Fibroblast บนแผ่นหรือโครงเซลลูโลสดัดแปลงเซลล์ถูกถ่ายลงในจานเลี้ยงเซลล์แบบ 24 หลุม ที่ความหนาแน่น 25,000 เซลล์/หลุม เป็นเวลา 16 ชั่วโมง จากนั้นจึงเปลี่ยนอาหารเลี้ยงเซลล์เป็นชนิดที่ไม่มีซีรัม เลี้ยงต่อไปอีก 24 ชั่วโมงแล้วจึงเริ่มเข้าสู่การทดลอง ในการทดลองจะเป็นการเปรียบเทียบระหว่างเซลล์ที่เลี้ยงในหลุมเลี้ยงเซลล์พลาสติกธรรมดา (Polystylene) และเซลล์ที่เลี้ยงอยู่บนแผ่นเซลลูโลส โดยจะการติดตามการเปลี่ยนแปลงการจัดเรียงตัวของเซลล์ในหลุมที่เวลา 0, 24 และ 48 ชั่วโมง การติดตามการเปลี่ยนแปลงจะทำโดยใช้กล้อง phase contrast microscope และทำการถ่ายภาพเซลล์เพื่อประกอบการวิเคราะห์ และใช้ Scanning electron microscope (SEM) เพื่อวิเคราะห์ลักษณะของการเจริญเติบโตบนวัสดุที่พัฒนาขึ้น

4) ศึกษาความสามารถในการเคลื่อนที่ของเซลล์ Keratinocyte และ Fibroblast บนแผ่นหรือโครงเซลลูโลสดัดแปลง การศึกษาความสามารถในการเคลื่อนที่ของเซลล์ในแนวระนาบ ทำโดยถ่ายเซลล์จำนวน 10,000 เซลล์ลงใน metal ring ที่มีรัศมีภายในประมาณ 0.5 มิลลิเมตร ที่วางทับอยู่บนแผ่นเซลลูโลส เลี้ยงเซลล์ในสภาวะนี้ต่อไปเป็นเวลา 16 ชั่วโมง ในอาหารเลี้ยงเซลล์ที่มีซีรัมร้อยละ 10 metal ring จะเป็นตัวกำหนดขนาดพื้นที่เริ่มต้นก่อนที่เซลล์จะเริ่มเคลื่อนที่ จากนั้นเอา metal ring ออกแล้วเปลี่ยนอาหารเลี้ยงเซลล์เป็นชนิดที่ไม่มีซีรัม แล้วเลี้ยงต่อไป โดยเก็บผลในช่วงเวลาที่กำหนด คือ ที่ 0, 7 และ 24 ชั่วโมง การวิเคราะห์การเคลื่อนที่ของเซลล์จะคำนวณจากพื้นที่ที่ถูกครอบคลุมโดยเซลล์ ในช่วงเวลาที่กำหนดดังกล่าว นอกจากนี้ทำการวิเคราะห์จำนวนเซลล์อีกครั้งหนึ่งด้วยเทคนิค MTT

- ศึกษาแนวโน้มการนำไปใช้ประโยชน์ในด้านอื่นๆเช่นการนำไปใช้เป็นวัสดุตรึงเซลล์จุลินทรีย์

- จัดทำรายงานสรุป จัดทำเอกสารเผยแพร่

ผลการทดลอง บทวิจารณ์

การปรับปรุงสมบัติของพอลิเมอร์อาจทำได้หลายอย่าง อย่างไรก็ตามพบว่าวิธีหนึ่งที่มีประสิทธิภาพมาก ทำโดยการนำพอลิเมอร์ที่มีสมบัติที่ต้องการมาผสมกันแล้วเชื่อมต่อกันโดยใช้เทคนิคทางเคมีหรือทางกายภาพ เพื่อให้ได้พอลิเมอร์ผสมแบบใหม่ที่มีสมบัติตามต้องการ โดยขั้นตอนต่างๆในขณะการขึ้นรูปและส่วนผสมขององค์ประกอบวัสดุ จะเป็นปัจจัยสำคัญในการกำหนดลักษณะโครงสร้างหรือลักษณะรูพรุนของโครงสามมิติ ตลอดจนสมบัติต่างๆทั้งสมบัติทางกายภาพ สมบัติเชิงกล สมบัติทางเคมี และสมบัติทางชีวภาพ งานวิจัยนี้สนใจพัฒนาสารประกอบแบคทีเรียเซลลูโลสแบบใหม่เพื่อขยายขอบเขตของการนำไปประยุกต์ใช้ โดยการนำพอลิเมอร์ที่มีสมบัติที่ต้องการมาผสมกัน โดยใช้ไบโอพอลิเมอร์ที่มีสมบัติที่เป็นประโยชน์แตกต่างกัน เช่น อัลจิเนท ไคโตรซาน เจลว่านหางจระเข้ เจลาติน และ ใยไหม โดยพบว่าที่ปริมาณการเติมสารจำนวนหนึ่งสารประกอบที่ถูกดัดแปลงมีลักษณะที่เป็นเนื้อเดียวกัน ซึ่งแสดงถึงการผสมเข้ากันได้ดีของสารประกอบ จากการวิเคราะห์โดยเครื่อง FTIR แสดงให้เห็นถึงการปฏิสัมพันธ์โดยเฉพาะระหว่างหมู่ไฮดรอกซิล (hydroxyl group) ของแบคทีเรียเซลลลูโลส และ หมู่ฟังก์ชัน (functional groups)ของสารพอลิเมอร์ต่างๆที่เติมเข้าไป โดยเป้าหมายเริ่มต้นของงานวิจัยนี้เพื่อศึกษาพัฒนาวัสดุแบบใหม่สำหรับใช้เป็นโครงเลี้ยงเซลล์เนื้อเยื่อ อาทิเช่น เซลล์ผิวหนังคน เซลล์เยื่อบุผิวในปากเป็นต้น โดยได้ทำการผลิตวัสดุโดยเริ่มจากการใช้พอลิเมอร์ผสมจากพอลิเมอร์ธรรมชาติโดยมีแบคทีเรียเซลลูโลสเป็นตัวหลัก ศึกษาขั้นตอนและปัจจัยต่างๆในการขึ้นรูป และทำการศึกษาลักษณะต่างๆที่สำคัญของโครงเลี้ยงเซลล์ เช่นโครงสร้าง รูพรุน สมบัติทางเชิงกล พันธะเคมีที่เปลี่ยนไป การอุ้มน้ำ ความสามารถในการย่อยสลายเป็นต้น จากนั้นศึกษาถึงปัจจัยสำคัญที่มีผลต่อการเจริญของเซลล์เป้าหมาย เช่นโครงเลี้ยงเซลล์จะต้องไม่มีความเป็นพิษต่อเซลล์ ส่งเสริมการเจริญเพิ่มปริมาณของเซลล์ และเซลล์มีลักษณะในรูปแบบที่เหมาะสม ลักษณะการเกาะและการแผ่ตัวของเซลล์บนวัสดุ โดยมีจุดมุ่งหมายเพื่อพัฒนาองค์ความรู้ของการสร้างโครงเลี้ยงเซลล์ เพื่อการนำมาประยุกต์ใช้ในการบำบัดรักษา เช่นการรักษาบาดแผลบริเวณผิวหนัง การรักษาสภาพความพิการหรือบาดแผลในช่องปากเป็นต้น ซึ่งผลการทดลองสรุปได้ว่าการเติมสารพอลิเมอร์เหล่านี้ส่งผลอย่างเด่นชัดต่อลักษณะโครงสร้างรูพรุน ความโปร่งแสงของวัสดุ สมบัติเชิงกล ความสามารถในการอุ้มน้ำ และลักษณะความเข้ากันได้กับเนื้อเยื่อของสิ่งมีชีวิต งานวิจัยนี้ยังได้มีการปรับปรุงสมบัติของวัสดุไบโอพอลิเมอร์ให้มีความพิเศษมากขึ้นเช่นให้มีสมบัติการยับยั้งจุลลินทรีย์ ปรับปรุงสมบัติให้ช่วยยับยั้งเซลล์มะเร็งผิวหนังเป็นต้น ตลอดจนศึกษาความสัมพันธ์ของโครงสร้างของวัสดุต่อการเจริญและการพัฒนารูปแบบของเซลล์ต้นกำเนิด นอกจากนี้ยังได้ศึกษาต่อไปถึงการประยุกต์ใช้ไบโอพอลิเมอร์ในด้านอื่นๆ เช่นการนำไปใช้เป็นวัสดุตรึงเซลล์จุลินทรีย์ จากผลการทดสอบจากงานวิจัย สรุปได้ว่าวัสดุไบโอพอลิเมอร์ที่ถูกดัดแปลงเหล่านี้มีศักยภาพในการนำไปประยุกต์ใช้ในหลายๆด้านแตกต่างกันไปตามลักษณะโครงสร้างและองค์ประกอบ เช่นสามารถนำไปพัฒนาต่อเป็นวัสดุชีวภาพทางการแพทย์ ชีววัสดุใช้ในงานวิศวกรรมเนื้อเยื่อ ชีววัสดุสำหรับนำส่งยา, สำหรับตรึงเซลล์และเอนไซม์ และสำหรับทำเป็นบรรจุภัณฑ์อาหาร

งานวิจัยนี้เป็นการทำงานโดยใช้องค์ความรู้หลายด้านมาประยุกต์เข้าด้วยกัน โดยได้รับความร่วมมือจากผู้เชี่ยวชาญด้านการเพาะเลี้ยงเนื้อเยื่อ จาก คณะทันตแพทย์ศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย คือ ศ. ทพ ดร. ประสิทธิ์ ภวสันต์ และ รศ ทญ.ดร. นิรชา สารชวนะกิจ โดยได้ทำงานร่วมกันในการทดสอบสารประกอบแบคทีเรียเซลลูโลสที่ทำเป็นโครงเลี้ยงเซลล์เยื่อบุช่องปาก เพื่อนำไปพัฒนารักษาบาดแผลในปาก นอกจากนี้ได้มีการปรับปรุงวัสดุให้มีคุณสมบัติเพื่อการเกาะติดของเซลล์ที่ดีขึ้นและมีสมบัติการยับยั้งจุลินทรีย์ อีกทั้งยังมีการพัฒนาสมบัติต่อเนื่องเพื่อการนำไปประยุกต์ใช้เพื่อยับยั้งเซลล์มะเร็งโดยได้รับความร่วมมือจาก ดร. ผ่องพรรณ ศิริพงษ์ หัวหน้ากลุ่มงานวิจัยสมุนไพร กลุ่มงานวิจัย สถาบันมะเร็งแห่งชาติ และยังได้ร่วมมือกับ Associate Professor Bi-min Zhang Newby จากภาควิชา Chemical and Biomolecular Engineering Department มหาวิทยาลัย The University of Akron ผู้เชี่ยวชาญด้านพอลิเมอร์และ การดัดแปลงพื้นผิว (surface modification) และ Dr. Liya Yin จากภาควิชาIntegrative Medical Sciences, Northeast Ohio Medical Universityในการปรับปรุงสมบัติพื้นผิวและการศึกษาผลของโครงสร้างของวัสดุต่อการเจริญและการพัฒนารูปแบบของเซลล์ต้นกำเนิด

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ผลงาน (Output) จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

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-หนังสือ (Book Chapter)

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-การจดสิทธิบัตร

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Characterization and biocompatibility of bacterial cellulose/alginate composite sponges with human keratinocytes and gingival fibroblasts

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ABSTRACT

The novel composite sponge of bacterial cellulose/alginate (BCA) has been developed for the use as mucosal flaps in oral tissue regeneration. The porous sponge was fabricated by a freeze drying process. The sponge matrix appeared organized in a three-dimensional network of nanofibrils. The FTIR spectra indicated the intermolecular interaction between bacterial cellulose (BC) and alginate. The in vitro studies with human keratinocytes (HaCat) and gingival fibroblasts (GF) demonstrated that the pure BC and the BCA sponges supported proliferations of the cells. However, in the wet state, only the BCA sponge with 30% alginate had a good tear resistance for sewing. The BCA composite sponge is a promising material for use as a non-adherent hydrogel dressing due to many advantages in terms of skin tissue compatibility, excellent water uptake ability, and high mechanical strength and stability in both water and PBS buffer.

1. Introduction

For the past ten years, a number of natural polymers have been studied for use as new alternative biomaterials. Bacterial cellulose (BC), which is synthesized by *Acetobacter xylinum* using glucose as a substrate, has been considered highly biocompatible with suitable properties allowing application in the tissue engineering and biomedical fields (Andrade, Pertile, Dourado, & Gama, 2010; Czaja, Young, Kawecki, & Brown, 2007). With the fine web-like network structure and high purity, BC provides significant benefits over plant cellulose. BC has advantages in terms of biocompatibility, non-toxicity, high mechanical strength, high swelling ability and high stability to pH variations. Compared to collagen or gelatin, BC does not contain any components of animal origin; additionally, it does not appear to cause allergic reactions. BC has been applied as artificial skin for patients with burns and ulcers (Fontana et al., 1990), temporary skin substitute for animals (Jonas & Farah, 1998), artificial blood vessels for microsurgery (Klemm, Schumann, Udhardt, & Marsch, 2001), material for a meniscal implant (Bodin et al., 2007) and as a potential scaffold for tissue engineering (Gao et al., 2011; Svensson et al., 2005).

Alginate, a linear polysaccharide (copolymer of (1-4)-linked β-mannuronic acid (M) and α-guluronic acid (G) monomers), is a natural polysaccharide derived primarily from brown seaweed. Due to its hydrogel properties and biocompatibility, alginate has also been widely used in biomedical applications, including wound management. The major function of alginate in tissue engineering applications is to provide mechanical integrity (Drury, Dennis, & Mooney, 2004). Alginate gel can form a gel on absorption of wound exudates, prevent the wound surface from drying out, minimize discomfort during removal (Thomas, 2000) and enhance the rate of healing of skin wounds (Jarvis, Galvin, Blair, & McCollum, 1987). Several therapeutic agents, including antibiotics, enzymes, growth factors and DNA, have already been successfully incorporated in alginate gels in order to maintain high biological activity (Smidsrod & Draget, 1996). Furthermore, alginate hydrogels have been applied as scaffolds for cartilage and bone regeneration (Alsberg, Anderson, Albeiruti, Franceshi, & Mooney, 2001).

To gain the beneficial properties of both BC and alginate, a novel porous sponge from a blend of BC and alginate was developed in this study. The structure, morphology, mechanical strength and biodegradability were characterized. In addition, for the further application as a mucosal flap in oral tissue regeneration, the biocompatibility of the alginate modified BC sponge was determined by examining its effect on HaCat and GF cells.

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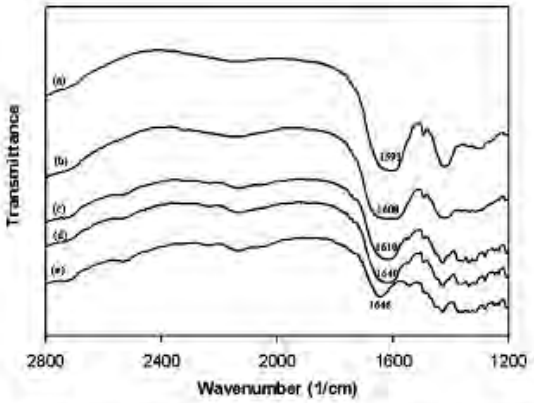

Fig. 1. FTIR spectra of (a) alginate, (b–d) BCA and (e) BC sponges in wave numbers ranging from 2800 to 1200 cm^{-1}. The weight ratios of BC/alginate are: (b) 30/70, (c) 50/50 and (d) 70/30.

2. Materials and methods

2.1. Preparation of the BCA composite sponge

The BC used in this study was the gel-like cellulose pellicle formed by *A. xylinum* AGR 60, kindly supplied by the laboratory of Prannote Tanonarate (the Institute of Food Research and Product Development, Kasetsart University, Bangkok). The medium for the inoculums was coconut-water containing 5.0% sucrose, 0.5% ammonium sulfate $(NH_4)_2SO_4$ and 1.0% acetic acid. Cell cultivation for BC biosynthesis was performed under static conditions as previously described (Sanchavanakit et al., 2006). The developed gel-like cellulose pellicle was then purified by washing with deionized (DI) water, treated with 1.0% (w/v) NaOH at 35 °C for 24 h to remove bacterial cells and rinsed with DI water until the pH was 7.0. The never dried BC was stored in DI water at 4 °C before use.

The BC pellicles (size 1 cm × 1 cm × 1 cm) were crushed to form BC slurry by using a homogenizer at room temperature. Sodium alginate of 1.5% (w/v) was dissolved in distilled water at room temperature to form a gel-like solution. Then, the BC slurry was thoroughly mixed with the alginate solution at weight ratios of BC/alginate at 100/0, 70/30, 50/50, 30/70, or 0/100 until homogeneous slurries were formed. Next, 50 g of the slurry mixtures were placed in Petri dish plates, cross-linked by an aqueous solution of 1.5 (w/v) $CaCl_2$ and rinsed with distilled water to remove the excess chlorides. Then, the mixtures were pre-frozen at −40 °C for 24 h prior to drying under vacuum pressure (~90 mTorr) at −40 °C for 24 h.

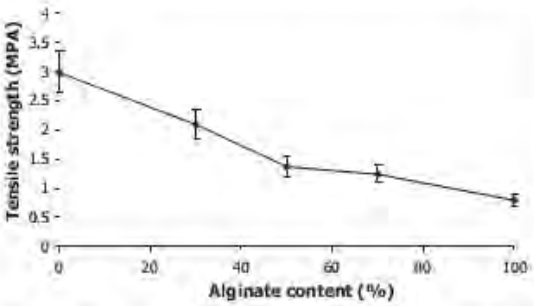

Fig. 2. The tensile strength of the BCA sponges as a function of alginate content (wt%).

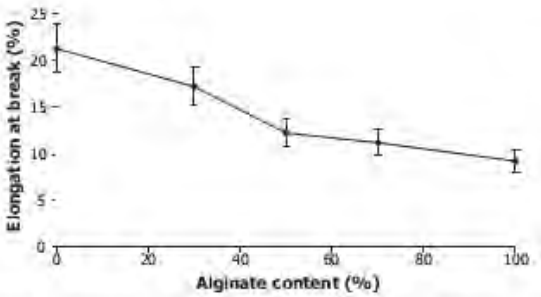

Fig. 3. The elongation at break of the BCA sponges as a function of alginate content (wt%).

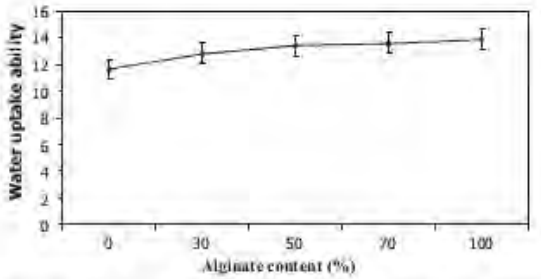

Fig. 4. The water uptake ability of the BCA sponges as a function of alginate content (wt%).

2.2. Characterization of the sponges

2.2.1. Elemental analysis

The calcium and sodium content were determined with an X-ray fluorescence (XRF) spectrometer (model ED2000, Oxford, United Kingdom).

2.2.2. Fourier transform infrared (FT-IR) spectroscopy

The structural information was collected using a Perkin Elmer Spectrum GX FT-IR Microscope (Waltham, MA). The FT-IR spectra of the samples were measured from 2800 cm^{-1} to 1200 cm^{-1} at a resolution of 4 cm^{-1}.

2.2.3. Mechanical testing

The samples were cut into strip-shaped specimens that were 10 mm in width and 10 cm in length (50 mm between the grips). A universal testing machine (INSTRON 5567, Rochester, NY) was used to determine tensile strength of the samples at the stretching speed of 2 mm/min. The ten-

Table 1
The sponge elemental content analyzed by X-ray fluorescence spectroscopy (XRF).

