รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาวัสดุยึดเกาะเซลล์เพื่อเพิ่มผลผลิตของกระบวนการหมักเอทานอลแบบต่อเนื่องโดย *Saccharomyces cerevisiae*

Development of cell carrier for improved productivity of continuous ethanol fermentation by *Saccharomyces cerevisiae*

โดย

รองศาสตราจารย์ ดร. เหมือนเดือน พิศาลพงศ์

ภาควิชา วิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

24 กรกฎาคม 2553

สัญญาเลขที่ RSA5080011

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สนับสนุนโดยทบวงมหาวิทยาลัย สำนักงานกองทุนสนับสนุนการวิจัย และจุฬาลงกรณ์มหาวิทยาลัย

กิตติกรรมประกาศ

โครงการวิจัยนี้ ได้รับทุนอุดหนุนการวิจัยจากทุนพัฒนานักวิจัย จากฝ่ายวิชาการ สำนักงานกองทุนสนับสนุนการวิจัย (สกว) ร่วมกับจุฬาลงกรณ์มหาวิทยาลัย (สัญญาเลขที่ RSA5080011) นอกจากนี้ยังได้รับการสนับสนุนและความร่วมมือจากบุคคลต่างๆ ทำให้งานวิจัยสำเร็จลุล่วงไปด้วยดี ดังต่อไปนี้

1. ศาสตราจารย์ ดร.สาวิตรี ลิ่มทอง ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ ให้ความอนุเคราะห์เซลล์ยีสต์เริ่มต้นสำหรับทำการทดลอง

2. คณะนิสิต ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ซึ่งมีส่วนช่วยในการทดลอง การวิเคราะห์ผล และการเตรียมเอกสารวิจัย

นาย Rusdianto Budiraharjo (ปริญญาโท)

นาย ฐาปนา สุวรรณมาโจ(ปริญญาโท)

นาย ภูวิศ บางรักษ์(ปริญญาโท)

น.ส. จิรวรรณ มงคลขจิต(ปริญญาโท)

นาย องอาจ สายบัวทอง(ปริญญาโท)

นาย นิติศักดิ์ กาญจนโมสิต(ปริญญาโท)

นาย อนุชิต รัตนพันธุ์(ปริญญาโท)

นาย จิรัณ กิ่งแก้ว (ปริญญาเอก)

ผู้วิจัยขอแสดงความขอบคุณอย่างยิ่งมา ณ ที่นี้

บทคัดย่อ

เนื่องจากวิกฤตการณ์โลกด้านพลังงานทำให้กระบวนการผลิตเอทานอลโดยการหมักได้รับความสนใจเป็นพิเศษ เนื่องจากเป็นแหล่งพลังงานหมุนเวียนที่ผลิตใหม่ได้เรื่อยๆจากชีวมวล ในปัจจุบันแกสโซฮอล์ อี-10 ซึ่งเป็นน้ำมันผสมในสัดส่วน 10 เปอร์เซ็นต์ของเอทานอล และ90 เปอร์เซ็นต์ของน้ำมันแกสโซลีน ได้ถูกใช้กันอย่างแพร่หลายสำหรับรถยนต์ในประเทศไทย และมีความพยายามที่ส่งเสริมการนำแกสโซฮอล์ อี-20 และ อี-85 เพื่อใช้สำหรับรถยนต์ในอนาคตอันใกล้ เมื่อพิจารณากระบวนการหมักเอทานอลแบบทั่วไปจะเป็นแบบกะ (batch) พบว่ามีข้อจำกัดหลายๆประการเช่น เป็นระบบที่มีความหนาแน่นของเซลล์ต่ำ ใช้เวลาผลิตค่อนข้างนาน แม้ว่ากระบวนการผลิตแบบต่อเนื่องโดยใช้เซลล์แขวนลอยจะช่วยเพิ่มความเร็วของการผลิตแต่ก็มีความยากลำบากในการดำเนินการตลอดจนยากในการรักษาระบบไม่ให้เกิดการปนเปื้อนจากจุลินทรีย์ภายนอก กระบวนการหมักสามารถปรับปรุงได้โดยใช้เทคนิคการตรึงเซลล์ การใช้เทคนิคการตรึงเซลล์จะช่วยให้ได้อัตราการผลิตผลิตภัณฑ์ที่เพิ่มขึ้นและการดำเนินการมีความเสถียรเพิ่มขึ้น ข้อดีหลักๆของการใช้เซลล์ที่ถูกตรึงเมื่อเปรียบเทียบกับเซลล์แขวนลอยคือ ระบบจะมีความเข้มข้นของเซลล์ที่เพิ่มขึ้น เซลล์ได้รับการปกป้องจากสารพิษ ตลอดจนช่วยลดค่าใช้จ่ายในส่วนกระบวนการแยกเซลล์ และการนำเซลล์กลับมาใช้ใหม่ อย่างไรก็ตามเทคนิคการตรึงเซลล์ยังไม่รับความนิยมใช้ในกระบวนการผลิตในระดับอุตสาหกรรม เนื่องจากปัญหาหลักๆคือ ข้อจำกัดทางการถ่ายเทมวลสารผ่านไปยังเซลล์ และความไม่เสถียรของวัสดุตรึงเซลล์เมื่อถูกใช้ในการดำเนินการหมักเป็นเวลานาน เพื่อปรับปรุงประสิทธิภาพของตัวพยุงเซลล์ งานวิจัยนี้จึงได้ศึกษาและพัฒนาวัสดุตรึงเซลล์ยีสต์ ชนิดใหม่ สามชนิด คือ 1) ตัวพยุงอัลจิเนทเสริมใยบวบ 2) ตัวพยุงอัลจิเนทเสริมอลูมินา และ3) ตัวพยุงรังไหมบาง ตัวพยุงเซลล์ทั้งสามแบบถูกนำมาประยุกต์ใช้ในการตรึงเซลล์ยีสต์ *Saccharomyces cerevisiae* M30 ในการหมักเอธานอล โดยใช้กากน้ำตาลเป็นแหล่งคาร์บอน พบว่าวัสดุตรึงเซลล์ทั้งสามชนิดมีคุณสมบัติที่เป็นประโยชน์หลายๆประการ เช่น มีความแข็งแรงเชิงกลสูง มีความเสถียร และ ให้ค่าการตรึงเซลล์ที่สูง โดยการใช้เซลล์ที่ถูกตรึงเหล่านี้จะทำให้ได้ผลผลิต เอทานอลที่ 1.3-1.7 และ 8.0-19.0 กรัม ต่อลิตรต่อชั่วโมง สำหรับกระบวนการหมักแบบกะและ แบบต่อเนื่องตามลำดับ พบว่ากระบวนการหมักเอทานอลแบบต่อเนื่องในถังปฏิกรณ์แบบเพคเบดโดยใช้เซลล์ที่ถูกตรึงสามารถทำงานได้อย่างมีประสิทธิภาพ และมีความเสถียรมากกว่า 30 วัน จากผลการทดลองแสดงให้เห็นถึงศักยภาพในการนำตัวพยุงเซลล์เหล่านี้ ไปใช้ในกระบวนการหมักเอทานอลที่มีการดำเนินการเป็นเวลานาน งานวิจัยนี้ยังได้ขยายขอบเขตการดำเนินงานไปถึงการพัฒนาชีววัสดุและการตรวจสอบคุณสมบัติ เพื่อใช้ในวิศวกรรมเนื้อเยื่อและกระบวนการแยกโดยใช้แผ่นเยื่อ

งานวิจัยนี้สามารถทำให้ผลิตผลงานวิจัยต่างๆดังนี้ (1) บทความวิจัยในระดับนานาชาติ 8 บทความ (2) สิทธิบัตร (ประเทศไทย) จำนวน 2 เรื่อง (3) บทความวิจัยในหนังสือประชุมวิชาการในระดับนานาชาติ 4 บทความ (4) บทความวิจัยในหนังสือประชุมวิชาการในระดับชาติ 3 บทความ และ (5) มีส่วนสนับสนุนกิจกรรมวิจัยในการผลิตมหาบัณฑิตจำนวน 7 คน และดุษฎีบัณฑิต 1 คน