Blending composition (BC/alginate)	Elemental content (%) by XRF	
	Na	Ca
100/0	–	–
70/30	–	0.88
50/50	–	2.49
30/70	–	2.96
0/100	–	5.57

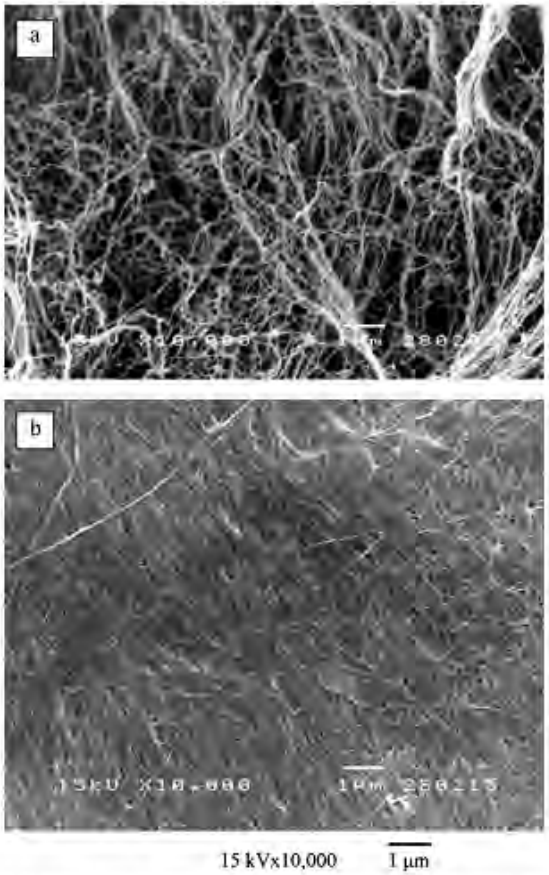

Fig. 5. SEM images of cellulosic fibers after homogenization (a) and surface morphology of the BCA sponge (b).

sile strength was the average value determined from five specimens.

2.2.4. Water uptake ability

To measure the water uptake ability (the equilibrium water content) of the sponge, pre-weighed dry sample was immersed in distilled water for 1 min. After the bulk water was removed by placing the wet sponge on the Petri dish for 1 min, the weight of wet sample was measured. All testing was run in triplicate, and the equilibrium water content was determined using the following equation:

$$\text{Water uptake ability} = \left(\frac{W_w - W_d}{W_d}\right)$$

where W_w and W_d represent the weight of wet and dry samples, respectively.

2.2.5. Scanning electron microscopy (SEM)

The morphology of BC, BCA and alginate sponges was analyzed by scanning electron microscope (SEM); model JSM-5410LV, JEOL (Tokyo, Japan). The freeze-dried samples were sputter coated with gold (JEOL JFC 1100 E ion sputtering device) and photographed at an accelerating voltage of 15 kV and magnifications of 35–10,000.

2.2.6. In vitro studies

Cells were cultured in a 24-well polystyrene plate using growth medium composed of Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin-B. The cells were incubated in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. At confluence, HaCat and GF cells were harvested using a suspension of 0.25% trypsin and subcultivated in the same medium with 12 and 4 dilutions, respectively.

HaCat and GF cells were used to evaluate adhesion and proliferation as a direct contact test. The sponges (13 mm in diameter and 2 mm in thickness) were immersed in 70% ethanol for 5 min for sterilization, followed with four successive solvent exchanges with deionized water. The sponges were then placed on a 24-well polystyrene plate (NUNC, Roskilde, Denmark), and 350 μl of culture medium (Sanchavanakit et al., 2006) was added to each well before cell seeding (6×10^4 cells/well). Cells were allowed to initially attach for 5 h. For the proliferation test, cells were seeded onto each of the matrices, and the cultures were harvested after 6, 24 or 48 h. The attached or proliferated cells were then quantified using the 3-(4,5-dimethythiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay.

Three hundred and fifty microliters of MTT solution (0.5 mg/ml in DMEM without phenol red, filter-sterilized) was added to each culture well. After incubation for 5 h, the MTT reaction medium was removed, and 900 μl of dimethylsulfoxide and 100 μl of glycine buffer (pH 10.5) were added. Optical densities were determined by spectrophotometer (Genesis 10 UV scanning, Rochester, NY) at 570 nm.

3. Results and discussion

3.1. Elemental analysis

The X-ray fluorescence (XRF) spectroscopy results (Table 1) showed no sodium (Na) peak. Therefore, NaOH was completely removed from the freeze-dried sponges. The XRF spectra indicated a calcium content of 0.88–2.96 wt% in the BCA sponges, depending on the alginate content. The calcium accumulation in the BCA and alginate sponges should be due to the formation of a calcium/alginate gel network by cross-linking with Ca^{2+} ions, as previously described (Phisalaphong, Suwanmajo, & Tammarate, 2008).

3.2. FT-IR spectrophotometric analysis

The FT-IR spectra of the BCA sponges at various blending compositions were measured from 2800 to 1200 cm^{-1}, as shown in Fig. 1. The BC spectra showed a band at 1646 cm^{-1}, which was attributed to the glucose carbonyl of cellulose. The BCA sponges exhibited the characteristic absorption bands with the appearance of no new peaks or the disappearance of any peaks associated with the parent molecules. The interaction between BC and alginate could be identified by the carbonyl and carboxyl group bands present in the range of 1900–1500 cm^{-1}. The carboxyl group bands for the BCA sponges of 70, 50 and 30% alginate were shifted from 1591 cm^{-1} (100% alginate) to 1608, 1610 and 1640 cm^{-1}, respectively. The shifts could be attributed to intermolecular interactions between the hydroxyl group of cellulose and the carboxyl group of alginate, which might disrupt the hydrogen bonding between cellulose fibers. Similar observations were previously reported in cotton cellulose/alginate blend membrane (Yang, Zhang, Peng, & Zhong, 2000) and BC/alginate blend membrane (Phisalaphong et al., 2008).

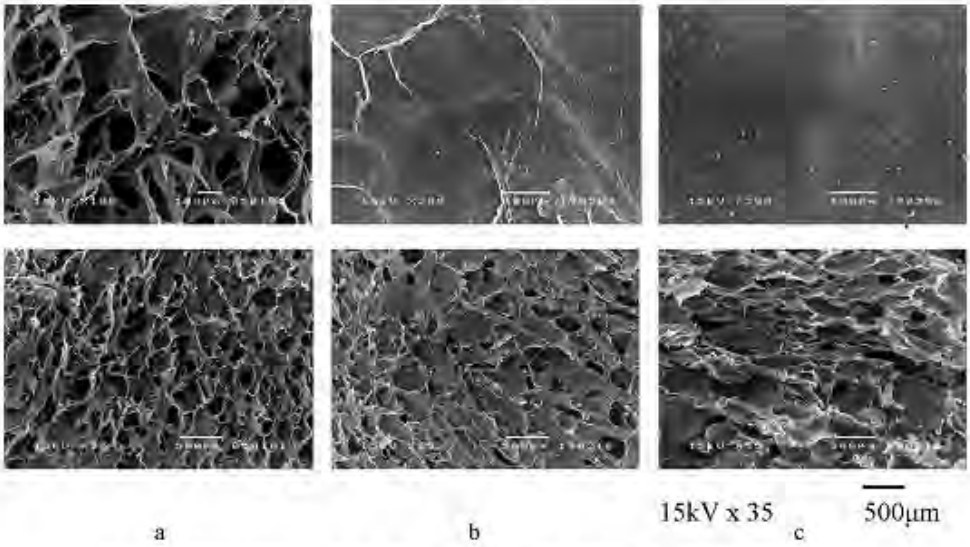

Fig. 6. SEM micrographs of surface (top) and cross-section (bottom) of (a) BC, (b) BCA and (c) alginate sponges.

3.3. Mechanical strength

Tensile strengths of freeze-dried sponges are shown in Fig. 2. The tensile strength of the BC sponge (0% alginate) was greater than that of the alginate sponge (100% alginate). For those of BCA sponges, the tensile strength decreased with an increase of alginate content. Similarly, the elongations at break of the BCA sponges also decreased with an increase of alginate content as shown in Fig. 3. It was previously reported that the presence of alginate decreases the mechanical properties of BC film (Kanjanamosit, Muangnapoh, & Phisalaphong, 2010; Phisalaphong et al., 2008; Wu et al., 2004), suggesting that the intermolecular hydrogen bonding of cellulose might be broken down or disrupted to form cellulose-alginate hydrogen bonds. The intermolecular interactions might reduce the crystallinity and mechanical strength of the composite material.

3.4. Water uptake ability and structural stability

Water uptake ability and structural stability of sponges play an important role in their practical use in biomedical applications. The water uptake ability of BCA sponges with different alginate concentration is shown in Fig. 4. The structural stability of the freeze-dried sponges was investigated by immersion in water and in a phosphate buffer solution (PBS).

The water uptake ability of the BC sponge was approximately 11.7 times of its weight in water. The freeze-dried sponges of BC swelled rapidly after the immersion in water or in PBS. However, because the BC sponge was composed of homogenized BC fibrils without a strong linking network, its structural stability was poor in the wet state. During the immersions, the BC sponges gradually collapsed and disintegrated within 2 and 3 h in PBS and in water, respectively.

The water uptake ability of the alginate sponge was 13.9 times of its weight, which was higher than that of the BC sponge.

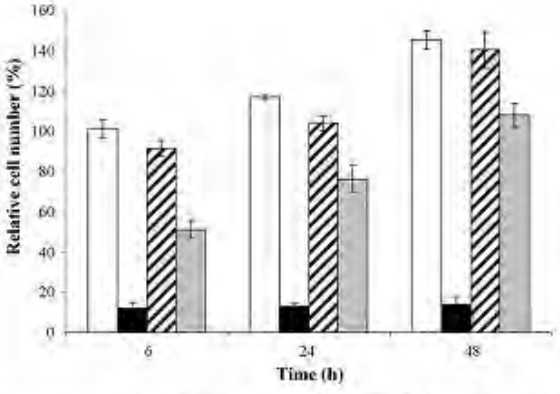

Fig. 7. Proliferations of HaCat on tissue culture plastic (□), the alginate sponge (■), the BC sponge (▨) and the BCA sponge (▤). Percentage of living cells was assessed at 6, 24, and 48 h of cultures by MTT assay.

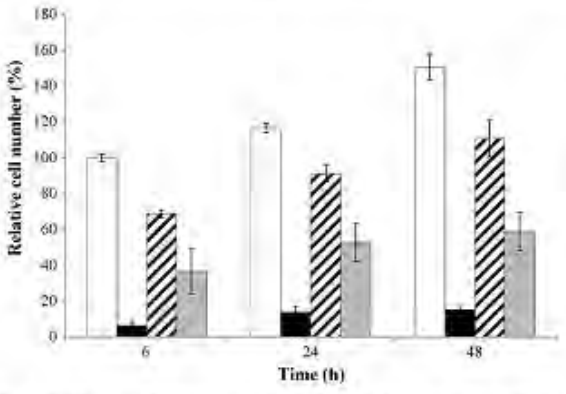

Fig. 8. Proliferations of GF on tissue culture plastic (□), the alginate sponge (■), the BC sponge (▨) and the BCA sponge (▤). Percentage of living cells was assessed at 6, 24, and 48 h of cultures by MTT assay.

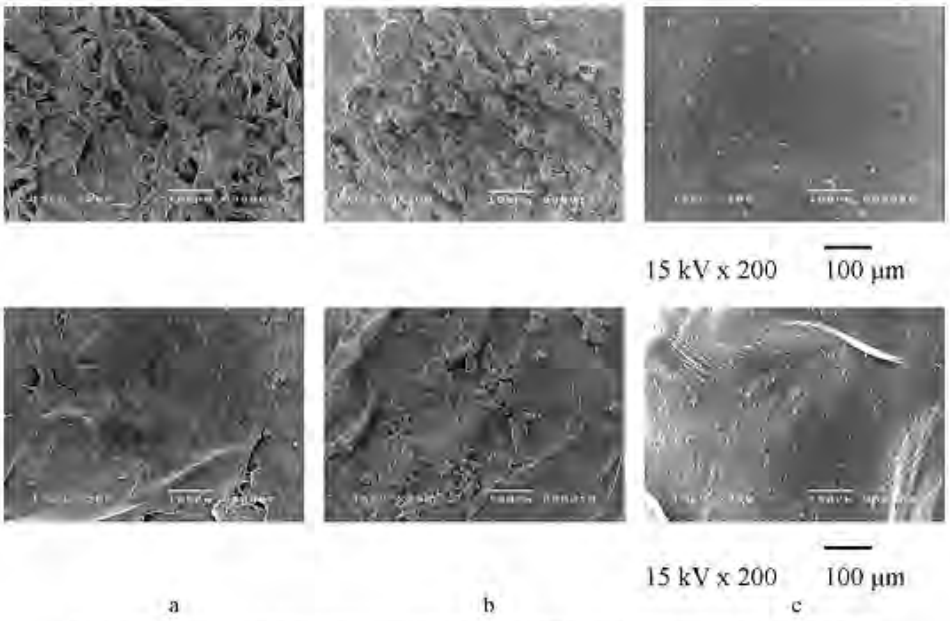

Fig. 9. SEM images of HaCat (top) and GF (bottom) on the (a) BC, (b) BCA and (c) alginate sponges at 48 h after seeding.

Water molecules are easily absorbed into alginate due to its highly hydrophilic property. The freeze-dried alginate sponge was stable in distilled water; however, it completely collapsed within 30 min in PBS. Drury et al., in 2004 reported that the dissolution of alginate hydrogel leads to the weakening of sponges based on alginate. Because the cross-linked calcium ions can be exchanged with other non-gel-inducing cations in PBS, the reduction in cross-linking is induced in the buffer solution causing erosion and destruction of alginate gel. Phosphate can sequester the calcium ions cross-linked with alginate, consequently destabilizing the calcium-alginate gel (Smidsrod & Skjak-Braek, 1990).

The supplement of alginate in the BCA sponges resulted in the increase in water uptake capacity. Blending with alginate could disrupt the hydrogen bonding between the cellulose fibers and result in the increase in the water uptake ability of the modified BC. The BCA sponges rapidly swelled and were stable in water. However, some structural disruption and disintegration of the sponges with an alginate content of more than 30% occurred after 2 h of immersion in PBS. The freeze-dried BCA sponge of 70% BC and 30% alginate was more stable and retained its overall size for the entire course of the examination (48 h). Because the structure of BCA sponge with 30% alginate could remain stable in water and PBS, it has better potential for use in a wide range of clinical settings. In addition, the excellent swelling ability of the sponge could facilitate adsorption of wound exudates.

3.5. Morphology

After the homogenization treatment, defibrillated BC appeared in form of groups of cellulose nanofibrils dispersed in aqueous solution (Fig. 5(a)). In order to fabricate the BCA sponge, the homogeneous mixture composed 30% alginate and 70% defibrillated BC was cast in Petri dish plates and was cross-linked in an aqueous solution of 1.5 (w/v) $CaCl_2$ before freeze-drying. The SEM image for close look of BCA sponge surface was shown in Fig. 5(b). After the cross-linking and freeze-drying, the water-insoluble connected network of BC-alginate was obtained. The conditions used in the cross-linking and freeze-drying processes strongly affected the structure of the sponge.

SEM micrographs of the BC, BCA and alginate sponges (Fig. 6) show the porous structure with the three-dimensional interconnection throughout the sponges. The BCA and alginate sponges had an asymmetric structure consisted of a top skin layer and sponge-like porous layer, similar to a functional wound dressing of chitosan sponge proposed by Mi et al., in 2003. The dense outer layer helps to prevent bacterial invasion and to avoid wound dehydration, whereas the porous support layer provides for the drainage of wound exudates and mechanical strength. The pore size of the sponges fell in the range of 100–500 µm, which is suitable for usage in tissue engineering (Yang, Leong, Du, & Chua, 2001).

3.6. In vitro study

Cell culture experiments with HaCat and GF cells were carried out to evaluate the biocompatibility of the sponges. Proliferations at 6, 24 and 48 h after seeding of HaCat and GF cells on the BC, BCA and alginate sponges, in comparison to those on the tissue culture plastic (polystyrene), are shown in Figs. 7 and 8, respectively. The BC and BCA sponges supported proliferations of HaCat and GF cells, while few cells of both cell types were detected on the alginate sponge. The proliferative rates of HaCat and GF cells were in the following order: on tissue culture plastic > BC > BCA > alginate. In a previous report by Svensson et al., in 2005, the proliferation tests of bovine chondrocytes showed that the percentage of relative cell viability on BC scaffolds were greater compared to those on tissue culture plastic and calcium alginate.

Morphologies of HaCat and GF cells on the BC, BCA and alginate sponges at 48 h after seeding are revealed in Fig. 9. HaCat cells responded to the BC and BCA sponges by exhibiting good attachment and spreading. The growing GF cells on the BC sponge demonstrated normal cell morphology with typical spindle shape and spread covering the material surface. GF cells only attached

onto the BCA sponge, however, the cells were still in round shape indicating that this modified material selectively supported spreading of HaCat, not GF. Very few cells of both cell types were observed on the alginate sponge.

Preliminary study on tear resistance was performed in water saturated BCA sponge by using 24 mm surgical needle (18-8 stainless steel, spring eye, cutting edge, 3/8 circle, Mani Inc., Tochigi, Japan) and braided silk US 3/0 (Pearsalls Ltd., Somerset, England) to suture material only together. The result showed that BCA was able to be sutured, since it resisted to the surgical needle and silk including force during the procedure. However further study, especially in vivo experiment, is required for definite information.

The BCA sponge has the potential to be used as a wound dressing material due to its biocompatibility, structural stability and good tear resistance. The BC sponge supported growth and spreading of HaCat and GF cells; however, it could not retain its shapes throughout the cell culture study (48 h).

4. Conclusions

A novel biocompatible porous sponge from the blend of BC and sodium alginate has been developed. The blend was fabricated in the form of a porous sponge by cross-linking with a $CaCl_2$ solution followed by a freeze drying process. FTIR analysis indicated that an intermolecular interaction exists between BC and alginate. The BCA sponge with 30% alginate has advantages in terms of biocompatibility, high mechanical strength, high water uptake ability and structural stability. The BCA sponges supported the proliferation of HaCat and GF cells. The BCA composite sponge was conceived as a temporary dressing and should be removed everyday of contact. It had an asymmetric structure consisted of a top skin layer and sponge-like porous layer. The dense outer layer helps to prevent bacterial invasion and to avoid wound dehydration, whereas the porous support layer provides for the drainage of wound exudates and mechanical strength. The pore size of the sponges fell in the range of 100–500 μm, which is suitable for usage in tissue engineering. In addition, the sponge had good tear resistance during sewing procedures. Therefore, the BCA sponge has a good potential to be used in the oral cavity to cover the surgical wound.

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Ethanol production by repeated batch and continuous fermentations of blackstrap molasses using immobilized yeast cells on thin-shell silk cocoons

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ABSTRACT

A thin-shell silk cocoon (TSC), a residual from the silk industry, is used as a support material for the immobilization of *Saccharomyces cerevisiae* M30 in ethanol fermentation because of its properties such as high mechanical strength, light weight, biocompatibility and high surface area. In batch fermentation with blackstrap molasses as the main fermentation substrate, an optimal ethanol concentration of 98.6 g/L was obtained using a TSC-immobilized cell system at an initial reducing sugar concentration of 240 g/L. The ethanol concentration produced by the immobilized cells was 11.5% higher than that produced by the free cells. Ethanol production in five-cycle repeated batch fermentation demonstrated the enhanced stability of the immobilized yeast cells. Under continuous fermentation in a packed-bed reactor, a maximum ethanol productivity of 19.0 g/(L h) with an ethanol concentration of 52.8 g/L was observed at a 0.36 h^{-1} dilution rate.