คำสำคัญ เอทานอล กระบวนการตรึงเซลล์ ชีววัสดุ เชื้อเพลิงชีวภาพ

Abstract

The production of a renewable energy from biomass, such as ethanol by fermentation, has received special attention as a consequence of the world energy crisis. Nowadays, gasohol E-10, a mixture of 10% ethanol and 90% gasoline has been widely used in vehicles in Thailand and there is an attempt to promote the use of E-20 or E-85 in the vehicles in the near future. Ethanol fermentation by conventional batch suffers from various constrains such as, low cell density and rather time consuming. Although continuous fermentation by suspended cell culture can be used to speed up the process, it is more difficult to operate and maintain it free of microbial contamination. Immobilized cell technology has been suggested as an effective mean for improved fermentation. The immobilization of cells leads to a high productivity, and good operational stability. The main advantages in the use of immobilized cells in comparison with suspended cells are the retention in a reactor of higher cell concentration, protection of cells against toxic substances and elimination of costly processes of cell recovery and cell recycle. However, the major problems of using immobilization technique in industrial scale are mass transfer limitation and instability in long term operation. For improved performance of immobilized cell carriers, three new types of the cell carriers for ethanol fermentation were developed in the current study, namely, 1) Loofa reinforced alginate carriers 2) Alumina doped alginate gel carrier and 3) Thin shell silk cocoon. These cell carriers were applied for the immobilization of *Saccharomyces cerevisiae* M30 in ethanol fermentation using sugar cane molasses as a C-source. The developed cell carriers provided many advantage characteristics such as, good mechanical strength, high stability and high immobilization yield. The ethanol productivities of 1.3–1.5 and 8.0-19.0 g/(L·h) were achieved by using the immobilized cultures in batch and continuous modes of operation, respectively. The ethanol fermentations in a continuous packed-bed reactor using the immobilized cultures worked efficiently and were stable over 30 days. The results demonstrated the potential use of the cell carriers in an ethanol fermentation system for a long period of time. In extending this work, biomaterial development and characterization for tissue engineering and membrane separation were carried out.

Based on this research, we can produce 8 international research articles, 2 Thai patents, 4 international conference proceedings, 3 national conference proceedings and support research activities for 7 master degree students and 1 doctoral degree student.

Keywords: Ethanol; Cell immobilization;Biomaterial; Biofuel

สารบัญ

หน้าสรุปโครงการ(Executive Summary)

ทุนพัฒนานักวิจัย

1. ชื่อโครงการ (ภาษาไทย) การพัฒนาวัสดุยึดเกาะเซลล์เพื่อเพิ่มผลผลิตของกระบวนการหมักเอทานอลแบบต่อเนื่องโดย *Saccharomyces cerevisiae*

(ภาษาอังกฤษ) Development of cells carriers for improved productivity of continuous ethanol fermentation by *Saccharomyces cerevisiae*

2. ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลข โทรศัพท์ โทรสาร และ E-mail

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3. สาขาที่ทำการวิจัย วิศวกรรมชีวเคมี

Keywords: Biochemical Engineering, Biomaterials, Biofuel

4. งบประมาณรวมทั้งโครงการ 1,059,000 บาท

งบประมาณจากสกว. = 794,250 บาท

งบประมาณจากจาก จุฬาลงกรณ์มหาวิทยาลัย =264,750 บาท

5. ระยะเวลาดำเนินการ 3 ปี

6. ได้เสนอโครงการนี้ หรือโครงการที่มีส่วนเหมือนกับเรื่องนี้บางส่วนเพื่อขอทุนต่อแหล่งอื่นที่ใดบ้าง

/ ไม่ได้เสนอต่อแหล่งทุนอื่น

o เสนอต่อ
ชื่อโครงการ
กำหนดทราบผล

7. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

เนื่องจากปัญหาความขาดแคลนของแหล่งเชื้อเพลิงปิโตรเลียม และความไม่แน่นนอนของราคาตลอดจนแนวทางพระราชดำริ เกี่ยวกับการสร้างพลังงานทดแทน จากวัสดุทางการเกษตรที่มีปริมาณมากและมีราคาถูกในประเทศ รัฐบาลจึงกำหนดเป็นนโยบาย เพื่อสนับสนุนการผลิตแอลกอฮอล์ใช้เป็นเชื้อเพลิงทดแทน โดยจากการทดลองนำน้ำมันเชื้อเพลิงเบนซินผสมเอทานอล (แก๊สโซฮอล์) จำหน่ายแก่ประชาชนตามปั๊มน้ำมันหลายแห่งพบว่าได้รับการตอบรับเป็นอย่างดี เนื่องจากเอทานอลสามารถผลิตได้ในประเทศโดยกระบวนการหมักใช้สารตั้งต้นทางเกษตรกรรมเช่น กากน้ำตาล หรือแป้งมันสำปะหลัง หรือผลพลอยได้จากเกษตรกรรมอื่นๆ การนำเอทานอลมาใช้ทดแทนเชื้อเพลิงน้ำมันบางส่วน จึงช่วยลดอัตราการนำเข้าของน้ำมันปิโตรเลียม และส่งผลดีต่อเกษตรกรของประเทศโดยตรง นอกจากนี้ จากการพิจารณาผลกระทบต่อสิ่งแวดล้อม รถที่ใช้น้ำมันผสมเอทานอล จะปล่อยควันเสียที่มีปริมาณของมลพิษต่ำลง โดย THCลดลงประมาณ 6.2-8.5%, CO ลดลงประมาณ 23.2-39.1% และ NOX ลดลงประมาณ 12.2-13.4% นอกจากนี้เอทานอลยังสามารถนำไปใช้เป็นสารตั้งต้นในกระบวนการผลิต เชื้อเพลิงที่สำคัญอื่นๆเช่น ไบโอดีเซล และเซลล์เชื้อเพลิง (Ethanol fuel cell) โดยเชื้อเพลิงต่างๆที่กล่าวมาข้างต้นเป็นเชื้อเพลิงที่มีแนวโน้มที่จะถูกนำมาใช้ทดแทนเชื้อเพลิงปิโตรเลียมในอนาคต ทั้งนี้การใช้เอทานอลยังให้ผลดีกว่าในด้านความเป็นมิตรต่อสิ่งแวดล้อม และยังผลิตได้ใหม่เรื่อยๆ (renewable energy) เอทานอลเป็นสารละลายที่มีคุณสมบัติเป็นตัวทำละลายที่ดี มีความปลอดภัย จึงถูกนำไปใช้ในอุตสาหกรรมหลายชนิดรวมทั้งทางการแพทย์ โดยมีความพิษต่อคน และ สิ่งแวดล้อมต่ำมากเมื่อเทียบกับสารละลายตัวอื่นๆรวมทั้งเมทานอล

ปัจจุบันการผลิตเอทานอลในประเทศไทยทำโดยกระบวนการหมักจากวัสดุทางการเกษตรเช่น กากน้ำตาลและ มันสำปะหลัง โดยใช้ จุลินทรีย์ *Saccharomyces cerevisiae* เช่น *S. cerevisiae* Sc 90 ส่วนใหญ่ทำการหมักแบบกะ (แบบbatch) หรือแบบกึ่งกะ (Fed batch) ซึ่งเป็นกระบวนการที่มีอัตราการเร็วการผลิตที่ต่ำ การพัฒนากระบวนการผลิตจึงมีความจำเป็นเพื่อให้ได้กำลังการผลิตในปริมาณมากเพียงพอกับการนำไปใช้เป็นเชื้อเพลิง การพัฒนากระบวนการผลิตเป็นแบบต่อเนื่องที่มีการจัดการที่เหมาะสมจะสามารถเพิ่มผลผลิตได้หลายเท่าของกระบวนการแบบไม่ต่อเนื่อง ทำให้สามารถลดต้นทุนการผลิตได้ การนำเทคนิคการตรึงเซลล์ใช้ร่วมกับกระบวนการหมักแบบต่อเนื่องจะช่วยเพิ่มเซลล์ในระบบ มีผลให้อัตราเร็วการผลิตผลิตภัณฑ์เพิ่มขึ้นหรือลดเวลาในการหมักลง และช่วยลดความเสี่ยงในการปนเปื้อนจากจุลินทรีย์ภายนอก