1. Introduction

Because of diminishing crude oil resources and increasing fuel costs in recent years, ethanol has re-emerged as an alternative to petrochemical-based liquid fuels. Ethanol is a renewable and clean fuel produced using biomass as the raw material. It is by far the most widely used biofuel for transportation worldwide [1]. In Thailand, gasohols E10 and E20 (with mixtures of 10% and 20% ethanol, respectively, in gasoline) have been widely used in vehicles, and there are attempts underway to promote the use of E85 in vehicles in the near future. According to the report on net renewable energy analysis in Thailand, a positive net energy of 5.95 MJ/L was obtained from the molasses-based ethanol system [2].

Traditional ethanol industries produce ethanol by batch or fed-batch processes. Cell immobilization in continuous fermentation has been proposed as an effective means of improving the ethanol production rate. Cell immobilization could lead to a higher biomass concentration and enhanced biological stability. The protection of immobilized cells against toxicity has been reported previously [3,4]. Almost all cell immobilization methods are based on adsorption or entrapment. Gel entrapment methods usually involve the problems of gel degradation and limitations of oxygen, nutrient and metabolite mass transfer. In contrast, natural adsorption is generally a simple and inexpensive procedure for cell immobilization without internal mass transfer limitations. Moreover, at the end of the fermentation, the carriers of yeast cells adsorbed onto agricultural byproducts can be modified into an animal feed co-product. Various biomaterials have been examined as support materials for yeast cell immobilization, including wood blocks [5], porous cellulose [6,7], apple cuttings [8], loofa sponges [9,10], and sorghum bagasse [3].

In this work we attempt to develop a new and effective method of yeast immobilization for ethanol production. A thin-shell silk cocoon (TSC), a residual from the silk industry, was used as a natural support material because of its advantages, which include its biocompatibility, light weight, high strength, low cost, chemical stability, high porosity and high surface area. TSC is the shell cocoon after removing the outer silk layer. It has been reported to have special microstructure, good mechanical strength when wet, resistance to enzymatic cleavage and high oxygen permeability [11]. Silk filaments adhere to form the cocoon shell. The filament is composed of two proteins: fibroin and sericin. Fibroin has a high content of the amino acids glycine and alanine. Sericin is characterized by its high content of serine and 18 amino acids, including essential amino acids [12]. There have been reports on the improved activity and stability of immobilized enzymes using silk fibroin as a support [13].

Raw silk is produced in many regions throughout Thailand. However, the silkworms are grown primarily in the northeast region. According to the report by EU-Thailand Small Projects Facility in 2007, the world production of raw silk was about 135 thousand tons in 2004. Thailand was the sixth largest raw silk producing country with the production about 1550 tons per year. In Thailand,

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a few amounts of TSCs are used as handicraft making raw material, as material for filling pillowcase or as scrub cocoons for traditional remedy. The leftovers are used as fish food and fertilizer. Owing to its substantial quantity, low price and advantageous properties, it has the potential to be used as immobilizing material for ethanol industries. Therefore, in this study, thin-shell silk cocoons were used for the natural immobilization of yeast cells for ethanol production in repeated batch and continuous fermentations of blackstrap molasses. To the best of our knowledge, this is the first report of the use of silk cocoons for yeast cell immobilization.

2. Materials and methods

2.1. Yeast strains, culture media and cell preparation

Saccharomyces cerevisiae M30, a high ethanol-producing flocculent strain, was used in this study. The stock culture was stored in potato dextrose agar (PDA) slants at 4 °C. Starter cultures were prepared by transferring a loop of the stock culture to 100 mL of the sterilized pre-culture medium, which was composed of 50 g/L reducing sugar from palm sugar, 0.05% w/v $(NH_4)_2SO_4$, 0.01% w/v KH_2PO_4 and 0.0035% w/v $MgSO_4.7H_2O$. The initial pH of the medium was adjusted to 5.0. Cell cultivation was carried out in Innova 4330 refrigerated incubator shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm and 33 °C for about 20 h. The late exponential-phase cells were harvested by decantation to obtain the stock cell suspension.

2.2. Cell immobilization

TSCs of mulberry silkworms were purchased from a local silk factory (Yala, Thailand) (Fig. 1). Cell immobilization was performed as follows. 2.5 g of TSCs and 250 mL of culture medium in 500 mL flasks were autoclaved separately for 15 min at 121 °C. 10 mL of stock cell suspension was then added to the sterilized culture medium. The sterilized TSCs were immersed in the culture medium and incubated at 150 rpm and 33 °C for 24 h to induce natural cell adhesion.

2.3. Fermentations

The five-cycle repeated batch fermentations using the suspended and TSC-immobilized cultures of *S. cerevisiae* M30 were carried out in duplicate using blackstrap molasses as the main substrate for ethanol fermentation. The experiment was initiated by transferring the suspended or immobilized cultures into 250 mL of culture medium containing 22–24% (w/v) of initial reducing sugar and 0.5 g/L $(NH_4)_2SO_4$ in a 500 mL Erlenmeyer flask. The flasks were shaken in the incubator at 150 rpm at 33 °C. The duration of each batch was 48 h. The experiment was monitored by removing 5 mL samples every 8 h for cell, sugar and ethanol analysis.

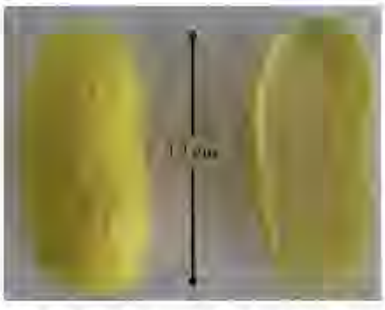

Fig. 1. Thin-shell silk cocoons (TSCs) of the mulberry silkworm.

Continuous fermentations were carried out in a 1 L (Ø = 5.7 cm; height = 43.4 cm) packed-bed reactor containing an immobilized cell bed (Fig. 2). The temperature of the system was controlled at 32 ± 1 °C by passing 28 °C cooling water inside the reactor jacket. The initial sugar concentrations of 220 and 240 g/L were continuously fed into the bottom of the reactor at dilution rates at 0.11, 0.16, 0.20 and 0.30 h^{-1}. Five milliliter samples were collected every 8 h. All samples were frozen before sugar, ethanol, and cell concentration analysis to analyze them simultaneously.

2.4. Analytical methods

Ethanol concentration was determined using gas chromatography (GC-7AG, Shimadzu, Japan) [14]. Residual sugar content was measured using the 3,5-dinitrosalicylic acid (DNS) method [15]. Free cell dry weight was determined from the absorbance at 660 nm measured by a UV-2450 UV–Visible spectrophotometer (Shimadzu Scientific Instruments, Inc., Japan) and converted to dry cell concentration using a corresponding standard curve. For cell dry weight determination, a 5 mL sample of the fermentation broth was centrifuged at 3000 rpm for 10 min. The cell pellet was resuspended in 0.1 N HCl and washed twice with distilled water and then dried at 90 °C for 48 h and then weighed. To determine the immobilized cell concentration, the carrier was cut into small pieces and stirred in deionized (DI) water for 1 h. After the carriers were removed, the immobilized cell concentration was determined similar to how it was for the free cells. The immobilization yield (Y_I) was determined from the ratio of immobilized cell concentration (X_I) to the total cell concentration (X_T), and X_F was the free cell concentration. The ethanol yield ($Y_{P/S}$) was determined from the ratio of ethanol accumulation to sugar consumption.

A series of SEM images was taken to provide a visual characterization of the cell carrier. The samples were frozen in liquid nitrogen, immediately snapped, vacuum-dried, sputtered with gold and then photographed. Images were collected on a JOEL (Tokyo, Japan) JSM-5410LV scanning electron microscope.

3. Results and discussion

3.1. Batch and repeated batch fermentation

Preliminary studies (data not shown) of 72-h batch fermentation have shown that an optimal ethanol concentration of 98.6 g/L with an ethanol yield ($Y_{P/S}$) of 0.47 (g/g) are obtained by TSC-immobilized cells (TSC-cell) at an initial reducing sugar concentration of 240 g/L. The ethanol concentration and conversion yield of the immobilized cells were 11.5% and 9.3% higher than those of the free cells. The inhibitory effect of high sugar concentration on ethanol fermentation by free cells has been previously reported [14]. It was found that, at high initial sugar concentrations, the system using TSC-cells was more efficient than when using free cells.

Ethanol production using TSC-cells was further examined by five-cycle repeated batch fermentation. The results of the ethanol production and reducing sugar consumption by the immobilized cells were compared to those using the suspended cells (SC). The duration of the experiments on each batch was 48 h, and the initial sugar concentration was 240 g/L. Table 1 summarizes the yields, end products and productivities of the repeated batch fermentations. In the first batch, the initial rates of ethanol production and sugar consumption in the TSC-cell culture were slightly lower than those in the SC culture. Nonetheless, the final end-product concentration, overall sugar consumption and ethanol yield ($Y_{P/S}$) were similar in both cultures. From the second to the fifth batch

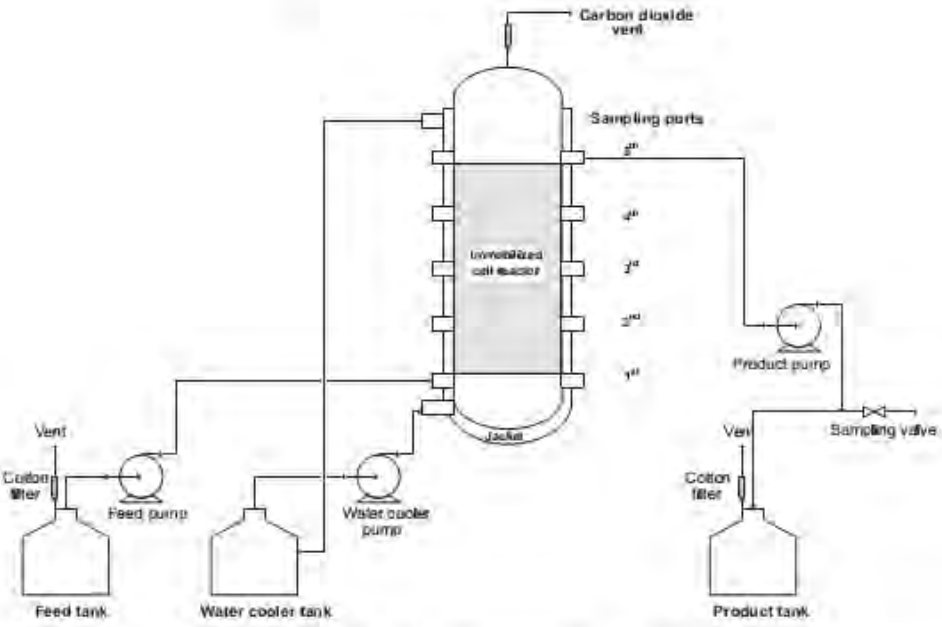

Fig. 2. Schematic diagram of the immobilized-cell packed-bed reactor.

Table 1
Concentrations and yields of end products and ethanol productivities during the five-cycle repeated batch fermentation using cultures of suspended cells (SC) and TSC-immobilized cells (TSC-cell).

Batch		Ethanol (P, g/L)	Cell concentration (g/L)			Y_i (%)	$Y_{P/S}$ (g/g)	Q_P (g/(L h))	Conversion of sugar into ethanol (%)
			X_F	X_I	X_T				
I	SC	88.8	3.5	–	3.5	–	0.47	1.85	91.98
	TSC-cell	88.1	0.6	4.9	5.5	89.1	0.47	1.84	91.98
II	SC	65.9	5.1	–	5.1	–	0.43	1.37	84.15
	TSC-cell	67.9	0.8	6.3	7.1	89.3	0.44	1.41	86.11
III	SC	79.8	6.5	–	6.5	–	0.45	1.66	88.06
	TSC-cell	88.3	1.0	7.9	8.9	89.6	0.47	1.84	91.98
IV	SC	72.3	7.1	–	7.1	–	0.46	1.51	90.02
	TSC-cell	87.6	1.2	10.6	11.8	90.4	0.48	1.83	92.89
V	SC	9.4	7.4	–	7.4	–	0.38	0.20	74.36
	TSC-cell	77.6	1.4	13.2	14.6	91.1	0.44	1.62	86.11

of TSC-cell culture, the ethanol production, sugar consumption and $Y_{P/S}$ appeared higher than those in the SC culture. A remarkably higher stability of the TSC-cell over SC was observed in the fifth batch. While the final ethanol concentration in the SC system dropped dramatically to 9.9 g/L in the fifth batch, a final ethanol concentration of 77.6 g/L was maintained in the TSC-cell system. High concentrations of ethanol have been reported to inhibit the growth and metabolism of free *S. cerevisiae* cells [14]. The enhanced cell stability of the immobilized cell culture compared to the free cell culture implies that the TSC carriers may have protected the yeast cells from toxic conditions during the fermentation process. The increased stability of the immobilized cells over the non-immobilized cells is attributed to the immobilization matrices' protective effects against the toxicity of metabolic products, as previously reported [3,4].

The final total cell concentration of the TSC-cell system was 14.6 g/L, which was approximately twice that of the SC system (7.4 g/L). An increase in the number of yeast cells adsorbed on the inner/outer surfaces and in the micro-porous structure of the fibril network of the TSC was observed. As shown in Fig. 3, the TSC cross-section exhibited a considerable increase in cell concentration from the initial time (0 h) to the end of the repeated batch fermentation (240 h). The cells had a normal oval shape with an average length of 2–5 μm. Cellular flocculation and adhesion to the TSC surfaces was observed. The average immobilization yield (Y_i) was 89.9%. The reuse of immobilized cells during repeated batch fermentation using cell carriers such as Ca-alginate matrices [16], Loofa sponges [10], and alginate–Loofa [4], has been previously reported.

3.2. Continuous fermentation

Continuous ethanol fermentation using TSC-cell culture in a 1 L packed-bed reactor was carried out at the following operating conditions: 32 ± 1 °C, feed sugar concentrations of 220 and 240 g/L and dilution rates of 0.04, 0.15, 0.24 and 0.36 h^{-1}. The steady-state ethanol concentration and productivity are shown in Fig. 4 as functions of the dilution rate. Table 2 summarizes the cell concentrations after 20 days of continuous operation along with the average immobilized yield (Y_i) and the average ethanol yield ($Y_{P/S}$).

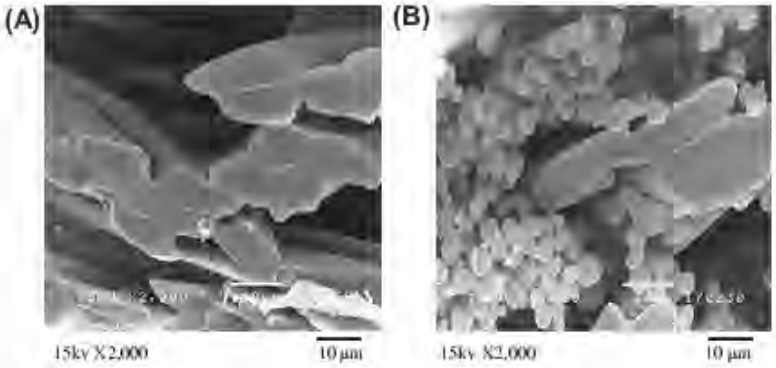

Fig. 3. A cross-section of a thin-shell silk cocoon (TSC) at the initial time (0 h) and after the five-cycle repeated batch fermentation (240 h).

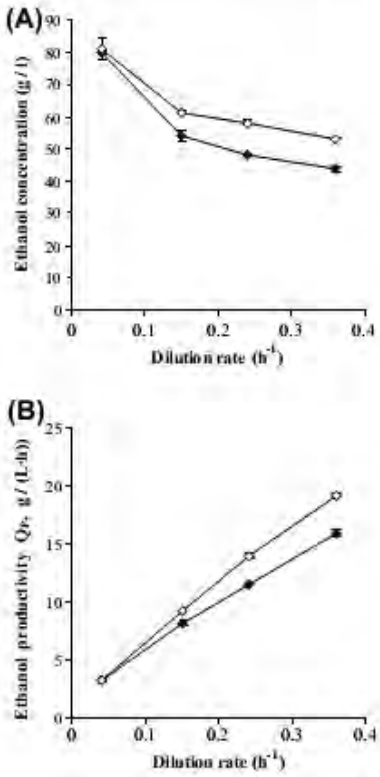

Fig. 4. The steady state ethanol concentration (A) and productivity (B) as a function of dilution rate during continuous-flow, packed-bed fermentations using TSC-immobilized cells with initial sugar concentrations of 220 (◇) and 240 (◆) g/L.

With a feed sugar concentration of 220 g/L at the dilution rates of 0.04, 0.15, 0.24 and 0.36 h^{-1}, the average ethanol concentrations at steady state were 80.7, 61.7, 58.1 and 52.8 g/L, respectively. The ethanol productivity increased approximately linearly with the dilution rate. In this study, a maximum productivity of 19.0 g/(L h) appeared at a dilution rate of 0.36 h^{-1}, which was 12.6-fold higher than that of the batch fermentation. At a dilution rate of 0.04 h^{-1} (HRT = 25 h), an ethanol concentration of 80.7 g/L was obtained, which was comparable to the average concentration from the fed-batch fermentation (48-h batch fermentations) but slightly lower than that of the single-batch fermentation (72-h batch fermentations).

Table 2
Concentrations and yields of end-products by TSC-cell fermentation after 20 days of continuous fermentation with a feed sugar concentration of 220 g/L at the dilution rates of 0.04 h^{-1}.

Concentrations and yields of end-products	Average values
Ethanol concentration (g/L)	80.6
Immobilized cell concentration (X_i, g/L)	40.0
Free cell concentration in reactor (X_{FR}, g/L)	5.5
Free cell concentration in effluent (X_{FE}, g/L)	0.5
Immobilized yield (Y_i, %)	87.0
Ethanol yield ($Y_{P/S}$, g/g)	0.48

The ethanol concentration and productivity were lower at a feed sugar concentration of 240 g/L. Although it was capable of producing 80.0 g/L ethanol with a feed sugar concentration of 240 g/L at a dilution rate of 0.04 h^{-1}, the ethanol productivities at dilution rates ranging from 0.15–0.36 h^{-1} were significantly lower than those obtained at the same dilution rates at a feed sugar concentration of 220 g/L. These results show that the high residual sugar concentration in the continuous system (especially at a high dilution rate) can trigger a negative inhibitory effect on cell activity.

The continuous ethanol fermentation in the packed-bed reactor was performed with a feed sugar concentration of 220 g/L at a dilution rate of 0.04 h^{-1} over a 20-day period without breakage of the carriers. The cell concentration data after 20 days of continuous fermentation are shown in Table 2 and indicate that the yeast cells were efficiently confined by the packed bed. Only 0.4–0.5 g/L of the free cells were in the effluent, whereas almost all of the cells were restrained in the packed bed reactor. After 20 days of continuous fermentation, the average immobilized yield from the continuous fermentation in the packed-bed reactor was 87.0%, which was slightly lower than that of the repeated batch operation. The free cells in the reactor appeared healthy and retained their normal oval shape. The cells mostly flocculated in big groups of 50–200 cells, whereas the free cells in the effluent were suspended as single cells or small groups of 3–5 cells (data not shown).

The maximum ethanol productivity, immobilized yield and cell density during continuous fermentation in the packed-bed reactor using TSC-cell cultures were relatively high compared to those of our previous work using alginate–loofa as a support material [17]. There have been reports on enhanced ethanol productivity by continuous fermentation with immobilized cells using other support materials, such as calcium alginate [18,19], calcium pectate [20] and polyurethane foam cubes [21].