ในปัจจุบันการผลิตเอทานอลโดยเซลล์ที่ถูกตรึงยังไม่ถูกนำมาใช้ในระดับอุตสาหกรรม เนื่องจากวัสดุและวิธีการตรึงเซลล์ยังมีสมบัติไม่ค่อยเหมาะสม เช่นไม่แข็งแรงมีราคาแพง หรือมีผลลบต่อกิจกรรมการผลิตของเซลล์จุลินทรีย์ นอกจากนี้บางส่วนเป็นวัสดุจากการสังเคราะห์ทางเคมีที่หลังจากเลิกใช้แล้วไม่สามารถกำจัดทิ้งได้โดยง่ายจึงทำให้เป็นปัญหาในการจัดการหรือมีผลลบต่อสิ่งแวดล้อมได้ในภายหลัง งานวิจัยนี้จึงมีความตั้งใจที่จะทำการศึกษา เพื่อพัฒนาให้ได้วัสดุตรึงเซลล์ที่มีความแข็งแรง มีราคาถูก มีความเสถียรต่อกระบวนการหมัก และมีผลดีต่อการหมัก โดยเน้นใช้วัสดุธรรมชาติซึ่งมีราคาถูกและมีปริมาณมากในประเทศเรา ซึ่งหลังจากเลิกใช้แล้วยังสามารถนำไปใช้เป็นประโยชน์ทางการเกษตรกรรมเช่นเป็นอาหารสัตว์ หรือ ทำเป็นปุ๋ย ทั้งนี้เพื่อช่วยลดต้นทุนของกระบวนการผลิตเอทานอลลง โดยเทคนิคที่

พัฒนาขึ้นนี้ยังอาจนำไปประยุกต์ใช้ในกระบวนการตรึงเซลล์เพื่อพัฒนากระบวนการหมักอื่นๆ นอกจากนี้ องค์ความรู้ต่างๆเกี่ยวกับชีววัสดุที่ได้รับจากการทำวิจัย ยังสามารถนำไปประยุกต์ใช้เพื่อการพัฒนาชีววัสดุ และการตรวจสอบสมบัติวัสดุ สำหรับงานอื่นๆ เช่นด้านวิศวกรรมเนื้อเยื่อ และกระบวนการแยกโดยใช้แผ่นเยื่อต่อไป

8. วัตถุประสงค์

8.1 พัฒนา ชีววัสดุและวิธีการตรึงเซลล์แบบใหม่ เพื่อ ให้ได้วัสดุที่มีความแข็งแรง มีราคาถูก ไม่เป็นพิษต่อสิ่งแวดล้อม และสนับสนุนการเจริญเติบโตตลอดจน การผลิตเอทานอลของยีสต์

8.2 พัฒนากระบวนการหมักเอทานอลแบบต่อเนื่องโดยใช้เซลล์ที่ถูกตรึง เพื่อลดต้นทุนการผลิตเอทานอล

8.3 สร้างองค์ความรู้ทางชีววัสดุที่สามารถนำไปประยุกต์ใช้ในระบบตรึงเซลล์หรือตรึงเอนไซม์อื่นๆ หรือ งานอื่นๆ เช่นด้านวิศวกรรมเนื้อเยื่อ และกระบวนการแยกโดยใช้แผ่นเยื่อต่อไป

9. ระเบียบวิธีวิจัย

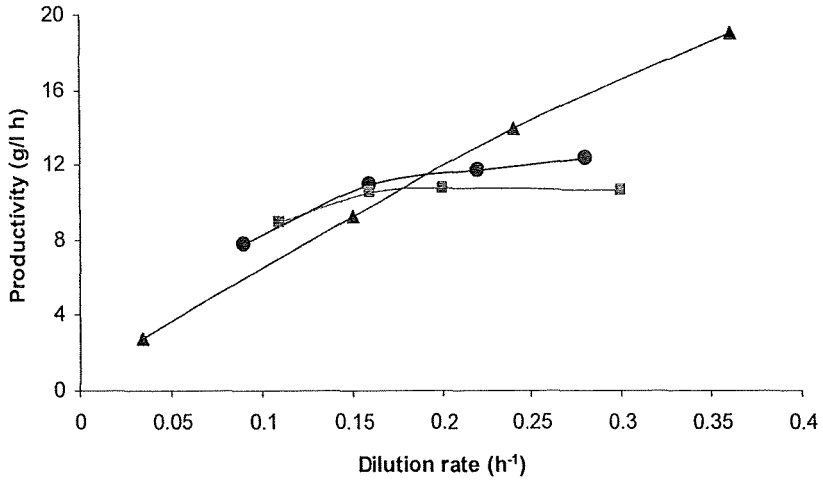
1) ศึกษาหลักการพื้นฐาน และรวบรวมงานวิจัยที่เกี่ยวข้อง
2) ทำการทดลองเตรียมวัสดุตรึงเซลล์ในรูปแบบต่างๆ
3) ทำการทดสอบเซลล์ที่ถูกตรึงในแบบต่างๆกับกระบวนการหมักเอทานอลแบบกะในระดับขวดเขย่าขนาด 500 มิลลิลิตร
4) ประเมินผล คัดเลือกวัสดุและวิธีการตรึงเซลล์ที่มีศักยภาพ
5) จัดทำถังปฏิกรณ์ในแบบเพคเบดขนาด 1 ลิตร
6) ทำการทดสอบเซลล์ที่ถูกตรึงในแบบที่พัฒนาขึ้น กับกระบวนการหมักเอทานอลแบบต่อเนื่องในถังปฏิกรณ์แบบเพคเบด
7) ทำการวิเคราะห์ผลเพื่อเสนอแนะกระบวนการผลิตเอทานอลที่เหมาะสม
8) ทดสอบ พัฒนาชีววัสดุ เพื่อใช้ประโยชน์ในด้านอื่นๆ
9) จัดทำรายงานสรุป และบทความวิจัยเพื่อเผยแพร่ผลงาน

10. ความเชื่อมโยงกับต่างประเทศ (หากมี)

ได้มีการวางแผนที่จะพัฒนาชีววัสดุต่อไป โดยร่วมกับนักวิจัยที่มีความเชี่ยวชาญด้านการพัฒนาพื้นผิววัสดุ ในปี 2554-2555 มีกำหนดที่จะส่งนิสิตปริญญาเอกซึ่งได้รับทุนโครงการกาญจนาภิเษก (RGJ) ไปทำวิจัยเพื่อพัฒนาชีววัสดุ ให้มีสมบัติดียิ่งขึ้น กับ Assoc. Prof. Bi-min Z. Newby, Chemical and Biomolecular Engineering Department, The University of Arkon ประเทศสหรัฐอเมริกา

เนื้อหางานวิจัย

เพื่อพัฒนาประสิทธิภาพของกระบวนการผลิตเอทานอล งานวิจัยนี้ได้ทำการพัฒนาชีววัสดุและวิธีการตรึงเซลล์แบบใหม่ เพื่อให้ได้วัสดุที่มีความแข็งแรง มีราคาถูก ไม่เป็นพิษต่อสิ่งแวดล้อม และสนับสนุนการเจริญเติบโตตลอดจนการผลิตเอทานอลของยีสต์โดยได้ทำการศึกษาและพัฒนาวัสดุตรึงเซลล์ชนิดใหม่ สามชนิด คือ 1) ตัวพยุงอัลจิเนทเสริมใยบวบ [1,2] 2) ตัวพยุงอัลจิเนทเสริมอลูมินา [3,4,5] และ3) ตัวพยุงรังไหมบาง[6] ตัวพยุงเซลล์ทั้งสามแบบถูกนำมาประยุกต์ใช้ในการตรึงเซลล์ยีสต์ *Saccharomyces cerevisiae* M30 ในการหมักเอทานอลใช้กากน้ำตาลเป็นแหล่งคาร์บอน ที่ความเข้มข้นเริ่มต้น 220-240 กรัมต่อลิตร ที่อุณหภูมิ 33 องศาเซนติเกรด โดยได้ทำการทดสอบเทียบประสิทธิภาพในการหมักเอทานอล กับระบบเซลล์แขวนลอย และระบบตรึงเซลล์แบบเดิมที่ตรึงเซลล์ภายในเม็ดเจลอัลจิเนท พบว่าวัสดุตรึงเซลล์ที่พัฒนาขึ้นใหม่ทั้งสามชนิดมีคุณสมบัติที่เป็นประโยชน์หลายๆประการเช่น มีความแข็งแรงเชิงกลที่สูง มีความเสถียร และ ให้ค่าการตรึงเซลล์ที่สูง ผลการวิจัยแสดงให้เห็นว่าระบบที่ใช้เซลล์ที่ถูกตรึงเหล่านี้ มีประสิทธิภาพดีกว่าการใช้เซลล์แขวนลอย หรือระบบที่ใช้อัลจิเนทเป็นวัสดุตรึงเซลล์แบบเดิม โดยระบบที่พัฒนาขึ้นนี้สามารถผลิต เอทานอลได้ที่ 1.3-1.7 จากกระบวนการหมักแบบกะ และ 8.0-19.0 กรัม ต่อลิตรต่อชั่วโมงจากกระบวนการหมักแบบต่อเนื่องที่อัตราการเจือจาง(Dilution rate) 0.1-0.35 ต่อชั่วโมง (ดังแสดงในรูปที่ 1) เมื่อเปรียบเทียบกับงานวิจัยอื่นๆที่ผ่านมา สรุปได้ว่ากระบวนการหมักเอทานอลแบบต่อเนื่องในถังปฏิกรณ์แบบเพคเบดโดยใช้เซลล์ที่ถูกตรึงที่พัฒนาขึ้นใหม่นี้สามารถทำงานได้อย่างมีประสิทธิภาพ มีความเสถียร และมีค่าการตรึงเซลล์ที่สูง จากผลการทดลองแสดงให้เห็นถึงศักยภาพในการนำตัวพยุงเซลล์เหล่านี้ไปใช้ในกระบวนการหมักเอทานอลที่มีการดำเนินการแบบต่อเนื่องเป็นเวลานาน