4. Conclusions

We have demonstrated the potential use of TSC for repeated batch and continuous ethanol fermentation. The immobilization of *S. cerevisiae* M30 was performed by adsorption on TSC. Many advantages, such as reusability, altered mechanical strength, cell regeneration and high immobilized yield, were achieved and resulted in stable operation with a high ethanol production and high biomass density. A comparative study of repeated batch fermentation showed that the TSC-cell system was more stable than the conventional free-cell system. A maximum ethanol productivity of 19.0 g/(L h) was obtained at a dilution rate of 0.36 h^{-1} with an average immobilized yield of 87.0% during the continuous fermentation of blackstrap molasses in a 1 L packed bed reactor. Since the applications of immobilized cells on TSC shows the potential benefits for ethanol production, the further investigation of the continuous ethanol fermentations using immobilized yeast cells on TSC in a pilot scale is ongoing in order to determine the applicability of commercial practices.

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High-temperature ethanol fermentation by immobilized coculture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*

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Suspended and immobilized cocultures of the thermotolerant yeast, *Kluyveromyces marxianus* DMKU 3-1042 and the mesophilic flocculent yeast, *Saccharomyces cerevisiae* M30 were studied for their abilities to improve production and stability of ethanol fermentation. Sugarcane juice and blackstrap molasses, at initial sugar concentrations of 220 g/L, were used as carbon sources. The results indicated that the coculture system could improve ethanol production from both sugarcane juice and blackstrap molasses when the operating temperature ranged between 33°C and 45°C. High temperature tolerances were achieved when the coculture was immobilized. The immobilized coculture was more effective in high-temperature ethanol fermentation than the suspended cultures. The coculture immobilized on thin-shell silk cocoon and fermented at 37°C and 40°C generated maximal ethanol concentrations of 81.4 and 77.3 g/L, respectively, which were 5.9–8.7% and 16.8–39.0% higher than those of the suspended cultures, respectively.

[Key words: Ethanol; Coculture; Sugarcane; Molasses; Immobilization]

Bioethanol is a renewable energy source that can be produced from agricultural feedstocks such as sugarcane juice, blackstrap molasses, cassava, potato, and corn. In Thailand, cane juice and blackstrap molasses are potential raw materials for ethanol fuel production. Cane juice contains 12–17% total sugars, of which 90% is sucrose and 10% is glucose and fructose (1). Blackstrap molasses contains 45–60% total sugars, of which approximately 50–55% is sucrose and 40–45% is glucose and fructose. Yeast can directly ferment glucose and fructose to produce ethanol. To ferment sucrose, yeast must first use the enzyme invertase to convert sucrose to glucose and fructose.

Thailand is one of the tropical countries where the average day-time temperature is usually high (between 30°C and 36°C) throughout the year. Therefore, ethanol fermentation at high temperatures has received much attention (2). The advantages associated with the production of ethanol at high temperatures include increased rate of productivity, reduced cooling costs and reduced risk of contamination. It has previously been demonstrated that a flocculent strain, *Saccharomyces cerevisiae* M30, is capable of producing ethanol in blackstrap molasses medium at the operating temperature ranging from 30°C to 35°C (3). However, its growth and ethanol producing activities are suppressed at temperatures higher than 37°C. High-temperature ethanol fermentation has been reported for *Kluyveromyces marxianus* (4–7). It has been demonstrated that *K. marxianus* DMKU 3-1042 was an effective strain for ethanol production at elevated temperatures up to 45°C when sugarcane juice was used as a raw material (2).

In an attempt to improve ethanol fermentation, cell immobilization techniques have been developed to increase the rate of ethanol production and to protect cells from factors that inhibit ethanol production (8–12). In our previous studies, immobilized cell carriers, such as alginate-loofa matrix (ALM) (10,11) and thin-shell silk cocoons (TSC) (12), were successfully used for flocculating *S. cerevisiae* M30 cells in both repeated batch and continuous ethanol fermentation. The immobilized yeast cells were able to remain viable and functioned normally, with high stability for long-term use. Alternatively, efforts for improved ethanol fermentation with cocultures in a single process have also been reported. The concept of cocultures has been widely utilized for the production of ethanol from low cost materials, such as cellodextrins and hemicellulose dextrins (13–15), glucose and xylose mixtures (16,17), sorghum carbohydrates (18,19) and starch (20–23), that are otherwise not easily converted into ethanol by monoculture.

In this study, a coculture of the mesophilic yeast, *S. cerevisiae* M30, and the thermotolerant yeast strain, *K. marxianus* DMKU 3-1042, was used to enhance ethanol production under a wide range of fermentation temperatures. The immobilization of coculture using TSC and ALM carriers was employed to enhance ethanol and temperature tolerances of the cells. Ethanol production by batch fermentation was measured from suspended and immobilized cells grown in coculture and monocultures. The traditional industrial raw materials blackstrap molasses and sugarcane juice were used as carbon sources.

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MATERIALS AND METHODS

Yeast strains, culture media and cell preparation *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 were used for ethanol fermentation. The stock cultures were stored in potato dextrose agar (PDA) slants at 4°C. Starter cultures were prepared by transferring a loop of the stock culture to 100 mL of the sterilized pre-culture medium containing 100 g/L reducing sugar from palm sugar, 0.5 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4 and 0.035 g/L $MgSO_4 \cdot 7H_2O$. The initial pH of the medium was adjusted to 5.0. Cell cultivation was conducted in an Innova 4330 refrigerated incubator shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm with 2.54 cm diameter circular orbit and 33°C for 20 h. The late exponential-phase cells were harvested by decantation to obtain the stock cell suspension with the average cell dry weight concentration of 8.3 g/L.

Cell immobilization on alginate-loofa matrix (ALM) To prepare the ALM-immobilized cells, 5 mL of cell suspension from monocultures or coculture (at a 1:1 volume ratio of *S. cerevisiae* M30: *K. marxianus* DMKU 3-10425) was added to 50 mL of 30 g/L sodium alginate solution to form an alginate-cell mixture. Then, 2.5 g of sterilized cubic sponges of loofa (19 mm × 19 mm × 2 mm) was dipped into the alginate-cell mixture. The gel carriers were transferred to 15 g/L $CaCl_2$ solution and left to harden in this solution with mild stirring for 15 min. The carriers were then rinsed 3 times with 9 g/L NaCl solution (11).

Cell immobilization on thin-shell silk cocoons (TSC) TSCs of mulberry silkworms from a local silk factory (Yala, Thailand) were also used as the other immobilized support. The sterilized TSCs of 2.5 g was added to 250 mL of pre-inoculum medium with 5 mL of cell suspension from monocultures or coculture (in a 1:1 ratio of *S. cerevisiae* M30: *K. marxianus* DMKU 3-10425) and incubated at 33°C in 500-mL shaking flasks with the rotation speed of 150 rpm for 24 h to induce natural cell adhesion (12).

Fermentations The batch fermentation was performed in duplicate using blackstrap molasses and cane juice as the main substrate for ethanol fermentation. The experiment was initiated by transferring 12.5 mL (5.0% v/v) stock cell suspension or immobilized cells on 2.5 g solid support (ALM or TSC) into 250 mL of media containing 220 g/L of initial reducing sugar and 0.5 g/L $(NH_4)_2SO_4$ in a 500-mL Erlenmeyer flask. Fermentation flasks were then shaken in the incubator at 150 rpm, 33°C for 72 h. The experiments were monitored by removing 3-mL samples every 8 h for sugar and ethanol analyses.

Analytical methods Ethanol concentration was determined using a gas chromatograph (model GC-7AG; Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector. To measure the total reducing sugar (RS) concentration, the sample solution was hydrolyzed with 33% (w/v) HCl at 100°C for 10 min, and neutralized with a NaOH solution. The RS content was then determined using the 3, 5-dinitrosalicylic acid (DNS) method. Cell dry weight concentrations were measured at the initiation and at the termination of fermentation. For determining dry weights of suspended cells, a 3-mL sample of fermentation broth was centrifuged at 3000 rpm for 10 min. The cell pellet was resuspended in 0.1 N HCl, washed twice with distilled water, dried at 90°C for 48 h and then weighed. Immobilized cell concentrations in loofa-reinforced alginate matrix (ALM) and thin-shell silk cocoon (TSC) were determined according to previously described methods (11,12). The immobilization yield (Y_I) was determined from the ratio of immobilized cell concentration (X_I) to the total cell concentration (X_T), and X_F was the free cell concentration. The ethanol yield ($Y_{P/S}$) was determined from the ratio of ethanol accumulation to sugar consumption.

A series of SEM images was taken to provide a visual characterization of the cell cultures. The samples were snap-frozen in liquid nitrogen, vacuum-dried, sputtered with gold and then photographed. Images were collected on a JSM-5410LV scanning electron microscope (JOEL, Tokyo, Japan).

RESULTS AND DISCUSSION

Suspended cell cultures SEMs of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 monocultures and the coculture after 24 h of cultivation in blackstrap molasses are shown in Fig. 1. *K. marxianus* DMKU 3-1042 has an oval shape with a diameter of 1–3 μm (Fig. 1A), whereas *S. cerevisiae* M30 is bigger with a diameter approximately 3–6 μm (Fig. 1B). Cell agglomeration in the coculture of *K. marxianus* DMKU 3-1042 and *S. cerevisiae* M30 was observed (Fig. 1C). Ethanol and cell concentrations from batch fermentations of the coculture of *S. cerevisiae* and *K. marxianus* in a volume ratio of 1:1 were compared with those from the monocultures (Fig. 2). The average initial cell concentrations were 0.4 g/L. After 72 h of fermentation, at temperatures ranging from 33°C to 45°C, the final ethanol concentration using the coculture was either higher or equivalent to that of *S. cerevisiae* or *K. marxianus* monocultures. At increasing operating temperatures from 33°C to 45°C, the average

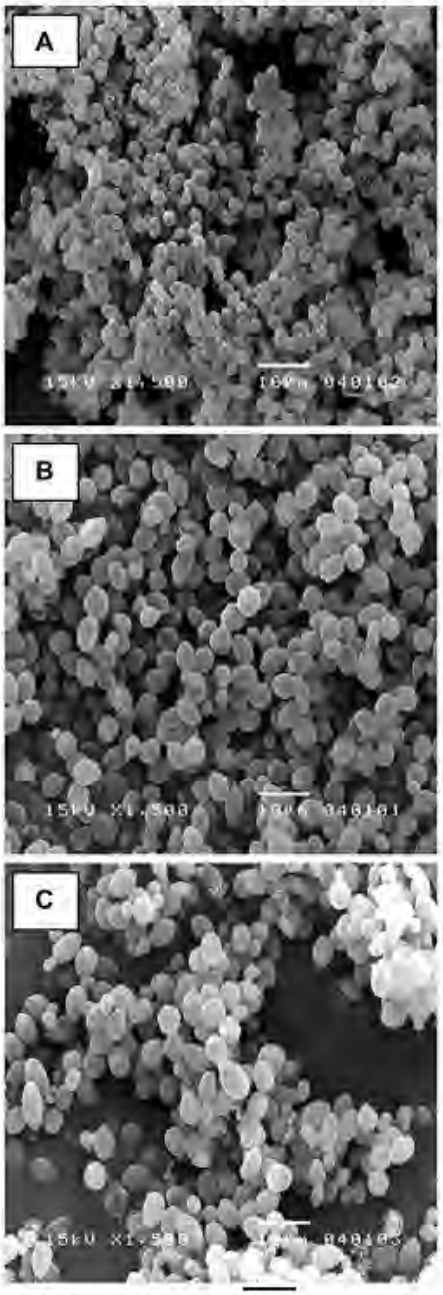

FIG. 1. SEM images of (A) the monoculture of *K. marxianus* DMKU 3-1042, (B) the monoculture of *S. cerevisiae* M30 and (C) the coculture after 24 h of batch fermentation.

final ethanol concentration of the coculture system was reduced from 103.9 to 62.3 g/L, respectively, when using the sugarcane juice medium and 87.9 to 40.0 g/L, respectively, when using the blackstrap molasses medium. The cell concentration in both

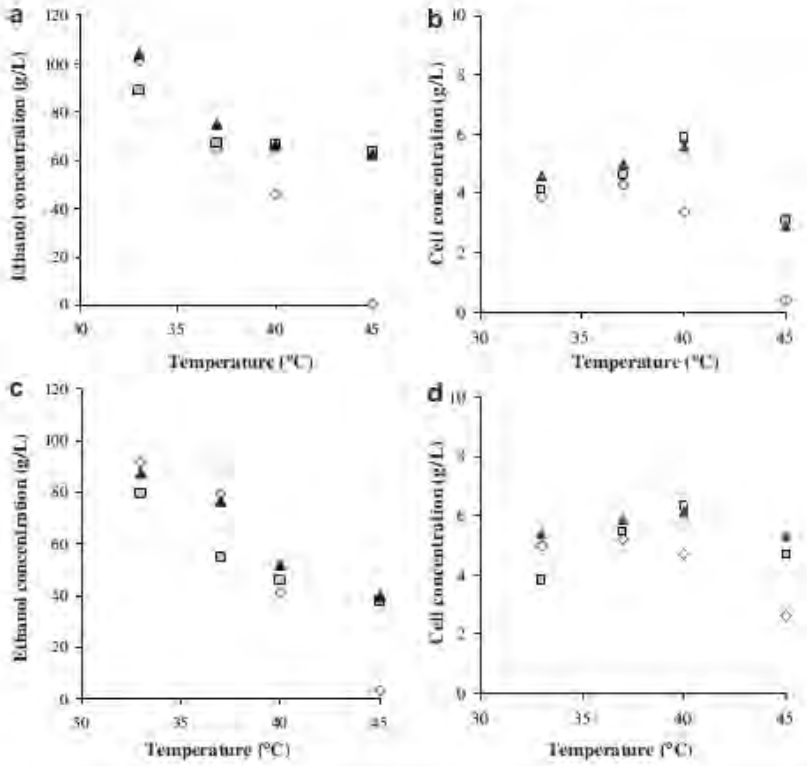

FIG. 2. Final concentrations of ethanol (a, c) and cells (b, d) after 72 h of batch fermentation at 33°C, 37°C, 40°C and 45°C using cane juice medium (a, b) and blackstrap molasses medium (c, d). Open squares, *K. marxianus* DMKU 3-1042; open diamonds: *S. cerevisiae* M30; closed triangles: the coculture.

coculture and monoculture of *K. marxianus* increased with an increase in temperature from 33°C to 40°C and subsequently decreased at 45°C. In the monoculture system of *S. cerevisiae*, a considerable decrease in cell concentration was observed with increasing temperature from 37°C to 45°C.

At the operating temperature range from 33°C to 37°C, the coculture and the monoculture of *S. cerevisiae* were more effective in producing ethanol than the monoculture of *K. marxianus*. *S. cerevisiae* M30 was more effective in ethanolic fermentation of blackstrap molasses when the fermentation temperature did not exceed 37°C. At high temperatures (range of 40–45°C), the coculture and the monoculture of *K. marxianus* were more effective in producing ethanol compared to the monoculture of *S. cerevisiae*. *K. marxianus* DMKU 3-1042 was able to produce ethanol at a high temperature of 40–45°C, particularly in sugarcane medium. It has been previously reported that *K. marxianus* DMKU 3-1042 was effective in producing ethanol at elevated temperature up to 45°C when sugarcane juice was used as a raw material (2). Therefore, the coculture of the thermotolerant yeast *K. marxianus* DMKU 3-1042 and the mesophilic yeast *S. cerevisiae* M30 could increase ethanol production under

TABLE 1. Yields and end products of batch ethanol fermentation at 37°C for 72 h using the cultures of suspended cells (SC), alginate-loofa-matrix-immobilized cells (IC-ALM) and thin-shell silk cocoon-immobilized cells (IC-TSC).

System			Ethanol (g L^{-1})	Cell (g L^{-1})				$Y_{P/S}$	Q_P (g L^{-1} h^{-1})
Cell Type	C-source	Cultures		X_F	X_E	X_T	Y_E		
SC	Cane juice	*K. marxianus*	67.0	5.3	–	5.3	–	0.40	0.93
		Coculture	74.9	5.0	–	5.0	–	0.40	1.04
	Blackstrap molasses	*K. marxianus*	54.5	4.8	–	4.8	–	0.38	0.76
		Coculture	76.9	5.3	–	5.3	–	0.43	1.07
IC-ALM	Cane juice	*K. marxianus*	70.0	1.7	3.9	5.7	0.69	0.40	0.97
		Coculture	73.2	1.9	4.1	6.1	0.68	0.41	0.98
	Blackstrap molasses	*K. marxianus*	66.1	0.4	3.6	4.0	0.89	0.37	0.92
		Coculture	80.4	1.3	3.3	4.6	0.71	0.40	1.12
IC-TSC	Cane juice	*K. marxianus*	79.8	0.5	4.8	5.3	0.91	0.40	1.11
		Coculture	81.4	0.3	4.9	5.2	0.94	0.41	1.10
	Blackstrap molasses	*K. marxianus*	65.0	0.5	4.4	4.9	0.90	0.38	0.90
		Coculture	81.4	0.2	5.0	5.2	0.96	0.41	1.13

X_F, X_E and X_T are the free cell, immobilized cell and total cell concentrations, respectively. Immobilized yield (Y_E) is the ratio of X_E to X_T. At $t = 0$, ethanol concentration (P_0) and sugar concentration (S_0) are 0 g/L and 220 g/L, respectively. Ethanol yield ($Y_{P/S}$) is the ratio of ethanol accumulation ($P - P_0$) to sugar consumption ($S_0 - S$). Q_P is the ethanol productivity.

TABLE 2. Yields and end products of batch ethanol fermentation at 40°C for 72 h using the cultures of suspended cells (SC), alginate-loofa-matrix-immobilized cells (IC-ALM) and thin-shell silk cocoon-immobilized cells (IC-TSC).

System			Ethanol (g L^{-1})	Cell (g L^{-1})				$Y_{P/S}$ (%)	Q_P (g L^{-1} h^{-1})
Cell Type	C-source	Cultures		X_F	X_I	X_T	Y_I		
SC	Cane juice	*K. marxianus*	66.4	4.8	–	5.8	–	0.42	0.92
		Coculture	66.2	5.5	–	5.5	–	0.43	0.92
	Blackstrap molasses	*K. marxianus*	45.5	5.7	–	5.7	–	0.34	0.63
		Coculture	52.0	6.1	–	6.1	–	0.37	0.72
IC-ALM	Cane juice	*K. marxianus*	66.3	1.5	3.4	4.9	0.69	0.38	0.92
		Coculture	66.5	0.9	2.2	3.1	0.71	0.39	0.92
	Blackstrap molasses	*K. marxianus*	64.7	1.6	3.3	4.8	0.68	0.38	0.90
		Coculture	65.6	1.7	3.6	5.4	0.68	0.39	0.91
IC-TSC	Cane juice	*K. marxianus*	77.2	0.3	2.6	2.9	0.89	0.44	1.07
		Coculture	77.3	0.7	3.0	3.7	0.82	0.44	1.07
	Blackstrap molasses	*K. marxianus*	69.1	0.5	2.5	3.0	0.84	0.39	0.96
		Coculture	72.2	0.7	3.9	4.5	0.86	0.42	1.00

X_F, X_I and X_T are the free cell, immobilized cell and total cell concentrations, respectively. Immobilized yield (Y_I) is the ratio of X_I to X_T. At $t = 0$, ethanol concentration (P_0) and sugar concentration (S_0) are 0 g/L and 220 g/L, respectively. Ethanol yield ($Y_{P/S}$) is the ratio of ethanol accumulation ($P-P_0$) to sugar consumption (S_0-S). Q_P is the ethanol productivity.

fermentation over a wide temperature range (33–45°C) in both sugarcane juice and blackstrap molasses medium.