รูปที่ 1 กราฟเปรียบเทียบอัตราการผลิตเอทานอลโดยใช้กระบวนการหมักแบบต่อเนื่องในถังปฏิกรณ์แบบเพคเบดที่อัตราเจือจาง (Dilution rate) 0.1-0.35 ต่อชั่วโมง โดยใช้เซลล์ที่ถูกตรึงทั้ง 3 ชนิดที่พัฒนาขึ้นในงานวิจัยนี้ (—■— =ตัวพยุงอัลจิเนทเสริมใยบวบ , —●— =ตัวพยุงอัลจิเนทเสริมอลูมินา, —▲— =ตัวพยุงรังไหมบาง)

นอกจากนี้ระหว่างการศึกษาวิจัยเพื่อพัฒนาชีววัสดุสำหรับการตรึงเซลล์ยีสต์ พบว่าชีววัสดุที่มีการปรับปรุงสมบัติบางชนิดสามารถนำไปประยุกต์ใช้ในด้านอื่นๆได้ด้วย งานวิจัยนี้จึงได้ขยายขอบเขตการดำเนินงานไปถึงการปรับปรุงชีววัสดุและการตรวจสอบสมบัติวัสดุที่มีศักยภาพในการประยุกต์ใช้ในด้าน วิศวกรรมเนื้อเยื่อ[7-9] และในการแยกโดยใช้แผ่นเยื่อ [10].

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ผลงาน(Output) ที่ได้จากโครงการ

ผลงานสิทธิบัตร(ไทย) ดำเนินการโดยสถาบันทรัพย์สินทางปัญญาแห่งจุฬาลงกรณ์มหาวิทยาลัย

1.การผลิตเอทานอลแบบต่อเนื่องโดยใช้ยีสต์ที่ถูกตรึงในตัวพยุงเจลเสริมใยบวบ เลขที่คำขอ 0901000435

2. กระบวนการสำหรับการหมักเอทานอลด้วยยีสต์ที่ถูกตรึงด้วยเปลือกรังไหมบาง เลขที่คำขอ 0901002997

ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

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8. Mongkolkajit,J., Phisalaphong, M. Alumina doped alginate gel as a cell carrier in ethanol fermentation process, Biotechnology for Biofuels (submitted).

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<u>บทความ Proceeding ในเอกสารการประชุมระดับนาชาติ</u>

1. Bangrak, P., Mongkolkajit, J., Phisalaphong,M., "Development of Entrapment-Loofa Matrix Carrier for Ethanol Production", the 17th Thailand Chemical Engineering and Applied Chemistry Conference, Chiangmai, Thailand.,October 2007. (ได้รางวัลการนำเสนอดี)
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<u>ผลงานอื่นๆที่เกี่ยวข้อง</u>

1. บทความเผยแพร่ในหนังสือพิมพ์ กรุงเทพธุรกิจ ฉบับ วันที่ 8 เมษายน 2551
2. บทความเผยแพร่ในวารสารวิจัย ระดับชาติ
   / เหมือนเดือน พิศาลพงศ์ และ จิรกานต์ เมืองนาโพธิ์, เชื้อเพลิงจากกระบวนการทางชีวภาพ, วิศวกรรมสาร (Thailand Engineering Journal), 2550 (60) 24-27.
3. บทความเรื่อง "จากโครงการส่วนพระองค์สู่งานวิจัยร่วมสถาบันการผลิตแอลกอฮอล์เพื่อนำไปใช้ทดแทนน้ำมันเชื้อเพลิง" โดย เหมือนเดือน พิศาลพงศ์ในหนังสือ "ตามรอยพระมหาวิศวกรบพิตร" หน้า 190-207 จัดทำโดย คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เพื่อเฉลิมพระเกียรติเนื่องในโอกาสมหามงคลเฉลิมพระชนมพรรษา 80 พรรษา, 5 ธันวาคม 2550

<u>การเชื่อมโยงกับต่างประเทศหรือรางวัลที่ได้รับ</u>

1. รับเชิญเป็น reviewer ในวารสารต่างประเทศ เช่น Biochemical Engineering Journal, Applied Polymer Science, Journal of Polymer Engineering, Journal of Chemical Technology and Biotechnology และ African Journal of Biotechnology
2. รางวัลวิจัยดีมาก ปี 2551 กองทุนรัชดาภิเษกสมโภช จุฬาลงกรณ์มหาวิทยาลัย
3. รางวัลดี ในการนำเสนอผลงานประเภท โปสเตอร์ การประชุมวิชาการวิศวกรรมเคมีและเคมีประยุกต์แห่งประเทศไทย ครั้งที่ 17

5. ผลงานที่นำเสนอในการประชุมในระดับนานาชาติ Regional symposium on Chemical Engineering (RSCE) ได้รับการคัดเลือกให้ตีพิมพ์ในวารสารวิจัยระดับนานานชาติ Asian Journal of Chemical Engineering e-journal
6. ได้รับเชิญให้เขียนบทความในหนังสือ

   Phisalaphong,M., Jatupaiboon, N. and Kingkaew, J. Biosynthesis of Cellulose Chitosan Composite in: Kim, Se-Kwon (Eds.), Chitin, chitosan, oligosaccharides and their derivatives, CRC Press, Taylor & Francis Group, New York, 6 (2010) 53–65.

ภาคผนวก A บทความวิจัย (Reprint, Manuscript)

No	Title	Year	Role	Journal	IF(2009)	Acknowledgement (in paper)
1	Alginate-Loofa as Carrier Matrix for Ethanol Production. [**Phisalaphong, M.***, Budiraharjo,R., Bangrak, P., Mongkolkajit, J., Limtong S.]	2007	Corresponding author	Journal of Bioscience Bioengineering	1.749	TRF-RSA5080011
2	Synthesis and Characterization of Bacterial Cellulose/ Alginate Blend Membrane. [**Phisalaphong, M.***, Suwanmajo, T., Thamarat P.]	2008	Corresponding author	Journal of Applied Polymer Science	1.203	TRF-RSA5080011
3	γ – alumina doped alginate gel for cell immobilization in fermentation processes. [Mongkolkajit, J., Pullsirisombat, J., Prichanont, S., Tantayakom,V., Prasitchoke, P., **Phisalaphong, M.***]	2010 (in press)	Corresponding author	Asian Journal of Chemical Engineering	-	TRF-RSA5080011
4	Novo aloe vera–bacterial cellulose composite film from biosynthesis. [Saibuatong, O., **Phisalaphong, M.***]	2010	Corresponding author	Carbohydrate Polymers	3.167	TRF-RSA5080011
5	Biocompatibility and growth of human keratinocytes and fibroblasts on biosynthesized cellulose - chitosan film. [Kingkaewa, J., Jatupaiboona, N., Sanchavanakitb, N., Pavasantb, P., **Phisalaphong, M.***]	2010	Corresponding author	Journal of Biomaterials Science	2.505	TRF-RSA5080011
6	Biosynthesis and Characterization of Bacteria Cellulose - Alginate Film, (Kanjamamosit, N., Muangnapoh, C. , **Phisalaphong, M.***]	2010	Corresponding author	Journal of Applied Polymer Science	1.203	TRF-RSA5080011
7	Continuous ethanol production using immobilized yeast cells entrapped in loofa reinforced alginate carriers, [Bangrak, P., Limtong, S., **Phisalaphong, M***]	2010 (revised & resubmitted)	Corresponding author	Brazilian Journal of Microbiology	0.622	TRF-RSA5080011
8	Alumina doped alginate gel as a cell carrier in ethanol fermentation process. [Mongkolkajit J., **Phisalaphong, M***],	2010 (submitted)	Corresponding author	Biotechnology for Biofuels	4.118	TRF-RSA5080011

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Alginate-Loofa as Carrier Matrix for Ethanol Production

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An alginate-loofa matrix was developed as a cell carrier for ethanol fermentation owing to its porous structure and strong fibrous nature. The matrix was effective for cell immobilization and had good mechanical strength and stability for long-term use. After a storage period of 4 months, yeast cells remained firmly immobilized and active.

[**Key words:** loofa sponge, alginate, yeast, ethanol production]

In response to energy crisis, ethanol has re-emerged as an alternative to, or extender for, petroleum-based liquid fuels. Ethanol production using an immobilized cell system offers many advantages such as higher productivity and protection of cells from inhibitions. Cell entrapment within alginate is one of the most widely studied because cell viability and activity are kept very high (1). However, the practical application of polymeric gel carriers including alginate beads has been limited by the problems of gel degradation, low physical strength, and severe mass transfer limitation (1–8). Furthermore, large-scale production of these carriers often requires complex and sophisticated equipment leading to high cost of production (2). On the other hand, loofa sponges, lignocellulosic matrices from *Luffa cylindrica*, were found to be promising cell carriers for ethanol production by flocculating cells (2–5). The sponges are light, strong, chemically stable, and composed of interconnecting voids within an open network of fibers. Because of the random lattices of small cross sections of the sponges coupled with high porosity, the sponges are suitable for cell adhesion. Continuous fuel ethanol production had been realized using yeast cells immobilized in loofa sponges in a bubble column configuration (5). However, a low-shear environment and large aggregates of cells were required in order to prevent excessive cell sloughing from the carriers (3, 9).

On the basis of the above, in the present work, we focused on developing a new cell carrier by combining alginate gel and loofa sponge namely, the alginate-loofa matrix (ALM). Ethanol production by repeated batch fermentation using yeast cells immobilized within the ALM was then examined and compared with that using suspended cells and cells immobilized in conventional calcium alginate beads.

MATERIALS AND METHODS

Yeast strains *Saccharomyces cerevisiae* M30 selected on the basis of its high efficiency in ethanol production from molasses at high temperature was used in this study.

Culture media and cell preparation Starter cultures were prepared by transferring cells from stock PDA slants to 150 ml of sterilized medium followed by incubation at 33°C, 150 rpm for 20 h. The medium for the starter culture contained 0.05% ammonium sulfate and 5% inverse sugar from palm sugar at pH 5.0. After that, the obtained cell suspension was concentrated by decantation and then transferred to the main culture.

Cells immobilized on alginate-loofa matrix Sodium alginate (3% w/v) solution was formulated by dissolving Na-alginate powder in 0.9% (w/v) NaCl solution. It was autoclaved for 5 min at 121°C and kept overnight at 4°C to facilitate deaeration. Cell suspension of 5 ml was then added to 50 ml of 3% (w/v) alginate solution to form an alginate-cell mixture. To form ALM, 2 g of sterilized cubic sponges of loofa (8×8×2 mm) was dipped into the alginate-cell mixture. The gel carriers were transferred to 1.47% (w/v) $CaCl_2$ solution and left to harden in this solution with mild stirring for 15 min. The carriers were then rinsed 3 times with 0.9% (w/v) NaCl solution. Carriers were prepared under aseptic conditions and the average size of ALM was 9×9×3 mm^3.

Fermentations Repeated batch fermentations were carried out in duplicate using a medium contained 0.05% ammonium sulfate and 21% (w/v) inverse sugar from cane molasses at pH 5.0. The prepared medium was sterilized at 121°C for 20 min. Experiments were initiated by transferring prepared cell suspension or immobilized cells into 250 ml of the medium in 500 ml Erlenmeyer flasks. Fermentation flasks were then shaken in the incubator at 150 rpm, 33°C for 48 h. The experiments were monitored by removing 2 ml samples every 6 h for cell, sugar and ethanol analyses.

Analytical methods Free cell dry weight was determined from the absorbance at 660 nm with a UV-2450 UV-visible spectrophotometer and converted to dry cell concentration on the basis of a corresponding standard curve. For immobilized cells, a known mass of cell carriers was dissolved in 0.05 M sodium citrate. After the sponge was removed, immobilized cell concentration was determined similarly for the free cells. The concentration of ethanol was determined by gas chromatography using a Shimadzu model GC $7A_G$ (Shimadzu, Kyoto) equipped with a flame ionization detector. To measure reducing sugar concentration, the sample solution was hydrolyzed in 33% HCl at 100°C for 10 min and neutralized with NaOH solution. Reducing sugar content was then determined by the dinitrosalicylic acid method (10).

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TABLE 1. Yields and end products of repeated batch ethanol fermentation for 48 h for each batch using the cultures of suspended cells (SC), Ca-alginate-immobilized cells (AB), and alginate-loofa-matrix-immobilized cells (ALM)

Batch	P (g/l)	X (g/l)		Y_I (%)	Y_S (%)	$Y_{P/S}$ (g/g)
		X_F	X_I			
I						
SC	91.7	3.5	–	–	86	0.47
AB	70.4	0.6	6.0	91	87	0.40
ALM	77.8	0.7	4.6	87	84	0.44
II						
SC	47.7	5.1	–	–	57	0.33
AB	72.7	0.7	8.5	93	86	0.40
ALM	75.9	0.9	6.1	87	86	0.40
III						
SC	97.4	6.5	–	–	85	0.46
AB	89.5	0.8	8.5	92	87	0.44
ALM	90.6	1.1	7.1	87	88	0.46

Immobilization yield (Y_I) is the ratio of immobilized cell concentration (X_I) to total cell concentration (X), and X_F is the free cell concentration. Sugar consumption yield (Y_S) is the ratio of sugar consumption (S_0-S) to the initial sugar concentration (S_0). Ethanol yield ($Y_{P/S}$) is the ratio of ethanol accumulation ($P-P_0$) to the sugar consumption.

Scanning electron microscopy (SEM) Samples of immobilized cells in alginate beads (AB) and ALM were frozen in liquid nitrogen, immediately snapped, vacuum-dried, and then sputtered with gold and photographed. Images were taken on a JEOL JSM-5410LV (JEOL, Tokyo) scanning electron microscope.

RESULTS AND DISCUSSION

Ethanol production using ALM as a carrier for *S. cerevisiae* M30 was examined by a 3-cycle repeated batch fermentation using cane molasses as the C source. The duration of each batch was 48 h. There were three cultures in this study: suspended cells (SC), Ca-alginate-immobilized (AB) cells and ALM-immobilized cells. The results of the fermentations are summarized in Table 1. For the first batch, after 48 h the ethanol concentration of the SC system was 91.7 g/l, whereas the final ethanol concentrations of immobilized cells (IC) in AB and ALM carriers were 70.4 and 77.8 g/l, respectively. At the end of the first batch, the total cell concentrations of IC cultures were higher than that of the SC culture. The increase in cell concentration in AB and ALM carriers owing to cell growth inside the carriers during the course of fermentation was observed previously (11). The final total cell concentration of the system with ALM carriers was 5.3 g/l with an immobilization yield (Y_I) of 87%, which was slightly lower than that of the system with AB carriers (X 6.6 g/l with Y_I 91%); however, the ethanol production was 10% higher.

Instability of SC culture was observed in the second batch. It was found that there was no lag phase in IC systems, whereas an approximately 30 h lag phase was observed in SC systems. Corresponding to its sugar consumption, the ethanol concentration of SC cultures in the second batch was only half of that of the first batch, whereas the ethanol productions by IC cultures were similar to those of the first batch. Free-cell concentrations of IC systems slightly increased from the first to the second batch, which can be attributed to cell leakage and growth in the medium.