In tropical countries, industrial fuel ethanol fermentation is typically performed using mesophilic yeast under controlled condition of temperature varying between 30°C and 35°C. The fermentation cooling cost has an important impact on fuel ethanol production costs. It was demonstrated that if the fermentation temperature could be raised by 5°C, it would greatly reduce the fuel ethanol production costs (24). As shown in this study, the coculture of *K. marxianus* DMKU 3-1042 and *S. cerevisiae* M30 resulted in enhancement of thermal stability and extended operating temperature range, which would help reduce heating and cooling requirements during the fermentation to delivering cost-effective fuel ethanol production. It was previously reported that the coculture system of *S. cerevisiae* and *K. marxianus* produced more ethanol from cheese whey powder solution compared to the monoculture system (25). Another study also reported that the coculture system of *S. cerevisiae* and *K. marxianus* was more effective than the monoculture system of *K. marxianus* for ethanol production from henequen juice and molasses (26).

Immobilized cell cultures Due to high year-round temperatures in tropical countries, ethanol and temperature tolerances are important characteristics of yeast cultures for commercial ethanol production in these areas. Our previous works demonstrated the improved ethanol tolerance in ALM-immobilized cell culture (10,11) and TSC-immobilized cell culture (12). In this study, the use of ALM and TSC-immobilized coculture of *K. marxianus* DMKU 3-1042 and *S. cerevisiae* M30 was investigated for their abilities to improve temperature tolerance. Without cooling system, the expected maximum temperature in ethanol fermentation is 37–40°C; therefore, the fermentation performances of suspended and immobilized cell cultures of *K. marxianus* DMKU 3-1042 grown at 37°C (Table 1) and 40°C (Table 2) were determined.

The results showed that both TSC and ALM had the potential to be used as cell carriers for monoculture of *K. marxianus* and coculture of *K. marxianus* and *S. cerevisiae*. At the operating temperatures of 37 and 40°C, the final ethanol concentrations in the immobilized cell cultures using either TSC or ALM were either equivalent to or higher than those of the suspended cell cultures. In blackstrap molasses medium, ethanol production showed significant improvements by using the immobilized cell cultures. The better performance of the immobilized cultures might be attributed to the benefit of the cell protection conferred by the immobilized systems when exposed to harmful conditions, such as high concentration of inhibitors and high temperatures (10–12). The results indicate that the thermotolerance characteristic could be improved by the use of ALM and TSC-immobilized cell systems.

Higher ethanol productions (up to 16.3% depending on the culture and culture medium) were observed in the TSC-immobilized cell system when compared with those of the ALM-immobilized cell system. Our previous work reported some increases in maximum ethanol productivity, immobilized yield and total cell density in a packed-bed reactor using TSC-immobilized *S. cerevisiae* M30 compared with those using ALM as a support material (12). The high biocompatibility and suitable porous structure of TSC are important characteristics that promote the yield and activities of the immobilized cells. In this study, under the controlled temperatures of 37°C and 40°C, the TSC-immobilized coculture was the most effective in producing ethanol from both sugarcane juice and blackstrap molasses. The maximum ethanol concentrations at 81.4 and 77.3 g/L were obtained at 37°C and 40°C with ethanol yields ($Y_{P/S}$) of 0.41 and 0.43, and immobilized yields (Y_I) of 0.95 and 0.84, respectively. No significant difference in final free and immobilized cell concentrations was obtained by using blackstrap molasses medium as compared to cane juice medium. Nonetheless, in blackstrap molasses medium, slightly higher total cell concentrations were observed in the coculture systems. The ethanol concentrations using the TSC-immobilized coculture at 37°C and 40°C were 5.9–49.4% and 16.8–58.6% higher, respectively, than those of suspended cell cultures, depending on the carbon source and the culture employed. The coculture was more effective than the monoculture of *K. marxianus*, especially when the blackstrap molasses medium was used.

Compared to their respective monocultures, the coculture of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 offered advantages in ethanol fermentation from sugarcane juice and blackstrap molasses medium and resulted in the better overall performance of ethanol production under the controlled temperature range of 33–45°C. In addition, the thermotolerance of the cells was increased when the coculture was grown on immobilization supports such as TSC and ALM. Based on the results of this work, TSC-immobilized coculture has potential applications in commercial ethanol fermentation, especially in tropical countries.

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Bacterial cellulose–alginate composite sponge as a yeast cell carrier for ethanol production

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ABSTRACT

A bacterial cellulose–alginate (BCA) sponge, fabricated by a freeze-drying process, was successfully used as a yeast cell carrier for ethanol fermentation. The BCA sponge exhibited several advantageous properties, such as high porosity, appropriate pore size, strong hydrophilicity and high mechanical, chemical and thermal stabilities. BCA has an asymmetric structure, with a thin, dense outer layer covering an interior of interconnected macropores that are distributed throughout the sponge, which is effective for yeast immobilization. At 48 h of the fermentation, the maximum ethanol concentration produced by the immobilized culture (IC) in the BCA carrier was about 100 g/L, which was approximately 13% and 45% higher than that from the suspended culture (SC) and from IC in Ca-alginate matrix, respectively. Repeated-batch ethanol productions using IC in BCA carriers were also more stable than those using SC or IC in Ca-alginate matrix. The results of a 15 cycle repeated batch operation demonstrated that the system with IC in BCA exhibited superior long-term stability for ethanol fermentation with the average ethanol productivity at 1.9 g/L h and the immobilized yield at 86%. The improved ethanol fermentation performance was mainly due to the water uptake ability and properly interconnected pore structure, which help to overcome limiting mass transfer.

1. Introduction

Immobilized culture (IC) technology has been applied to both repeated batch and continuous ethanol fermentations to improve ethanol productivity, increase cell density, retain the stability of the cells' activities and protect cells from inhibitors [1,2]. The inhibition effect of the substrate and ethanol on the cell activities, lowering both cell and ethanol yields has been extensively studied [3,4]. During ethanol fermentation, the existence of either substrate or product inhibition in a long-term operation reduce the activity of yeast cells, resulting in lower ethanol production. In this case, immobilized cell systems with proper carriers could help yeast cells tolerate inhibitors or unsuitable conditions better than free cell systems, resulting in higher and more stable ethanol productivity [5]. Many immobilization techniques have been applied in ethanol fermentation systems such as entrapment [6,7], adsorption [8,9] and self-aggregation [10]. The entrapment of cells within beads of calcium alginate is a commonly used technique for immobilizing living cells, especially in smaller scale laboratory settings, because it is simple and maintains high cell viability and activity [11]. However, the drawbacks of alginate gel beads, including its poor mechanical properties, its rapid degradation and its dense structure, have limited its applications in larger scale production in industrial fermentation processes [12–14]. During fermentation, the evolution of carbon dioxide influences alginate gel (AL) by expanding and causing rapid disruption. Consequently, AL is not a stable cell carrier for use in long-term ethanol fermentation [14]. The chemical and physical properties of AL could be improved by blending it with some special polymers such as polyvinyl alcohol [15,16] and polyethylene oxide [17,18]. Sodium alginate-graft-poly (N-vinyl-2-pyrrolidone (NaAlg-g-PVP) beads was developed and applied for ethanol fermentation [19]. Due to the hydrophilic property of N-vinyl-2-pyrrolidone, this polymeric bead showed better mass transfer characteristics compared with conventional alginate beads. The incorporation of alumina into AL improved the porous structure of the carrier and had a positive influence on yeast cell activity during ethanol fermentation [12]. In addition, improved mechanical and biological properties could be obtained from a composite of bacterial cellulose (BC) and AL [20,21]. Our novel composite sponge made of BC and AL was fabricated by a freeze-drying process, and it has been successfully developed for use as mucosal flaps in oral tissue regeneration [22]. The freeze-dried sponge with a blending composition of 70/30 BC/AL was stable in both water and PBS and retained its overall size after being used as a cell scaffold.

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The bacterial cellulose (BC) was synthesized by *Acetobacter xylinum* in the form of a pellicle, a fine web-like network of cellulose fibers. BC has been reported to have many unique properties, including a high crystallinity, high water retention, high tensile strength and high purity. It has also been reported to have high biocompatibility and low toxicity [23,24].

To obtain suitable immobilization carriers, characteristics of the carrier such as pore structure, water content, hydrophilicity and electrical charge should be considered [25,26]. Ethanol production from IC can be improved by a polymeric matrix carrier with high hydrophilicity [19]. However, excessive hydrophilicity and excessively large pores could lead to the decrease of polymer strength and linkage of immobilized cells [27]. Yu et al. [13] also reported that a larger number of pores in sorghum bagasse facilitated the mass transfer of substrates and products in IC. In addition, the higher electrical conductivity of the modified sorghum bagasse promoted ethanol production and cell adhesion [28].

In the present study, a bacterial cellulose–alginate composite sponge (BCA) was fabricated via freeze-drying to carry yeast cells for ethanol fermentation. The BCA sponges had an asymmetric structure, consisting of a top dense skin layer and a sponge-like porous layer. The optimal ratio of bacterial cellulose to alginate and the suitable concentration of alginate were investigated. The ethanol productions by batch and repeated batch fermentation using BCA-immobilized culture were compared with those of suspended culture (SC) as well as immobilized culture (IC) entrapped within alginate gel (AL). The stability of BCA in a long-term operation was also studied.

2. Materials and methods

2.1. Microorganism and culture media

The yeast strain used throughout the experiments was *Saccharomyces cerevisiae* M30, which was provided by the laboratory of Dr. Savithree Limthong (Department of Microbiology, Kasetsart University, Bangkok). The stock culture was maintained on a Potato Dextrose Agar (PDA) slant at 4 °C. Starter cultures were prepared by transferring cells from stock PDA slants to 150 mL of sterilized culture medium in a 500 mL Erlenmeyer flask. The culture media was composed of 100 g/L reducing sugar in the form of palm sugar, 0.05% (w/v) $(NH_4)_2SO_4$, 0.01% (w/v) KH_2PO_4 and 0.0035% (w/v) $MgSO_4{\cdot}7H_2O$. The initial pH of the culture media was adjusted to 5.0 by the addition of either 0.1 M NaOH or 0.1 M HCl solution. The cell culture was then cultivated in an Innova 4330 Refrigerated Incubator Shaker from New Brunswick Scientific (NJ, USA) at 33 °C and 150 rpm for 24 h. The stock cell suspension was obtained by decantation of the cell culture at the late exponential phase.

2.2. BCA sponge preparation and cell immobilization

The BCA sponge was prepared using the following three-step method: (1) homogenizing the Na-alginate-BC solution, (2) cross-linking with a $CaCl_2$ solution to form a gel and (3) freeze-drying the BCA hydrogel. First, the sodium alginate solution was formulated by dissolving Na-alginate powder in deionized (DI) water. The purified BC pellicles (size 1 cm × 1 cm × 1 cm) were homogenized in a blender and were then mixed with the Na-alginate solution in a weight ratio of 50/50, 60/40 and 70/30. To form the cross-linked BCA hydrogel, the homogeneous slurry mixture was poured into a tray and was cross-linked by the addition of a 0.12 M $CaCl_2$ solution for 24 h. Next, the BCA hydrogel was rinsed with deionized (DI) water 3 times and was submerged in DI water overnight to removing any excess Ca^{2+}. The BCA hydrogel was then placed in a −40 °C freezer for 24 h and dried under vacuum pressure (< 100 mTorr) for 48 h. The dried BCA sponges were cut into rectangular shapes of 2.0 cm × 2.0 cm × 0.2 cm (≈0.116 g) and were sterilized in an autoclave for 15 min at 121 °C. Then, the 2.5 g sterile BCA sponges were mixed with 250 mL of sterile culture medium in a 500 mL Erlenmeyer flask. Finally, 10 mL of stock cell suspension was added to the culture medium and incubated at 150 rpm and 33 °C for 24 h to induce natural cell adsorption.

2.3. Batch and repeated batch fermentations

The IC in the BCA sponges were each transferred into a 500 mL Erlenmeyer flask, containing 250 mL of sterile main medium with 220 g/L of initial sugar from cane molasses and 0.05% (w/v) $(NH_4)_2SO_4$ at a pH of 5.0. All experiments were carried out at 33 °C and 150 rpm for 72 h and were performed in duplicate. The experiment was monitored by aseptically removing 2 mL of sample every 8 h for cell, sugar and ethanol analysis. For repeated batch fermentations, after 48 h of each initial batch, the fermentation broth was withdrawn, the ethanol and sugar concentrations were determined, and the BCA sponges were transferred to fresh medium to begin the next batch.

2.4. Analytical method

A gas chromatograph (Model GC-7 AG) from the Shimadzu Co. (Shimadzu, Japan), equipped with a flame ionization detector, was used to analyze the ethanol concentration. To measure the total sugar concentration, the sample solution was hydrolyzed with 370 g/L HCl in a boiling water bath for 10 min. After hydrolysis, the sample was neutralized by 200 g/L NaOH. The total sugar content was then determined by a YSI (Model 2700) SELECT Biochemistry Analyzer from YSI Incorporated (OH, USA). To calculate the free cell concentration, the absorbance at 660 nm was measured by a UV-2450 UV–Visible spectrophotometer (Shimadzu Scientific Instruments, Inc., Japan) and then converted to the dry cell concentration using a corresponding standard curve [29]. To determine the IC concentration, the carrier sponge was cut into small pieces and stirred in DI water for 1 h. Then, the carrier was removed and the concentration of cells in the resulting suspension was determined similarly to the free cell calculation. Cell concentrations were reported in dry cell mass (g) per volume of fermentation broth (L). The immobilization yield (Y_I) was determined from a ratio of the immobilized cell concentration (X_I) to the total cell concentration (X_T). The ethanol yield ($Y_{P/S}$) was determined from a ratio of ethanol accumulation to sugar consumption.

2.5. Characterization

2.5.1. Scanning electron microscope (SEM)

An examination of the structural properties of the carriers was performed by scanning electron microscopy (SEM). The carriers were frozen in liquid nitrogen, immediately snapped, and vacuum-dried. Then, the carriers were sputtered with gold and photographed. The coated specimens were examined under SEM using a JOEL JSM-5410LV microscope (Tokyo, Japan).

2.5.2. Equilibrium water content

The equilibrium water content (EWC) was determined by immersing the pre-weighed dry sample of BCA in DI water at room temperature until equilibration occurred. The BCA was then removed from the water. After excess water on the surface of the BCA was blotted off with Kimwipes paper, the weight of the swollen BCA was determined. This procedure was repeated until there was no further weight change. All tests were run five times, and the

equilibrium water content was determined using the following equation:

$$\text{Water uptake ability} = \frac{W_w - W_d}{W_d}$$

where W_w and W_d represent the weight of wet and dry samples, respectively.

2.5.3. Mechanical properties measurement

The mechanical strength of the carriers was measured by a Universal Testing Machine-H 10 KM (Hounsfield, USA). The test conditions followed the standard procedure ASTM D882. The samples were cut into strip-shaped specimens of 10 mm in width and 10 cm in length (with 50 mm between the grips). The stretch rate was 2 mm/min. The tensile strength was averaged over five specimens.

3. Results and discussion

3.1. Batch fermentation

Batch fermentation using cane molasses as the main carbon source was carried out at 33 °C and 150 rpm for 72 h. In our preliminary study, it was found that the ethanol fermentation performance by using yeast cells immobilized in BC by adsorption method was relatively poor with the cell immobilization yield lower than 5% (data not shown). Therefore, BCA sponge was developed for improved yeast fermentation performance. For a conventional alginate matrix, the optimal alginate concentration was ~2.0% by wt. [12,30]. Therefore, in this study, an alginate solution of 2% by wt. was used to formulate the AL and BCA carriers. As shown in Table 1, the ethanol concentration produced by the SC system was significantly higher than that from the system of IC in AL. A maximum ethanol concentration of 87.9 g/L was obtained at 48 h of the batch fermentation from the SC system, which was 28.5% higher than that of IC in the AL carriers. Besides, the productivity and production yield ($Y_{p/s}$) of SC system were 1.83 g/(L h) and 0.48, respectively, which were higher than those of IC in AL carrier (1.42 g/(L h) and 0.35, respectively). A diffusion limitation of the cells into the substrate has often been observed for IC in AL carriers because of AL's dense structure [12]. The preference of cells to grow near the surface instead of in the middle of a gel matrix has been previously reported [5,11,31]. Growing yeast cells in an environment with insufficient nutrients causes a low sugar uptake rate and low ethanol production [32]. Low mechanical strength and rapid degradation are other large drawbacks of an alginate gel [31]. Some leakage of cells was also found on the surface of the alginate gel (figure not shown). However, the ethanol fermentation by IC in BCA carriers showed comparable productivity to that of the SC system. This result indicates that BCA is a superior carrier of IC for ethanol production. The designed BCA structure, consisting of a thin film with micro pores on the outer surface and large pores distributed in the spongier interior, was found to be effective for yeast immobilization [5,33]. Due to the high porosity of the BCA carrier, a higher mass transfer rate could be achieved, which allowed for the relatively higher ethanol concentration.

Table 1 shows a comparison between the ethanol concentrations produced by SC, IC in AL carriers, and IC in BCA carriers at 48 h of the fermentation with different ratios of BC/AL (50/50, 60/40 and 70/30). The maximum ethanol concentrations of the system with IC in BCA were slightly higher (2.2–4.7%) than those obtained from the SC system. It has been previously reported that the immobilization of yeast with proper carriers leads to higher cell densities, protects cells from both inhibitors and toxins, and allows for high ethanol productivity [5,12,29,31,34]. Y_1 values of IC in BCA were slightly higher than that of IC in AL. Although cell entrapment technique is efficient for high initial cell loading into cell carriers, large numbers of yeast cells attached on the surface of AL were observed.

The SEM images of the fresh BCA (0 h) and the BCA carrier at the end of the batch fermentation are shown in Fig. 1. The BCA had an asymmetric structure that consisted of a thin surface layer with interconnected hollow spaces in the interior, which facilitated the transmission of substrates and products between the carrier and the medium. After being used for 72 h in ethanol fermentation, large numbers of yeast cells adhered to the inner and outer surfaces of the BCA sponges; cell agglomeration in the hollow spaces of the carrier was also observed, indicating that the substrates were adequate for cell growth. In a previous study of cellulose carriers, the structure consisting of small pores distributed on the outer surface and large pores distributed in the interior was found to be effective for yeast immobilization [25].

The structural characteristics of the carrier, such as porosity and water uptake ability, are important factors that influence cell adhesion and ethanol productivity [27]. The blending composition influences the structure and properties of composite materials. From SEM observation (cross-sectional view), the BCA carriers, with BC/AL ratios varying from 50/50 to 70/30 using 2% (by wt.) alginate solution, presented an interconnected porous structure with an average pore size of 500–1000 μm. The water uptake ability of the BCA carriers varied from 6.0 to 12.2, depending on the AL content. The tensile strengths and elongations of the BCA carriers varied from 2.8 to 4.4 MPa and 10.7 to 13.0%, respectively. A decline in tensile strength was observed with increasing AL content. Alginate is very hydrophilic; as more alginate was incorporated into the BCA film, the film became more hydrophilic [21]. The presence of alginate in the BCA sponge could also enhance the molecular motion of cellulose in the blend and perturbed the strong hydrogen bond of pure cellulose resulting in the reduction in mechanical strengths. The increase in water uptake ability and the drop in mechanical strength in BC/AL composites with increasing AL content have been previously reported [21,22]. In our experimental results, the highest ethanol concentration and ethanol conversion yield ($Y_{p/s}$) at 92.0 g/L and 0.48, respectively, were obtained from IC in a BCA carrier with a BC/AL weight ratio of 60/40. Ethanol production from IC in BCA carriers with BC/AL ratios of 50/50 and 70/30 were approximately 1.5% and 2.5% lower, respectively, than that of the optimal ratio (60/40). Therefore, the blending composition of 60/40 BC/AL was selected for the fabrication of our BCA sponge.

3.2. Effect of alginate concentration

A decrease in the mass transfer diffusivity of the gel matrix with increasing alginate concentrations has been reported [5,12,30]. However, a gel matrix formulated with a low concentration of alginate gel (≤1.5%) is too soft and easily degrades when it is used in a shaking incubator or a packed-bed reactor. The effects of the AL concentration on the fabrication of BCA at the blending BC/AL ratio of 60/40 was examined by batch ethanol fermentation for 48 h as shown in Table 2.

The results of ethanol fermentation by IC in BCA with different concentrations of AL could be divided into 2 groups: (1) low-medium AL concentrations (1.5–2.5% by wt.) and (2) high-medium AL concentrations (3.0–6.0% by wt.). The maximum ethanol concentrations produced from the first group were approximately 6% higher than in the SC system, whereas the ethanol concentrations from the second group were approximately 13% higher than in the SC system. The observed physical properties and pore structure of the prepared BCA sponges with different concentrations of AL are summarized in Table 3. It was found that shrinkage in the BCA structure during the cross-linking step was influenced by the AL concentration. The sponges formulated with an AL solution at a low concentration (less than 3.0%) were thin and lacked firmness;

Table 1
Concentrations of products, yields of cell immobilization (Y_i), yields of ethanol ($Y_{P/S}$) and ethanol productivities (Q_P) at 48 h of batch fermentation using the suspended culture (SC) and immobilized culture entrapped within AL and BCA fabricated with different BC/AL ratios.

System	Ethanol (g/L)	Residue sugar (g/L)	Cells (g/L)		Y_i (%)	$Y_{P/S}$ (%)	Q_P (g/(L h))
			Free cell	Immobilized cell			
SC	87.9 ± 3.0	31.6 ± 0.1	4.6 ± 0.1	–	–	47.7 ± 1.7	1.83 ± 0.06
AL	68.4 ± 2.9	37.9 ± 1.3	1.6 ± 0.1	3.6 ± 0.5	69.9 ± 0.9	34.6 ± 4.1	1.42 ± 0.18
BCA							
50/50	90.7 ± 0.1	22.5 ± 1.7	1.5 ± 0.1	3.5 ± 0.3	70.7 ± 2.1	46.2 ± 0.4	1.89 ± 0.01
60/40	92.0 ± 3.1	26.3 ± 2.6	1.8 ± 0.1	5.1 ± 0.8	74.4 ± 1.5	48.4 ± 2.3	1.92 ± 0.07
70/30	89.8 ± 1.2	25.7 ± 2.2	1.9 ± 0.0	5.0 ± 0.3	73.7 ± 1.9	45.8 ± 1.2	1.87 ± 0.03

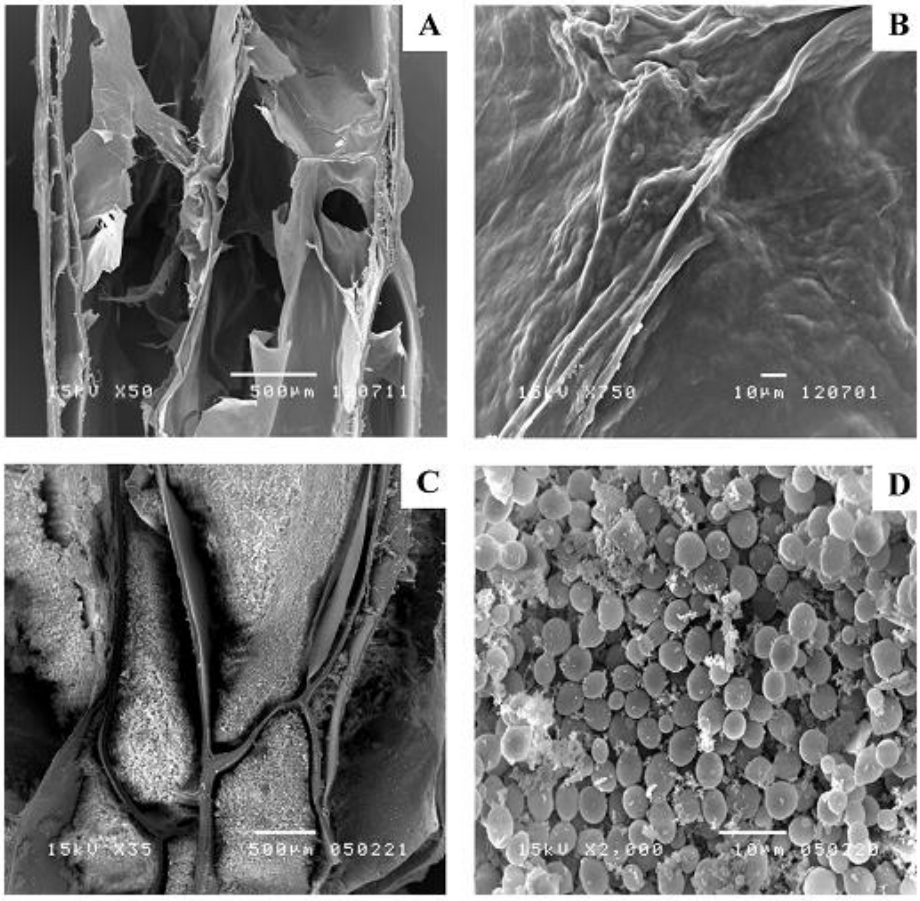

Fig. 1. SEM images of fresh BCA carrier (0 h): (A) cross section and (B) surface and BCA carrier after 72 h of batch fermentation: (C) cross section and (D) a closer look of yeast cells in a hollow space.

Table 2
Concentrations of products and yields of cell immobilization (Y_i), yields of ethanol ($Y_{P/S}$) and ethanol productivities (Q_P) at 48 h of batch fermentation using the immobilized culture entrapped within BCA fabricated with different alginate concentrations [AL].

BCA (60/40)	Ethanol (g/L)	Residue sugar (g/L)	Cells (g/L)		Y_i (%)	$Y_{P/S}$ (%)	Q_P (g/(L h))
			Free cell	Immobilized cell			
[AL] (% by wt.)							
1.5	93.4 ± 3.2	31.2 ± 0.1	2.8 ± 0.2	5.3 ± 1.2	65.3 ± 3.8	49.5 ± 1.7	1.95 ± 0.07
2.0	93.8 ± 1.2	31.7 ± 0.6	2.5 ± 0.0	4.8 ± 0.5	65.8 ± 2.8	49.8 ± 0.5	1.95 ± 0.03
2.5	93.6 ± 0.1	33.0 ± 0.0	2.7 ± 0.2	5.5 ± 0.0	66.7 ± 1.6	50.1 ± 0.0	1.95 ± 0.01
3.0	99.6 ± 1.7	32.6 ± 0.6	2.2 ± 0.4	12.2 ± 2.1	84.3 ± 2.7	53.1 ± 0.7	2.08 ± 0.03
4.0	99.7 ± 3.6	31.5 ± 1.1	2.4 ± 0.2	7.9 ± 0.6	76.8 ± 3.1	52.9 ± 1.6	2.08 ± 0.08
5.0	99.0 ± 2.2	33.6 ± 0.8	2.1 ± 0.2	6.4 ± 0.7	75.7 ± 0.1	53.1 ± 1.4	2.06 ± 0.05
6.0	99.1 ± 1.4	35.2 ± 0.6	3.2 ± 0.6	4.6 ± 0.4	59.3 ± 2.5	53.6 ± 0.9	2.06 ± 0.03

Table 3
Effect of alginate concentrations [AL] on physical properties and porous structure of BCA.

[AL] (% by wt.)	BCA						
	1.5	2.0	2.5	3.0	4.0	5.0	6.0
Thickness (mm)		0.1–0.2				0.2–0.3	
Porous structure		Interconnected				Multi-layer	
Tensile strength (MPa)	5.5 ± 1.4	5.5 ± 1.6	5.8 ± 1.3	7.3 ± 2.2	6.8 ± 1.7	7.9 ± 2.1	4.1 ± 0.9
Elongation (%)	10.7 ± 1.4	10.2 ± 3.0	10.4 ± 1.3	10.5 ± 1.9	10.1 ± 1.0	10.0 ± 0.5	10.7 ± 0.5
Water uptake ability	5.9 ± 0.3	7.1 ± 0.7	7.8 ± 0.6	8.6 ± 0.9	8.1 ± 0.3	7.9 ± 0.5	6.4 ± 0.1

only slight shrinkage of their structure was observed during the cross-linking step. The main disadvantages of the thin BCA sponges were poor mechanical properties and instability. On the other hand, the sponges fabricated with an AL solution at a higher concentration (more than 3.0%) showed considerable shrinkage during the cross-linking step and formed a multilayer structure instead of an interconnected porous structure. Accordingly, at an AL concentration greater than 3.0%, the water uptake ability decreased. The interconnected porous structure and high water uptake ability were shown to promote cell growth, cell immobilization and ethanol productivity. The highest immobilized cell density and ethanol concentration were obtained from IC in BCA formulated by AL at 3.0% by wt., where the immobilized cell concentration, immobilized yield (Y_I) and ethanol concentration were at 12.2 g/L, 84.3% and 99.6 g/L, respectively. Therefore, the suitable AL concentration for BCA sponge formation was found to be 3.0% by wt. In a previous study, the diffusion of substances was not only influenced by the structure of the carrier but also affected by the carrier's hydrophilicity [27]. The hydrophilicity or hydrophobicity can affect the distribution and availability of substrates and products [35].

3.3. Repeated batch fermentation

The BCA carrier fabricated by the 60/40 mixture of BC/AL (using a 3.0% by wt. AL solution) was used as a cell carrier for 5 repeated batch ethanol fermentations. Repeated batch fermentation was carried out under similar conditions as batch fermentation, with each batch lasting 48 h. Fig. 2A and B shows a comparison of the ethanol concentration and residue sugar concentration profiles, respectively, between repeated batch systems with SC, IC in the AL carrier, and IC in the BCA carrier. The cell concentrations and immobilization yields after the fifth cycle of the repeated batch fermentation are summarized in Table 4.

The highest ethanol concentration and highest total cell concentration were obtained from the system of IC in BCA. Instabilities of the SC and the IC in AL systems were observed in the fifth batch. For long-term fermentation, the SC culture exhibited a poorer tolerance to higher sugar and ethanol concentrations than did IC [36]. In the AL carrier, degradation and breakage of the carriers undergoing long-term operations resulted in the leakage of cells and a reduction of ethanol production in the fifth batch. A degradation of the AL surface has been previously reported to cause a decrease in the immobilization yield after a four-cycle repeated batch [5]. Previously, degradation of the AL gel after the second batch of ethanol fermentation was reported [37]. A comparison of our results showed that the BCA carrier was more stable, as it retained its overall carrier size and ethanol productivity at about 1.7–1.9 g/L h for the entire course of the examination. In addition, at the end of the fifth cycle of the repeated batch, the total cell concentration was 25.8 g/L, which was 69.0% and 24.4% higher than that of the SC and the IC in AL systems, respectively. The incorporation of BC enhanced the mechanical strength of the BCA carrier [20]. The existence of substrate or product inhibition in long-term repeated batch fermentation or continuous fermentation can deactivate yeast cells; therefore, the ethanol production efficiency becomes lower. In this case, the protection of the cells by immobilization in a suitable support material can lead to a significantly improved tolerance to inhibitors and unsuitable conditions over the free cell system [5].

The difference in ethanol productivity in IC systems could mainly due to the difference in the internal structure and properties of polymer carrier, such as surface charge, hydrophilicity, and swelling ability of polymer carrier [27]. Previously, BC hydrogel was applied as a yeast cell carrier [14]. It was reported that *S. cerevisiae* adsorbed mostly on the surface of BC pieces and might diffuse partially to the inside structure. However, owing to the dense structure of BC hydrogel, after 2 days of the incubation, yeast cell number in the biocatalyst decreased as a result of the lack of substrates for yeast metabolism. It was found in this study that yeast cells could attach and grow from the surface to the inside of BCA. The growth and aggregation of yeast cells were observed throughout the entire space of the BCA carrier. Therefore, the diffusional resistance to substrates/products (ethanol) transport was minor. Consequently, in the repeated batch fermentation using IC

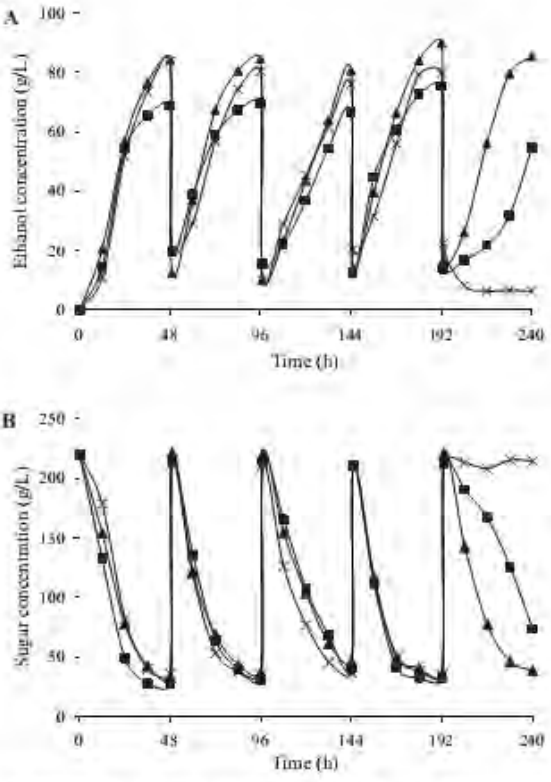

Fig. 2. Profiles of ethanol (A) and residue sugar (B) of repeated batch fermentation (48 h for each batch) using the suspended culture (×) and immobilized culture entrapped within AL (■) and BCA (▲).

Table 4
Cell concentrations and immobilization yields (Y_i) using the suspended culture (SC) and immobilized culture entrapped within AL and BCA after the fifth cycle of the repeated batch fermentation.

System	Free cell (g/L)	Immobilized cell (g/L)	Total cell (g/L)	Y_i (%)
SC	8.0 ± 0.7	–	8.0 ± 0.7	–
AL	4.2 ± 0.5	15.2 ± 0.7	19.5 ± 0.3	78.2 ± 2.5
BCA	3.2 ± 0.1	22.6 ± 0.9	25.8 ± 0.9	87.6 ± 0.6

in BCA, the very high cell density was obtained. The cell density during repeated batch fermentation using IC in BCA was relatively high compared to that of our previous work using thin shell silk cocoon [29] and alginate–loofa [31] as support materials, whereas the ethanol productivity was comparable to that of IC on thin shell silk cocoon and relatively higher than that of IC in alginate–loofa.

3.4. Stability of BCA

A 15 cycle repeated batch system (30 days) was used to investigate the long term-stability of the BCA carrier for ethanol fermentation. The final ethanol concentrations of each batch are fluctuated between 87.4 and 96.8 g/L during the 30 days of the repeated batch fermentation. The average ethanol concentration produced by using the IC in the BCA carrier was at 92.1 g/L. Therefore, the IC and their BCA carrier could be reused for at least 30 days, retaining high activity. In a study of IC in sugarcane stalks, it was reported that after an 8 cycle repeated batch fermentation (over 12 days), ethanol fermentation gradually decreased [33]. The difference in the obtained results might also arise from differences in yeast strains and system conditions. The results of our repeated batch experiment demonstrated that the BCA carrier's mechanical properties could withstand the shear force applied during the shaking process and the evolution of carbon dioxide during ethanol fermentation. No significant degradation or breakage was observed. The BCA carrier also exhibited good porous structure and good water uptake ability. Therefore, it represented a highly effective and stable cell carrier for use in long-term repeated batch and continuous ethanol fermentations. Besides, the BCA sponge has a good potential to be applied as cell carrier for other microorganisms, animal cells and plant cells.

4. Conclusions

A new method for the immobilization of microbial cells has been developed in this study. Our biocompatible porous cell carrier, BCA, was fabricated from BC and AL via crosslinking of alginate chains by calcium salts and freeze drying. BCA was found effectively used as a yeast cell carrier for ethanol fermentation due to its advantageous porous structure, mechanical strengths, stability and hydrophilic character. The outer, thin layer covering the interior interconnected macroporous structure of the BCA sponge was found to be very effective for yeast immobilization while minimizing internal mass transfer limitations. The BCA carrier also showed good mechanical properties, withstanding the shear force in the mixing process and the evolution of carbon dioxide during ethanol fermentation. Repeated-batch ethanol productions using IC in BCA carriers were more stable than those using SC or IC in Ca-alginate matrix. An examination using 15 cycle repeated batch fermentation demonstrated that BCA was capably reused as a carrier for at least one month.

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Effect of oxygen plasma treatment on bacterial cellulose-alginate composite sponge as a yeast cell carrier for ethanol fermentation

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Keywords: bacterial cellulose, alginate, sponge, ethanol fermentation, oxygen plasma

Abstract. A bacterial cellulose-alginate composite sponge (BCA) was developed for use as a cell carrier in ethanol fermentation. Its hydrophilicity was improved by oxygen plasma treatment. Due to the etching effect in plasma application, the external surface roughness of treated BCA was increased, resulting in a decrease of advancing water contact angle. However, oxygen plasma treatment might not be able to create sufficient hydrophilic functional groups on the internal pore surface of BCA, where the yeast cells would be immobilized during fermentation. As a result, under batch fermentation, no significant difference in ethanol production obtained from the immobilized cell systems using the treated and untreated BCAs.

Introduction

Immobilized cell (IC) systems have been extensively applied in ethanol fermentation because of high cell density, short operational time and low recovery cost [1]. Alginate gel is one of the most common materials used as a yeast cell carrier. However, the dense structure of the gel is the barrier which causes mass transfer limitation of substrates to the immobilized cells [2]. Also, fast degradation and low mechanical strength of the gel limit long-term operation in industrial processes. In our previous works, the sponge composed of bacterial cellulose (BC) and alginate gel was developed for use as a wound dressing material [3] and a cell carrier for ethanol fermentation [4]. Because BCA had asymmetric structure consisted of nanoporous layer on the outer and interconnected microporous layer in the interior, it was a good support material for immobilization of yeast cells in ethanol fermentation. From a previous report, the increase in hydrophilicity of cell carriers could result in the accelerations of water and nutrients diffusion into the hydrogel [5]. To improve hydrophilicity of the BCA, the sponge was treated by oxygen plasma. The chemical structure, morphology, contact angle and uptake abilities of the plasma-treated BCA (P-BCA) were investigated. Ethanol production by batch fermentation using IC in the P-BCA was compared with that using IC in the BCA and the suspended culture (SC).

Experimental details

BCA surface modification by oxygen plasma treatment. BCA was fabricated by crosslinking of alginate chains by calcium salts and freeze drying [4]. BCA in square shape of 2×2 cm^2 with a thickness of 0.2 cm was treated in a plasma chamber (PC-1100, Samco, US) for 15 min each side with the oxygen gas flow rate of 40 SCCM and the pressure of 15 kPa.

Materials characterizations. BCA and P-BCA were identified for the chemical structural information by FT-IR Spectrometer (SX-170, Nicolet, US) in the region of 4000-400 cm^{-1}. The surface morphology was examined using scanning electron microscopy (JSM-5410LV, JOEL, Japan). The samples were coated with gold and photographed at an accelerating voltage of 15 kV. The advancing water and ethanol contact angle were assessed by Washburn capillary rise technique, as described in [6].

Water/ethanol uptake ability was determined by immersing the pre-weighted of dried samples in distilled water/98% of ethanol at room temperature until equilibration. The weight of the swollen samples was measured. All testing was run in 5 times. The water uptake ability was determined by using the following equation:

$$\text{water/ethanol uptake ability} = (W_w - W_d)\rho_m / (W_d \rho_l). \quad (1)$$

where W_w and W_d represent the weight of wet and dry samples, respectively and ρ_l and ρ_m represent the density of material and liquid, respectively, by assuming $\rho_m = \rho_{cellulose} = 1.25$.