In the third batch, all the systems exhibited high ethanol productions without any occurrence of the lag phase. In most cases, the majority of sugar was consumed within 36 h with the final ethanol concentrations being 97.4, 89.5 and 90.6 g/l for SC, AB-immobilized cell and ALM-immobilized cell cultures, respectively. The ethanol concentration profile followed the trend of a normal microbial growth curve. At the end of the third batch, partial gel degradation on the surfaces of AB and ALM carriers was observed. However, the immobilization yields (Y_I) remained constant.

A marked instability of the SC culture in the repeated batch fermentation was observed from the comparison of its final ethanol concentration from batch to batch, which may be attributable to the negative effect of high ethanol concentration on cell activity and viability. In contrast, the ethanol production of IC cultures in AB and ALM carriers were relatively stable. It was suggested from previous studies (2, 7) that the matrix of carriers can protect yeast by functioning as a fortification against toxins and inhibitors. In terms of immobilization yield (Y_I), ALM carriers exhibited a slightly lower immobilization capacity (average Y_I=87%) than AB carriers (average Y_I=92%). However, the final ethanol concentration and the ethanol yield factor ($Y_{P/S}$) were comparable. Changes in physical or chemical parameters such as temperature and the concentrations of sugar and ethanol affected cell growth and product formation during fermentation (12). To improve yield, substrates, metabolite products, and conditions should be maintained or controlled at optimal levels under steady-state condition by continuous bioreactor fermentation.

A series of SEM images were taken to provide visual description and information of fermentation systems. The SEM images of the initial AB and ALM are shown in Fig. 1. From the external surface, most of the yeast cells were covered by alginate film; only a few cells were absorbed on the surface of the carriers. The AB and ALM carriers displayed different gel morphologies. ALM carriers were composed of stacked layers of thin alginate films whereas the structure of AB carriers were dense and less porous (Figs. 1 and 2). Consequently, fewer cells were observed in the central part

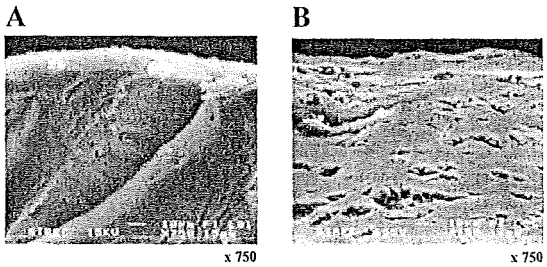

FIG. 1. Cross section of fresh carriers after the entrapment of yeasts (0 h): (A) alginate bead, AB; (B) alginate-loofa matrix, ALM. Bars: 10 μm.

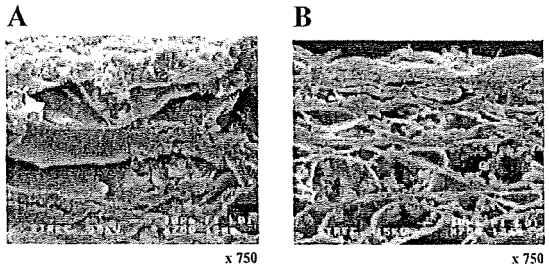

FIG. 2. Cross section of carriers after the third batch (144 h): (A) alginate bead, AB; (B) alginate-loofa matrix, ALM. Bars: 10 μm.

of AB carriers. The preference of cells to grow near a surface other than the middle of a gel bead was previously reported (1, 11). The high concentration of substrates near the surface as a consequence of mass transfer limitation was believed to be the main driving force for this phenomenon.

High porosity and better cell distribution were observed in ALM carriers. Because of their highly porous structure, mass transfer limitation in ALM carriers was less severe than that in AB carriers despite their larger size. An examination of the center of ALM carriers revealed that yeast cells were located in the middle of ALM carriers although ALM carriers were relatively large (about 70 times the size of AB carriers). Despite the partial degradation of alginate films after several fermentation cycles, cells were still firmly immobilized due to the aggregation of yeasts and their adhesion to the ALM matrix. Many cells in the space between a loofa fiber and an alginate film were observed (Fig. 3). In a previous study of cellulose carriers (13), 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.

The beneficial properties of IC systems, such as the protection of cells from solvent inhibitions and promotion of cell productivity as demonstrated in this study, were also reported elsewhere (14). The ability of cells to grow in an immobilized state made it possible for cell regeneration under hostile conditions such as a high ethanol concentration. In this study, the regeneration and protection of entrapped cells by ALM were proposed as the main factors that work synergistically to preserve cell activity. Thus, a stability of ALM-immobilized cell culture higher than that of SC culture was achieved.

Ogbonna *et al.* (4, 5) reported that loofa sponge alone can

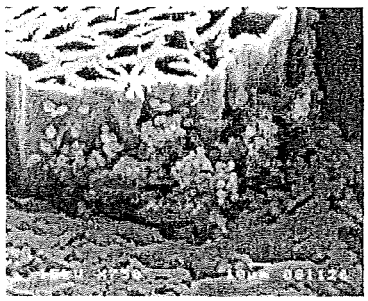

FIG. 3. Yeast cells in a space between loofa fiber and alginate gel of alginate-loofa matrix (ALM) carrier. Bar: 10 μm.

be used to achieve 99% immobilization of a flocculating yeast strain for ethanol production in column-type bioreactors. On the other hand, the results from our preliminary study in conventional shake flask cultures at 150 rpm showed that loofa sponge alone was not effective for the immobilization of yeast cells owing to the large pores of loofa sponges (500–1000 μm) with respect to the size of yeast cells (3–7 μm). Therefore, in a high-shear environment arising from agitation or high fluid velocity, excessive cell detachment from the sponges was observed regardless of the shape or size of sponges used. The difference in the obtained results might arise from differences in the yeast strain used and system characteristics.

To investigate storage effects, cell cultures from the third batch (a total time span of 144 h) were stored for 4 months at 4°C and reused. Ethanol productions by a 4-cycle repeated batch fermentation (a total time span of 192 h) using the stored cultures of AB-immobilized cells, ALM-immobilized cells and SCs, were examined. Overall, the IC cultures remained stable; a maximum ethanol concentration of 70–80 g/*l* was achieved with an ethanol yield ($Y_{P/S}$) of 0.44–0.49. The instability of SC cultures was again observed in the first and the fourth batches as shown in Table 2. In comparison with the results shown in Table 1, the average productivities of the stored AB- and ALM-immobilized cell cultures (Table 2) slightly decreased. However, compared with the SC cultures, the stability and average ethanol productivity of the ALM-immobilized cell culture were significantly improved. After the 4-cycle repeated batch, a higher degree of gel degradation occurred on the surface of the car-

TABLE 2. Ethanol concentration in repeated batch fermentation using 4-month-stored cultures of suspended cells (SCs), Ca-alginate-immobilized cells (AB), and alginate-loofa-matrix-immobilized cells (ALM)

Batch	Time (h)	Ethanol concentration (P, g/*l*)		
		SC	AB	ALM
I	24	4.4	76.6	75.5
	48	65.7	76.0	76.0
II	24	63.3	60.3	66.3
	48	72.0	86.1	80.7
III	24	47.6	69.5	69.0
	48	86.1	76.5	71.3
IV	24	2.1	61.8	65.1
	48	3.7	70.0	70.4

riers; however, the majority of cells were still attached to each other within the matrix. The immobilization yields (Y_I) of AB and ALM carriers slightly decreased to 86% and 81%, respectively. From the results together with the strong and chemical stable nature of loofa sponge, ALM has good mechanical strength, durability, and stability for long-term use.

In conclusion, ALM was successfully developed and applied in repeated batch ethanol fermentation. The carriers were fabricated simply by entrapment of a peripheral loofa sponge that was previously dipped in an alginate-cell mixture. The porous structure conferred the new carriers with better mass transfer characteristics. An ALM with a size of $9 \times 9 \times 3$ mm^3 was effective for cell immobilization, which is comparable to a 2-mm-diameter alginate bead. The immobilization yield of the new carriers was approximately 87%. Ethanol production using these carriers was proven to be more stable than that using SC cultures. After storage for 4 months, the ALM-immobilized cell culture was still active, and the stability of IC cultures being higher than that of SC culture was confirmed. As shown in this study, the ALM carriers have many advantages including regeneration ability, reusability, stability, altered mechanical strength, and high ethanol productivity. The results demonstrated the potential use of ALM carriers in an ethanol fermentation system for a long period of time. To improve productivity and yield, the evaluation of ALM as carrier matrix in a packed column for continuous fermentation is on going.