Ethanol fermentation. Starter culture was obtained by transferring cells from an agar slant into 150 mL sterilized cultivation medium. Cell culture was cultivated in a refrigerated incubator shaker (Innova 4330, New Brunswick Scientific, US) at 150 rpm, 33ºC for 20-24 hours. The cells were concentrated by decantation to obtain stock cell suspension.

To immobilize cells into the materials, stock cell suspension of 10 mL and 2.5 g of the sterilized BCA and P-BCA in square shape of 2×2 cm^2 with a thickness of 0.2 cm were added to 250 mL sterilized culture medium and incubated at 33ºC, 150 rpm for 20-24 hours. For batch ethanol fermentation, cane molasses was used as a substrate for ethanol fermentation. Experiments were initialed by transferring suspended cell culture or immobilized cell culture into 250 mL of the medium. Fermentation flasks were then shaken in the incubator at 150 rpm, 33°C for 72 h. The experiments were monitored by removing 2 mL samples every 8 h for sugar and ethanol analyses. Ethanol concentration was analyzed by a gas chromatograph (GC-7 AG, Shimadzu, Japan) equipped with a flame ionization detector.

Results and discussion

Characterizations of the materials. After BCA was treated with oxygen plasma, it was immediately analyzed for chemical functional structure using FTIR spectra. FTIR spectra adsorption bands of BCA and P-BCA were compared (Figure not shown). It was noticed that there was no alteration of carbonyl and carboxyl groups in P-BCA when compared with BCA. The spectra bands of BCA and P-BCA were very similar. Generally, during the plasma process, hydrogen atom on polymeric chain is abstracted or polymeric chains are split, and free radicals are created by plasma. Afterward, the free radicals are activated to incorporate with a gas [7]. The incorporation of cellulose and oxygen mechanism (oxidation reaction) was proposed by Calvimontes et al. [8], and carbonyl and/or carboxyl groups would increase after cellulose was treated with oxygen plasma [8-10]. However, in this work, oxygen plasma was unable to create detectable hydrophilic functional groups on the surface of BCA. Due to the complex 3D structure of BCA (the intermolecular interaction between hydroxyl group of cellulose and carboxyl group of alginate [3]), the incorporation of cellulose and oxygen mechanism might not be the same as those of pure cellulose materials.

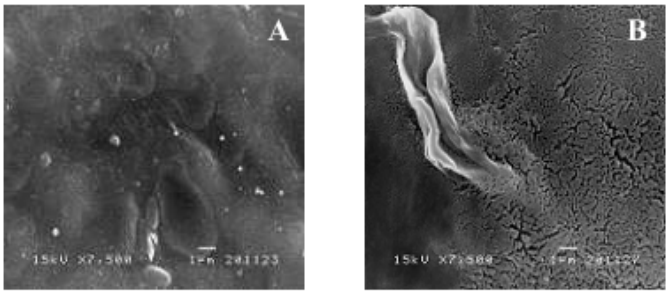

Fig. 1 SEM images of BCA surface (A) and P-BCA surface (B).

The surface morphologies are shown in Fig. 1. Surface area of P-BCA was damaged by oxygen plasma treatment as shown in multiple surface cracks in Fig. 1B. The etching effect on material surface has been observed by applying plasma [9, 11, 12]. The surface of P-BCA appeared to be rougher as compared to that of BCA. A rougher surface might enhance cell adsorption. However, due to the nanoporous and relatively denser outer layer of the BCA sponge, the amount of plasmatic oxygen ions reaching the interior surface of the sponge could be limited.

Table 1. Advancing water and advancing ethanol contact angles obtained by WCR using tubes packed with 500-2000 μm powders

Material	Advancing contact angle (°)	
	Water	Ethanol
BCA	75.8 ± 2.3	31.2 ± 3.5
P-BCA	51.7 ± 1.0	34.5 ± 7.8

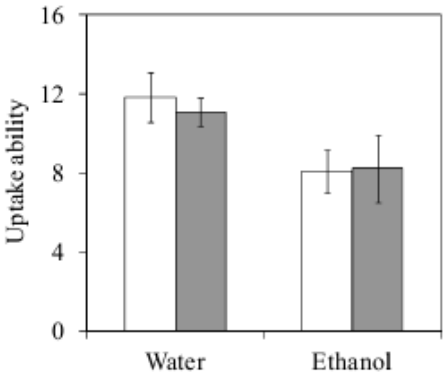

Fig. 2 Uptake ability of BCA (white) And P-BCA (gray)

The contact angle was used to identify the improvement of the material hydrophilicity. The advancing water and advancing ethanol contact angles are summarized in Table 1. The advancing water contact angle of P-BCA was significantly lower than that of BCA (≈24° difference). But, the advancing ethanol contact angles were similar. These results showed that the hydrophilicity of P-BCA was enhanced by oxygen plasma treatment. According to the results of FT-IR and SEM, a cause of the improvement of P-BCA hydrophilicity is likely the increase of surface roughness. Zemljic et al. [13] claimed that the major effect on the increase hydrophilicity of the material treated with plasma was the formation of new functional groups but might also be the morphology change and/or cleaning process.

BCA and P-BCA were immersed in water/ethanol to determine the uptake abilities and the results are presented in Fig. 2. There was no significant difference in water/ethanol uptake abilities between treated and untreated BCA, which could probably be the result of little to, no formation of hydrophilic functional group in the interior of the BCA sponge with oxygen plasma treatment.

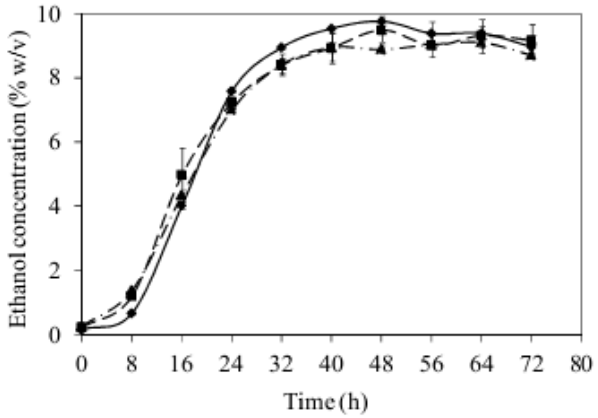

Fig. 3 Profiles of ethanol concentration of batch fermentations using SC (solid line) and ICs in BCA (dashed line) and P-BCA (dot-dash line)

Ethanol fermentation. Ethanol production using IC in P-BCA was compared with those of IC in BCA and suspended cells, as shown in Fig. 3. Ethanol concentrations obtained from IC in P-BCA were almost equivalent to those in BCA. The ethanol fermentation by IC in both BCA and P-BCA carriers showed comparable productivity to that of the SC system. The main reason for no substrate limitation in the IC systems was that the inter-connected macro porous structure in the interior of the BCA facilitated the transmission of substances and products between the carrier and the medium. The etching of plasma on BCA surface did not show significant effect on ethanol production.

Summary

The hydrophilicity of BCA sponge was improved by oxygen plasma treatment. It was shown that the water contact angle was decreased because of increasing surface roughness by etching effect. No formation of new hydrophilic functional groups on P-BCA was detected. There was no significant difference in water/ethanol uptake abilities between treated and untreated BCA. Consequently, there was no significant difference in ethanol production obtained from the systems of IC in P-BCA and BCA. Oxygen ions might not be able to incorporate with the 3D chemical structure of BCA due to its complex chemical and physical structure.

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Effect of Molecular Weight of Chitosan on Antimicrobial Properties and Tissue Compatibility of Chitosan-impregnated Bacterial Cellulose Films

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ABSTRACT

Bacterial cellulose-chitosan (BC-C) films were developed by immersing purified BC pellicles in acetic acid solutions containing chitosan of varying molecular weights (MWs). Effects of low, medium and high MWs of chitosan on physical, biological and antimicrobial properties of the composite films were investigated. The cumulative chitosan absorption capacities with MW of 30,000, 80,000 and 200,000 were 38.43 mg/cm^3, 24.65 mg/cm^3 and 23.89 mg/cm^3 of dry BC film, respectively. The cumulative release profiles of chitosan from the films strongly depended on the MW of chitosan and pH of solution. The order of release of chitosan from the BC-C films was dependent on MW as follows: MW 30,000 > MW 80,000> MW 200,000. All BC-C films showed the antimicrobial abilities against *Staphylococcus aureus* and *Aspergillus niger* and supported for adhesion, spreading and proliferation of both human skin keratinocytes and fibroblasts. The antibacterial activity against *S. aureus* of the BC-C with the high MW chitosan (200,000) was higher than those of the medium and low MWs. On the other hand, the BC-C films with the low MW chitosan (30,000) promoted the growth of human skin cells more than those of the medium and high MWs.

Keywords: Bacterial cellulose, chitosan, release, Keratinocytes, Fibroblasts

1. Introduction

Cellulose, a linear polysaccharide, is the most abundant polymer in nature. It forms the basic structural matrix of the cell wall of nearly all plants, algae and fungi. Several bacteria can produce solid extracellular cellulose, as reported from the genera *Acetobacter* (formerly *Gluconacetobacter*), *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Azotobacter*, *Rhizobium*,

Sarcina, Salmonella, and Escherichia [1]. *Acetobacter xylinum* is the most widely used bacterium for the synthesis of nanocellulose fibers, which are also known as bacterial cellulose (BC). Plant cellulose and BC are chemically similar (β-1, 4 glucans), but the degree of polymerization (DP) differs. The DP is approximately 13,000 to 14,000 for plant cellulose and approximately 2,000 to 6,000 for BC [2]. With its ultra-fine network structure, BC has unique properties, including a high water-holding capacity, high crystallinity, hydrophilicity, high purity, and high tensile strength.

BC and BC composites have a wide range of potential applications in many fields, for instance, as mixing agents or viscosity modifiers in the food industry, as fibers in industrial paper [2,3], as immobilization matrices of proteins and as separation membranes or chromatography substances [2]. The recognized applications of BC in the biomedical field included wound dressings [4-6], artificial skin [2,7,8], dental implants, vascular grafts, catheter-covering dressings, dialysis membranes, coatings for cardiovascular stents, cranial stents, tissue replacement, controlled-drug release carriers [9], membranes for tissue-guided regeneration [7,9], vascular prosthetic devices [10], membranes in antibacterial applications, membranes/scaffolds for tissue engineering [7,11], bone tissue engineering [12,13], artificial blood vessels [8,14] and scaffolds for the tissue engineering of cartilage [15].

Chitosan has also been recognized for applications in various fields, including the biomedical area. Chitosan is an amino-polysaccharide obtained by the deacetylation of chitin. The structure of chitosan is composed of b-(1, 4)-linked GlcN and GlcNAc units. Chitosan has special properties, including polyoxy salt formation, ability to form films, chelated metal ions and optical structural characteristics [16]. It is known for its absorption of exude and its important biological properties such as anti-fungal, anti-microbial, anti-viral and wound-healing

properties. Chitosan has potential applications as a wound management aid to reduce scar tissue and could be used to inhibit fibroplasias in wound healing and promote both tissue growth and differentiation in tissue culture [17]. Its biological properties, such as its bacteriostatic and fungistatic properties, are particularly useful for wound treatment. The main factors affecting the antibacterial activity of chitosan are its molecular weight and degree of N-deacetylation [18,19]. However, the disadvantages of thin films of chitosan are that they are fragile and brittle in the dry state. Its characteristic properties have been extensively reviewed [20,21].

This study aims to develop films combing the extremely good physical properties of BC with the special biological and antimicrobial properties of chitosan. BC films were incorporated with chitosan of different MWs by immersing method. The effect of chitosan of low (30,000), medium (80,000) and high (200,000) MWs on the physical, biological and antimicrobial properties of the BC-C films were examined. The absorption capacity and release characteristics of chitosan from the BC-C films were also studied. Furthermore, antimicrobial properties of the BC-C films and the effects of the films on the growth and attachment of human skin cells were investigated.

2. Materials and Methods

2.1. Microbial Strains

The *Acetobacter xylinum* strain AGR 60 kindly supplied by Mr. Pramote Tammarat, the Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand was used in this study.

2.2. Culture medium and bacterial cellulose preparation

The medium for the inoculums was coconut-water supplemented with 5.0% (w/v) sucrose, 0.5% (w/v) ammonium sulfate $(NH_4)_2SO_4$, and 1.0% (v/v) acetic acid. The culture medium was sterilized at 110 ^{o}C for 5 min. Preculture was prepared by transferring cells from a stock culture to 500 ml medium, and incubated statically at 30 ºC for 7 days. The preculture broth was added to the main culture medium and 75 ml activated medium was placed in a glass Petri dish (Ø 14.5 cm) and incubated at 30°C for 7 days. All samples were first purified by washing with distilled water and then they were treated with 1% (w/v) NaOH at room temperature to remove bacterial cells followed by a rinse with distilled water until pH came to neutral. Afterward, the wet purified BC pellicle was stored in distilled water at 4°C.

2.3. Impregnated bacterial cellulose-chitosan film preparation

The wet, purified BC pellicles were immersed in 2.0 % (w/v) chitosan (MW of 30,000 and 80,000) and 1.5 % (w/v) chitosan (MW of 200,000) in a 1 % (w/v) acetic acid solution for 36 h. The degree of deacetylation (DAC) of all chitosan samples was 85%. After that, the chitosan-impregnated BC pellicles were air-dried by placing them on a Teflon sheet at room temperature. To remove the remaining acetic acid from the BC-chitosan (BC-C) composite film, the film was rinsed in distilled water at room temperature several times until the pH of water became neutral. The films of BC-C free acetic acid were then air-dried at room temperature (30°C) and stored in plastic film at room temperature.

2.4. Characterization of the film

Measurements were made following the previously reported procedures [22]. For surface morphology, scanning electron micrographs (SEM) were taken using a Jeol (Tokyo, Japan) JSM-5410LV scanning electron microscope. The pore size, porosity and pore size distribution of the BC film were determined by a Brunauer-Emmett-Teller (BET) surface area analyzer (Sorptomatic 1990, Quantachrome Instruments, Florida, USA). Fourier transform infrared (FTIR) spectroscopy (Spectrum One, Perkin Elmer, Massachusetts, USA) is used primarily to identify the chemical structure of the samples. X-ray diffraction (XRD) patterns of the BC and the BC-composites were determined with a diffractometer (Bruker AXS Model D8 Discover, USA. The Mechanical properties, tensile strength, Young's modulus and elongation at break of BC and BC-C dried films, were measured by Universal Testing Machine (Hounsfield H 10 KM, Redhill, England). The test was conducted in accordance with a standard test method, ASTM D882.

2.5. Water absorption capacity

The water absorption capacity (WAC) was determined by immersing the pre-weighted of dried samples in distilled water at room temperature until equilibration. The sample was then removed from the water. After excess water at the surface of the sample was blotted out with Kimwipes paper, the weight of the swollen sample was measured and the procedure was

repeated until there was no further weight change. The water content was calculated with the following formula:

$$WAC(\%) = \frac{W_h - W_d}{W_d} x100$$

where W_h and W_d denoted the weight of hydrate and dry sample, respectively.

2.6. Water vapor and oxygen transmission rates

The water vapor transmission rate (WVTR) of the BC and BC-C films with area of 50.00 cm^2 was determined with the desiccant method. The test was conducted in accordance with a standard test method, ASTM E96. The determination of WVTR was done at 38 ºC and 98% relative humidity. The test specimen was sealed to the open mount of test dish containing a desiccant, and the assembly placed in a controlled atmosphere. Periodic weighting was performed to determine the rate of water vapor movement through the specimen into the desiccant.

The oxygen transmission rate (OTR) was determined with an oxygen permeation analyzer, Illinois Instruments Model 8000 (Johnsburg, IL). The test was conducted in accordance with a standard test method, ASTM D3985. The determination of OTR was done at 23 ºC and 0 % relative humidity. The film was held in such a manner that it separated two side of test chamber. One side was exposed to a nitrogen atmosphere, while the other side was exposed to an oxygen atmosphere. A. Testing was completed when the concentration of oxygen in the nitrogen side was constant.

2.7. In vitro released studies

The release characteristics of chitosan content from the BC-C films were investigated by the total immersion method [23]. The relevant buffer-simulated gastric fluid USP (pH 1.2), acetate buffer (pH 5.5), and phosphate buffer (pH 7.4) were used as a releasing medium. Each specimen (square sheet of 2.5 x 2.5 cm^2) was immersed in 30 ml of the releasing medium at 37 °C. During the immersion period (0 to 268 h), 1 ml medium was withdrawn every 2 h on the 1st day and every 12 h on the other days, and an equal amount of the fresh medium was refilled. The amounts of chitosan content in the sample solutions were determined using the ninhydrin method [24]. To determine the actual amount of chitosan in the BC-C composite films, each 2.5 x 2.5 cm^2 of the samples was hydrolyzed by 37% HCl at 100 °C for 30 min. After that, 20% NaOH was added to neutralize the solution, and then the sample solution was centrifuged at 2500 rpm for 30 min. The amount of chitosan content in the supernatant was determined using the ninhydrin method.

2.8. Antimicrobial ability testing

The antimicrobial properties of BC and BC-C films were examined against *Escherichia coli* (gram-negative bacteria), *Staphylococcus aureus* (gram-positive bacteria) and *Aspergillus niger* (fungus) by using the agar diffusion test. The films were cut into round-shaped samples 38 mm in diameter and the test was conducted in accordance with AATCC Test Method 39-1989. Before the antibacterial and antifungal assay, all BC, BC-C, and BCA films were sterilized by UV irradiation for 20 min in each side. The incubation was carried out at 37 °C for 24 h under

aerobic conditions for *E. coli* and *S. aureus*, while the test for *A. niger* was performed on an AGAR plate at 30°C for 7 days.

2.9. In vitro cells study

The cytotoxicity of BC and BC-C films against L929 mouse fibroblasts was determined. In addition, the tissue compatibility of BC and BC-C films was evaluated by cell adhesion and proliferation of human skin keratinocytes (HaCaT) and human fibroblasts (GF) on each material. The test samples were punched into round-shaped samples of 14 mm diameter. The samples were sterilized by UV irradiation for 20 min in each side and transferred aseptically to 24-well culture plates. Proliferations of cells on the films were determined by MTT assay. The experiments were conducted in triplicate. The studies were performed following the procedures similar to those previously reported [5].

3. Results and Discussion

3.1 BC film impregnated with chitosan

To improve the antimicrobial and biological properties of BC films for use as biomaterials for medical applications, BC-C films were prepared by immersing wet and purified BC pellicles in 2.0 % (w/v) chitosan-acetic acid solution (MW of 30,000 and 80,000) and 1.5 % (w/v) chitosan-acetic acid solution (MW of 200,000). BC-C3, BC-C8 and BC-C20 refer to the samples of BC films impregnated with chitosan having MW of 30,000, 80,000, and 200,000, respectively.

Overall, the absorption profiles of chitosan of different molecular weights were similar (Figure not shown). The absorption rate was high in the first 6 h; afterward, it gradually decreased. After 24 h of absorption, the cumulative concentration of chitosan was nearly constant. Chitosan having an MW of 30,000 could penetrate into the BC network better than those of MW 80,000 and 200,000. The total contents of chitosan in BC-C3, BC-C8, and BC-C20 films were 38.43±5.02 mg/cm^3, 24.65±0.48 mg/cm^3, and 23.89±2.54 mg/cm^3 of dried film, respectively. Therefore, molecular weight of chitosan significantly influenced chitosan absorption capacity of the BC films.

3.2 Morphology

The appearance of the BC-C films was different from the pristine BC film. The BC-C films were thicker and denser. The thicknesses of BC and BC-C3, BC-C8 and BC-C20 films were 71±2 µm, 81±2 µm, 103±9 µm, and 94±2 µm, respectively. The SEM images in Figure 1 show the surface morphologies of the BC and the BC-C3, BC-C8 and BC-C20 films. The BC film was composed of well-organized nanofibrillar networks with a relatively more uniform pattern than those of the BC-C films. From the BET analysis, the average pore diameters and pore volume of the BC-C films were smaller than those of the BC film (Figure 2). Therefore, chitosan could penetrate into the pore structure and fill the pores. Overall, the pore diameter and the surface area of the BC-C films relatively decreased with increasing MW of chitosan (Table 1). The descending order of pore volume was ranked as follows: BC > BC-C3 > BC-C8 > BC-C20. Previously, it was also reported that chitosan could penetrate into the empty spaces of the BC

The diffraction patterns of BC-C3, BC-C8, and BC-C20 films demonstrated the three main peaks, similar to the pattern of BC, but the peak in the $(1\bar{1}0)$ reflection plane slightly shifted to a wider angle (data not shown). Compared with those of the unmodified BC, the crystallinities of BC-Cs were relatively lower, which should be owing to intermolecular interaction between chitosan molecules and cellulose fibrils. It has previously been suggested that the incorporation of polymer into the BC structure could affect the preferential orientation of the $(1\bar{1}0)$ reflection plane, resulting in the incomplete orientation of the plane, which in turn leads to a decrease in crystallinity [28].

Tensile strength, elongation at break and Young's modulus of the BC-C films were slightly lower than those of the BC film. The incorporation of chitosan into the BC pellicle might increase the amorphous phase fraction, which could weaken the affinity of the binding of the films. As discussed in the XRD results, the incorporation of chitosan could cause a decrease in crystallinity, leading to a decrease of the mechanical strength of the films. From the decreased values of the elongation at break of the BC-C films, it could be concluded that the flexibility and endurance of the BC-C films were relatively less in comparison to those of the BC film.

Water absorption capacity (WAC) is one of important properties of biomaterials that are used in the medical field, especially for wound dressing. A significant reduction ($P<0.05$) in the WAC of the BC-C films to approximately 56 - 65 % of that of the BC film was observed. Incorporation of chitosan into the BC film formed a more dense structure; therefore the water penetration and water-holding capacity of the film were lower, leading to a decrease in the WAC.

The water vapor transmission rate (WVTR) is also an important characteristic of wound dressing. Generally, the water loss for normal skin is 204.0±115.2 $g \cdot m^{-2} \cdot day^{-1}$, while that for the

fibril network and form intermolecular hydrogen bonding interactions between the BC and chitosan molecules [25,26].

3.3 Fourier transform infrared spectroscopy

The FTIR spectra of the BC and BC-C films were measured from 4000–400 cm^{-1}, as shown in Figure 3. The BC spectra showed a broad band at a wave number of 3,418 cm^{-1}, attributed to the O-H stretching vibration; a band at 2,895 cm^{-1} represents the aliphatic C-H stretching vibration [25], a band at approximately 1641 cm^{-1} represents the H-O-H bending of the absorbed water, and a band between 1,155-1032 cm^{-1} is attributed to the polysaccharide structure [27]. For chitosan powder, an observed band at 1599-1598 cm^{-1} is attributed to the amide group. The spectra of BC-C films exhibited all of the characteristic bands of BC coupled with the appearance of a new band between wave numbers of 1,563 and 1,561 cm^{-1}, which represented the amide group of the chitosan molecule. The shift of the characteristic bands of chitosan indicated an intermolecular bonding interaction between the amino groups of chitosan and the hydroxyl groups of BC.

3.4 Physical Properties

The physical and mechanical properties of BC and BC-C films are shown in Table 1. The average crystallinity index (C.I.) value was calculated from the X-ray diffraction pattern. The pattern of BC exhibits three main peaks at $2\theta = 14.50°$, 16.72°, and 22.74°, attributed to the $(1\bar{1}0)$, (1 1 0), and (2 0 0) reflection planes of the cellulose I structure, respectively [28,29].

injured skin can range from 207±26 g·m^{-2}·day^{-1} for a first-degree burn to 5138±202 g·m^{-2}·day^{-1} for a granulating wound [30]. The WVTR of the BC film was higher than those of the BC-C films with a statistically significant difference (P<0.05). In the case of the BC-C films, the WVTR was increased with the increasing MW of chitosan. It could be explained that the incorporation of chitosan into the cellulose network resulted in the denser structure and the decrease in pore diameters, leading to a decrease of the WVTR. On the other hand, the incorporation of chitosan, especially with high molecular weight, might also enhance the hydrophilic properties of the BC-C films. Therefore, the WVTR of the BC-C20 film was higher than those of the BC-C8 and the BC-C3 films, respectively. The WVTR of the BC and the BC-C films are comparable to those of commercial wound dressings, such as Bioprocess®, Biobrane II®, Op-site® and gauze [30,31].

3.5 Release of chitosan from BC-C films

In this study, effect of pH-dependent solubility on release behavior of chitosan from the BC-C films was investigated. The cumulative releases of chitosan from the BC-C films in buffers with pH 1.2, pH 5.5 and pH 7.4 are shown in Figure 4 A, B and C, respectively. Molecular weight of chitosan has a significant effect on the release rate and cumulative release of chitosan from the BC-C films. The maximum amounts of chitosan released from BC-C3, BC-C8, and BC-C20 in the buffer solution with pH 1.2 were 27.17 ±1.85, 22.07±0.45 and 17.98±1.32 mg·cm^{-3} of dried films, respectively; 27.03±1.64, 20.61±1.33 and 11.34±1.31 mg·cm^{-3}, respectively in the buffer solution with pH 5.5 and 0.60±0.07, 0.46±0.12 and 0.27±0.14 mg·cm^{-3}, respectively in the buffer solution with pH 7.4. In the acidic buffer solutions, all types of the BC-C films showed a

rapid release of chitosan during the initial 24 h of the immersion. Afterward, the release rate of chitosan from the BC-C films into the acidic buffer solutions of pH 1.2 and 5.5 declined and the cumulative releases of chitosan became constant at approximately 72 and 96 h of immersion time, respectively. The release rate of chitosan of MW 30,000 was higher than those of MW 80,000 and MW 200,000, respectively. Therefore, the level of release of chitosan from the BC-C films depended on its molecular weight and the pH value of the solution. Since chitosan is only soluble in acidic solutions below pH of approximately 6.5 (below the pKa of chitosan), it was slightly released from the BC-C films in the buffer solution with a pH of 7.4. The poor solubility of chitosan was due to the precipitation or gelation of chitosan in solutions with pH ≥ 7.0 [16].

3.6 Antimicrobial ability

BC alone does not have antimicrobial activity. The incorporation of chitosan in the BC-C films enhanced the antimicrobial properties, as presented in Table 2. The BC-C films showed significant inhibitory effect on the growth of *S. aureus*, whereas the BC film did not. However, from the test, both BC and BC-C films had no inhibitory effect on the growth of *E. coli*. It was previously reported that the efficiency of inhibition of chitosan was higher for gram-positive than for gram-negative bacteria [32]. Moreover, MW and concentration of chitosan were found to be important factors affecting the antibacterial property of chitosan. The BC-C20 film showed a stronger inhibitory effect on *S. aureus* than those of the BC-C3 and BC-C8 films. The result indicated that all types of the BC-C films had significant inhibitory effect on the growth of *A. niger*. It was suggested that the antimicrobial abilities of chitosan might be due to the positive charges presented in the molecule of chitosan interacting with the negative charges from the

residues of macromolecules in the membranes of microbial cells [33]. The antimicrobial activities against the gram-positive bacteria and fungus were found to increase with increasing chitosan MW, whereas the opposite effect was observed with the gram-negative bacteria [34].

We have previously reported that BC-chitosan composite films prepared from a biosynthesis process had no significant antimicrobial ability (by agar diffusion test) [35]. Similarly, it was found that chitosan-starch films had no inhibitory effect against all tested microorganisms since chitosan was incapable of diffusing through the agar media [36]. Therefore, the structure of films and the interaction between chitosan and films significantly affected the antimicrobial activity of the films containing chitosan. With the immersion method used in this work, a higher content of chitosan in the BC film was obtained and the BC-C film was capable of release of chitosan. As a result, the BC-C film showed antimicrobial activity against *S. aureus* and *A. niger*.

3.7 In vitro cell study

The cytotoxicity of BC-C films against L929 mouse fibroblast cells was evaluated. The percentages of living cells with the extraction medium of BC, BC-C3, BC-C8, and BC-C20 films incubated for 24 h, in comparison with those cultured with the fresh medium, were 95.5±3.0 %, 91.6±6.3 %, 97.0±3.1 %, and 92.9±2.9 % with an extraction medium concentration of 5 $mg \cdot ml^{-1}$ and 95.2±8.7 %, 97.8±3.3 %, 95.1±3.8 %, and 92.6±5.2 % with an extraction medium concentration of 10 $mg \cdot ml^{-1}$, respectively. The results indicated that the BC and BC-C films had no toxicity against L929 mouse fibroblast cells. There is no statistically significant difference in mean values from the cytotoxicity test of the BC and BC-C films ($P>0.05$).

The ability to support skin cell adhesion and proliferations is the foremost characteristic of potential dressing materials. The proliferations of HaCaT and GF cells on the BC-C film are shown in Figures 5. The percentages of living cells after seeding on the BC and BC-C films from 0 to 24 and 48 h were gradually increased for both HaCaT and GF cells. At 48 h after seeding, both HaCaT and GF cells proliferated on the BC and BC-C films, with a significant difference in the mean value relative to those of the control polystyrene plate ($P < 0.05$). In addition, the cell number of GF on the BC-C3 film was significantly higher than those cultured on the BC film and relatively higher than those of the BC-C8 and BC-C20 films. The improved proliferations of HaCaT and GF cells on the BC-C films might be owing to the interaction of positively charged amino groups of chitosan and negatively charged cell membranes [37].

The morphologies of HaCaT and GF cells on the polystyrene plate and the BC, BC-C3, BC-C8, and BC-C20 films at 24 h after seeding are revealed in Figures 6. The HaCaT cells could attach to BC and BC-C films with different extend of spreading. The well-spread HaCaT cells were observed on the polystyrene plate and the BC-C films, whereas the cells with less-spread were observed on the BC film. The scanning electron micrographs demonstrated similar morphology of GF cells as a flattened, spindle-like shape indicating well attachment and spreading on the polystyrene plate and the BC-C films. The result indicated that the incorporation of chitosan into BC films promoted adhesion, spreading and proliferation of both GF and HaCaT. Previously, it was reported that MW and level deacetylation (DAC) of chitosan had effect on growth of human skin cells [38]. Low-MW chitosan (MW 13,000) with high-DAC (85%) stimulated fibroblast proliferation more than that of high-MW chitosan or low-DAC. Our previous work showed better spread and adhesion of human skin cells on the composite films of BC and chitosan developed by biosynthesis (BCC), with the comparable cell proliferation

compared to those on the BC films [22]. Herein, with chitosan release capability, the BC film impregnated with chitosan MW of 30,000 (BC-C3) was more efficient at stimulating the proliferation of human skin cells than those of the BC film. It was previously suggested that some factors in serum such as growth factors might potentiate the proliferation effect of chitosan [38].

4. Conclusion

The BC-C composite films were developed by immersing wet BC pellicle in chitosan-acetic acid solution. Chitosan with MWs of 30,000, 80,000 and 200,000 could penetrate into the BC fibril network and fill the pores. The FTIR result indicated the intermolecular interaction between the amino groups of chitosan and the hydroxyl groups of cellulose. Some mechanical properties, such as the tensile strength, Young's modulus and elongation at break relatively decreased compared with the unmodified BC film. However, with the chitosan release capability, the BC-C films exhibited important beneficial biological and antimicrobial properties for wound healing. The BC-C films showed antimicrobial abilities against *S. aureus* and *A. niger* and promoted adhesion, spreading and proliferation of both human keratinocytes and fibroblasts. The BC-C3 film (impregnated with chitosan MW of 30,000, DAC 85%) could promote proliferation of both human keratinocytes and fibroblasts more effectively than those of the BC and the other BC-C films.

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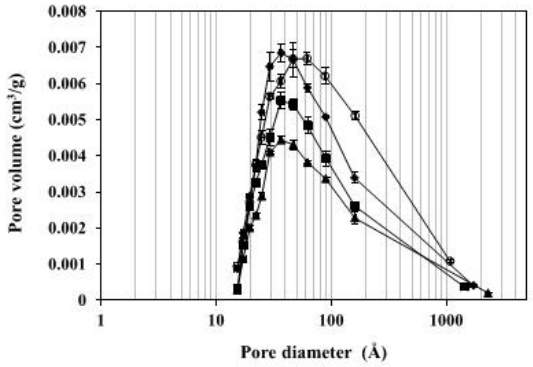
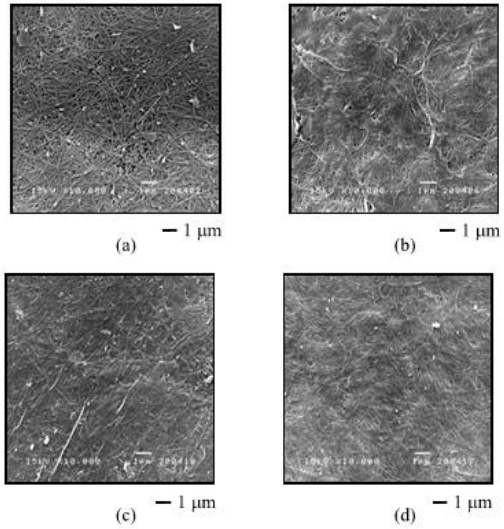


Figure 2.



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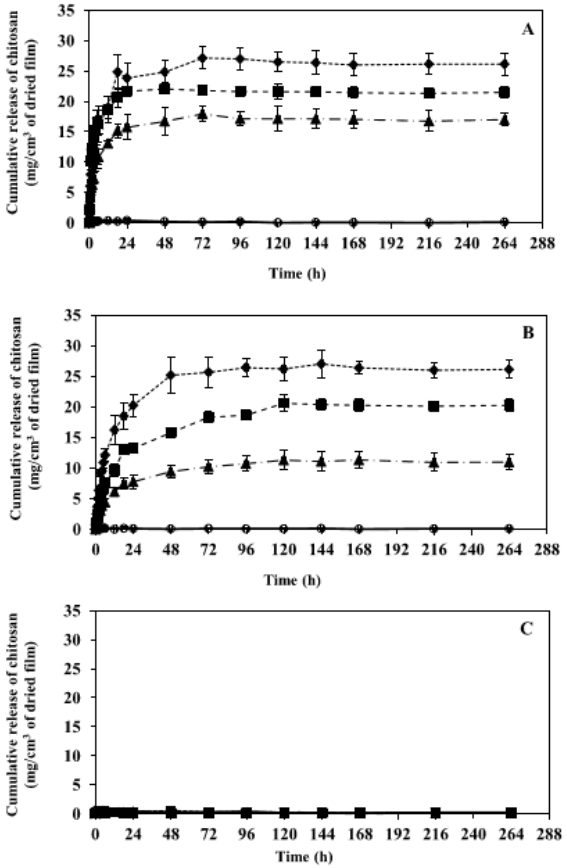

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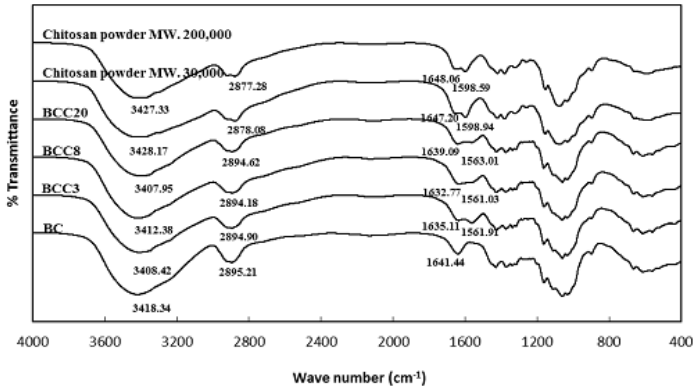


Figure 3.

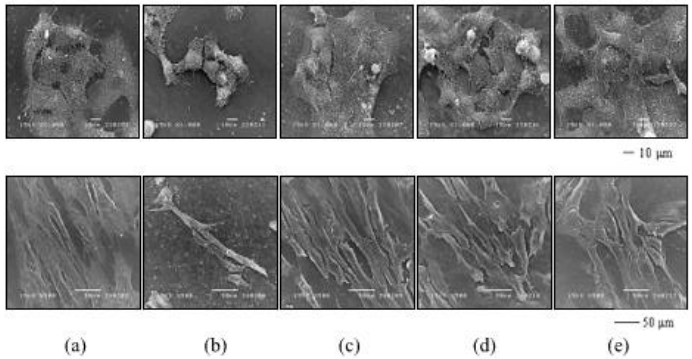
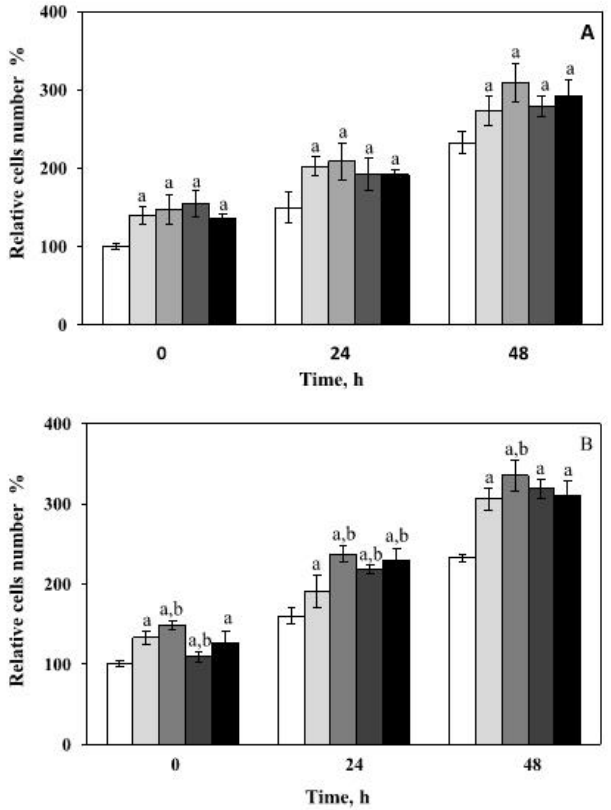


Figure 6.



[a] significant difference in mean value relative to the control polystyrene plate ($P < 0.05$),

[b] significant difference in mean value relative to the control BC film ($P < 0.05$)).

Figure 5.

Table 1 The physical and mechanical properties of BC and BC-C films.

Property	BC	BC-C3	BC-C8	BC-C20
Crystallinity Index (%)	86.47	78.07	79.21	77.52
Tensile strength (MPa)	94.14	71.00	81.47	87.79
Young's modulus (MPa)	746	640	628	690
Break strain (%)	26.79	23.37	24.63	21.60
Average pore diameter (nm)	100.0	56.7	51.8	49.8
Surface area (m^2/g)	4.20	4.40	3.39	2.64
Water absorption capacity (%)	330	186	174	216
Water vapour transmission rate ($g/m^2/day$)	1049	529	778	830
Oxygen transmission rate ($cm^3/m^2/day$)	2.63	1.74	1.55	1.42

Table 2 Antimicrobial properties of BC and BC-C films against *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger*.

Bacteria	Sample	Clear zone (mm)
Escherichia coli	BC	0
	BC-C3	0
	BC-C8	0
	BC-C20	0
Staphylococcus aureus	BC	0
	BC-C3	43.00 ± 1.25
	BC-C8	42.66 ± 0.90
	BC-C20	46.33 ± 0.77
Fungus	**Sample**	**Observed growth (Grade*)**
Aspergillus niger	BC	5
	BC-C3	2
	BC-C8	2
	BC-C20	2

*Grade was used as a measurement of fungal growth: 0 = none, 1 = only apparent under microscope, 2 = trace (<10%), 3 = light growth (10-30%), 4 = medium growth (30-60%) and 5 = heavy growth (> 60%).

List of Figures