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Synthesis and Characterization of Bacterial Cellulose/Alginate Blend Membranes

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ABSTRACT: Bacterial cellulose and alginate in an aqueous NaOH/urea solution were used as substrate materials for the fabrication of a novel blend membrane. The blend solution was cast onto a Teflon plate, coagulated in a 5 wt % $CaCl_2$ aqueous solution, and then treated with a 1% HCl solution. Supercritical carbon dioxide drying was then applied for the formation of a nanoporous structure. The physical properties and morphology of the regenerated bacterial cellulose and blend membranes were characterized. The blend membrane with 80% bacterial cellulose/20 wt % alginate displayed a homogeneous structure and exhibited a better water adsorption capacity and water vapor transmission rate. However, the tensile strength and elongation at break of the film with a thickness of 0.09 mm slightly decreased to 3.38 MPa and 31.60%, respectively. The average pore size of the blend membrane was 10.60 Å with a 19.50 m^2/g surface area. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 107: 3419–3424, 2008

Key words: biopolymers; blends; membranes; synthesis

INTRODUCTION

Cellulose is Earth's major biopolymer from plants and other living systems such as plankton, algae, fungi, and bacteria. Bacterial cellulose (BC), produced by *Acetobacter xylinus*, is distinctly different from cellulose derived from plants and the others. BC is devoid of lignin, hemicellulose, and other complex carbohydrates. Because of the entangled mesh of nanofibrils compacted in the form of an ultrafine network, BC displays unique properties, including high mechanical strength, high water absorption capacity (WAC), and high crystallinity.[1–5] It is incredibly hydrophilic, absorbing 60–700 times its weight in water.[1,6] The diameter of BC is about 1/100 of that of plant cellulose, and Young's modulus of BC is higher than that of general organic fibers.[5]

Regenerated cellulose membranes blended with other natural polymers such as alginate (Al),[7–11] konjac glucomannan,[12] chitosan,[13] and chitin[14] to modify their properties for applications in many separation fields have been extensively reported. Al, a heteropolysaccharide extracted from marine brown algae, has been widely explored as a substrate material for blend membranes and a thickener for cellulose fibers. A new blend membrane from cellulose (cotton linter) and Al showed improved performance for the dehydration of ethanol/water in a pervaporation process.[9] The mechanical properties of the Al membrane were found to be significantly improved by the introduction of cellulose and Ca^{2+} bridges. The crystalline state of the blend membrane from cellulose (cotton linter) and Al prepared in an aqueous NaOH/urea solution was broken completely, and the crystallinity of the blend membranes decreased with an increase in the Al ratio.[10] Although studies of blend membranes from plant-derived cellulose with other biopolymers have been extensively synthesized and characterized,[7–15] very few, if any, studies using BC as a source of cellulose have been reported.

Because BC displays unique properties considerably different from those of plant cellulose, in this work, we focused on developing a new nanostructure blend membrane from BC and Al in an aqueous NaOH/urea solution. The surface morphology, pore structure, tensile strength, WAC, and water vapor permeability of the blended membranes were examined and compared with those of regenerated bacterial cellulose (RBC) films.[16] To the best of our knowledge, this type of blend combination was first prepared in this work.

EXPERIMENTAL

BC

The BC used in this study was the gel-like cellulose pellicle formed by *A. xylinus* cultures on the surface

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of media containing 0.5% ammonium sulfate, 5.0% sucrose, and 1.0% acetic acid in coconut water. The sheets of BC were purified by washing with deionized (DI) water and then were treated with 1% (w/v) NaOH at 35°C for 24 h to remove bacterial cells and rinsed with DI water until the pH was 7. Afterward, the BC film was air-dried at room temperature (30°C) and stored in plastic film before use.

Preparation of the membranes

Preparation of the BC/Al blend film

To prepare a slurry of BC, 3 wt % BC was dissolved in a 4 wt % NaOH/3 wt % urea aqueous solution and stirred for 10 min at room temperature. The BC slurry was then cooled to −5°C in a freezer and held at −5°C until it became a solid frozen mass for 12 h. The frozen solid was then allowed to thaw and was stirred extensively at room temperature to obtain a clear BC solution. After that, the insoluble parts were isolated by centrifugation at 6000 rpm and 10°C for 30 min. Sodium alginate (3 wt %) was dissolved in distilled water at room temperature to form a gel-like solution. Then, the prepared solution of BC was mixed with the Al solution to produce mixtures having BC/Al weight ratios of 100/0, 80/20, 60/40, 40/60, 20/80, and 0/100. The mixtures were stirred energetically at room temperature for 24 h to form clear solutions. The casting solutions were spread over a Teflon plate. The thickness of this solution was controlled at 2.3 mm by manual adjustment of the height of the casting blade. The thickness of the membranes was measured with a micrometer (Mitutoyo, Tokyo, Japan) at various parts of a particular membrane. All of the casting solutions were coagulated in a 5 wt % $CaCl_2$ aqueous solution for 30 min and treated with a 1% HCl solution for 10 min. After that, the membranes were washed with DI water until the drain water reached a pH of 7, and then they were air-dried at room temperature.

Preparation of the nanoporous structure

Supercritical drying was applied to the preparation of a porous structure. First, the films were dipped in DI water for 24 h. After that, to replace water with ethanol, the swollen films were immersed in 10, 30, 50, and 70% (w/v) ethanol for 30 min in each step, and then they were immersed in 100% (w/v) ethanol for 1 h. Lastly, the swollen membranes were dried by supercritical CO_2 drying. In the drying procedure, the films were placed in a vessel inside a high-pressure cell with an inner diameter of 10 cm. The cell was immediately filled with supercritical CO_2 and controlled at 40°C and 1200 psi (the critical point of CO_2; critical pressure = 1072 psi, critical temperature = 31°C). The temperature and pressure were selected so that the CO_2 and ethanol inside the membrane were fully miscible. Subsequently, the cell was flushed by the addition of fresh CO_2 for 2 h to replace the residual ethanol inside, and then the system was slowly depressurized at a constant rate of 150 psi/min to remove the CO_2.

Characterization of the membranes

Elemental analysis of the membranes

The membranes were cut into particles and vacuum-dried for 24 h before the analysis of the elemental contents. The contents of nitrogen in the membranes were determined with a Leco (St. Joseph, MI) CHN-2000 elemental analyzer. The contents of calcium and sodium were determined with an Oxford Ed (Oxford, UK) 2000 X-ray fluorescence (XRF) spectrometer.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was used primarily to identify the chemical structure of the membrane. The FTIR spectra of the membranes were recorded with a Nicolet (Madison, WI) SX-170 FTIR spectrometer.

WAC

To determine WAC, the dried membranes were immersed in DI water at room temperature until equilibration. After that, the membranes were removed from the water, and excess water at the surface of the membranes was blotted out with Kimwipes paper. The weights of the swollen membranes were measured, and the procedure was repeated until no further weight change was observed. The water content was calculated with the following formula:

$$\text{WAC}\ (\%) = \frac{W_h - W_d}{W_d} \times 100 \qquad (1)$$

where W_h and W_d are the weights of the hydrate and dry membrane, respectively.

Tensile property testing

All the membranes under study in the dry form were tested for the tensile strength and elongation at break. The film samples were cut into strip-shaped specimens 10 mm wide and 10 cm long. The maximum tensile strength and break strain of RBC films were determined with a Lloyd (Southampton, UK) 2000R universal testing machine. The test conditions

followed ASTM D 882. The tensile strength and break strain were the average values determined from 10 specimens.

Scanning electron microscopy (SEM)

The films were frozen in liquid nitrogen, immediately snapped, vacuum-dried, and then sputtered with gold and photographed. Images were taken on a JOEL (Tokyo, Japan) JSM-5410LV scanning electron microscope.

Water vapor permeability measurement

The water vapor transmission rate (WVTR) of the dry RBC membrane and the dry RBC/Al blend membrane with an area of 50 cm^2 were determined with a Lyssy (Zollikon, Switzerland) L80-4000 water vapor permeation tester. The test conditions followed ISO 15106-1. The determination of WVTR was done under 38°C and 90% relative humidity. As water solubilized into the membrane and permeated through the sample film, nitrogen gas swept and transported the transmitted water vapor molecules to a calibrated infrared sensor. The response was reported as a transmission rate.

Brunauer–Emmett–Teller (BET) surface analysis

The pore size and surface area of the membranes were determined with a BET surface area analyzer. To remove moisture from the film samples, the samples were placed in sample cells, which were then heated up to 373 K for 2 h and cooled to room temperature before the BET analysis. The BET pore size and surface area were determined with N_2 adsorption at 77 K in a Micromeritics (Atlanta, GA) ASAP 2020.

RESULTS AND DISCUSSION

Membrane compositions

Elemental analysis revealed the absence of nitrogen in the fabricated membranes. Therefore, urea was completely removed from the membrane during the coagulation and washing. The XRF spectra indicated that the calcium content was less than 0.005 wt % in the blend membrane with $\leq$40 wt % Al and increased to 0.010–0.019 wt % in the blend membranes with greater than 40 wt % Al. No sodium peak was observed in any of the membranes, and this suggested that NaCl and NaOH were completely removed from the developed membranes. A small amount of Al/calcium complex as calcium bridges was previously reported in Al membranes.[9,17] Therefore, the calcium accumulation in the BC/Al blend membranes should be due to the formation of a calcium/Al gel network by crosslinking with Ca^{2+} ions.

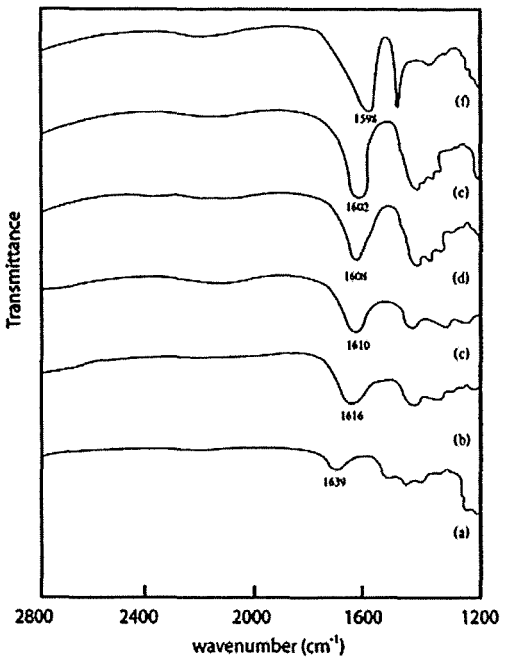

Figure 1 FTIR spectra of the BC/Al blend membranes. The BC/Al ratios were (a) 100/0, (b) 80/20, (c) 60/40, (d) 40/60, (e) 20/80, and (f) 0/100.

FTIR analysis of the blend membrane

FTIR spectroscopy was used to investigate the nature of mixing between the two biopolymers in this study. The FTIR spectra of the RBC, Al, and blend membranes were measured at wave numbers ranging from 2800 to 1200 cm^{-1}. The FTIR spectra of the RBC membrane [Fig. 1(a)] showed a band at 1639 cm^{-1}, which was attributed to glucose carbonyl of cellulose. The strong band of the Al membrane at 1598 cm^{-1} [Fig. 1(f)] indicated the presence of the carboxyl group. The FTIR spectra of BC/Al blend membranes [Fig. 1(b–e)] were characterized by the presence of absorption bands in the pure components, whose intensities were roughly related to the blending ratio. The carboxyl group bands for the blend membranes with BC/Al at 20/80, 40/60, 60/40, and 80/20 ratios were shifted from 1598 to 1602, 1608, 1610, and 1616 cm^{-1}, respectively. The result, therefore, implied that there might be some specified interaction between the hydroxyl group of cellulose and carboxyl group of Al. Similar observations were previously discussed in the preparation of cotton cellulose/Al blend membranes.[9]

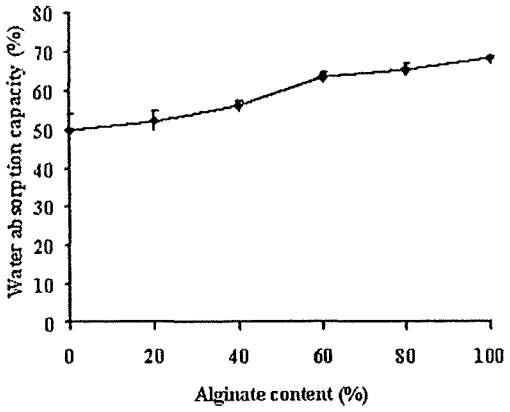

Figure 2 WAC (%) of the RBC/Al blend membranes as a function of the Al content.

WAC

WAC of the membranes as a function of the Al content is illustrated in Figure 2. WAC of the RBC membrane was 49.67%, whereas WAC of the Al membrane was 68.40%. The increase in the Al content in the membranes resulted in an increase in the water absorption ability of the blend membranes. The hydrogen bonds in regenerated cellulose formed very tightly packed crystallites, and these crystals could be so tight that water hardly penetrated them. Water molecules were found to be easily absorbed into the Al membrane crosslinked with glutaraldehyde because of its high hydrophilic property.[18] The blending of RBC with Al reduced hydrogen bonding of cellulose chains in the membranes, resulting in the increase in the water absorption ability of the membranes. Besides the composition of the membranes, the drying conditions also affected the water absorption of the membranes. In a previous report,[19] the water retention values of wet-state BC, freeze-dried BC, BC air-dried up to 100°C, and typical plant cellulose (cotton linters) were 1027, 629, 106, and 60%, respectively. In our works,[6,16] WAC of natural BC films after air drying at 30°C was 509%, whereas WAC of the fabricated RBC films decreased to 49.67%, which was about that of typical plant celluloses.

Mechanical properties of the membranes

Figure 3 presents the tensile strength of the blend membranes as a function of the Al content. The tensile strengths of the RBC membrane and the Al membrane with the thickness of 0.09 mm were 4.32 and 1.01 MPa, respectively. Increasing Al from 20 to 80% decreased the tensile strength of the blend membranes from 3.38 to 1.67 MPa. Figure 4 shows the elongation at the break of the blend membranes as a function of the Al content. The elongation at break of the RBC membrane was 35.20%, whereas the elongation at break of the Al membrane was 15.50%. In the same way, it was found that the elongation at break of the blend membranes decreased from 31.60 to 19.20% with the increase in the Al content from 20 to 80 wt %.

The effect of the Al content on the mechanical properties of the blend membranes was similar to that of cotton cellulose/Al blend membranes.[7,10] The sodium alginate membrane was mechanically weak, but its mechanical properties were obviously improved by the introduction of cellulose and cross-linking with Ca^{2+}. The interaction between cellulose and Al molecules based on their structural similarities was supposed to be hydrogen-bonding formation between the hydroxyl groups of cellulose and the carboxyl groups of Al. The presence of Al chains could enhance the molecular motion of cellulose in the blend and perturbed the strong hydrogen bond of pure cellulose because of the formation of new intermolecular interactions between the two biopolymers.[9] The morphological structure, tensile proper-

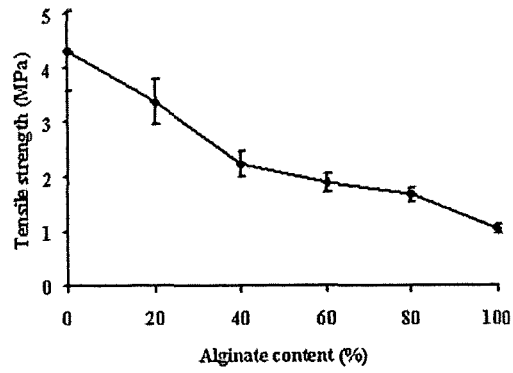

Figure 3 Tensile strength of the BC/Al blend membranes as a function of the Al content.

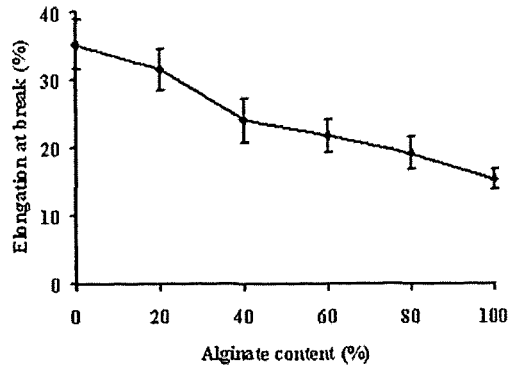

Figure 4 Elongation at break of the BC/Al blend membranes as a function of the Al content.

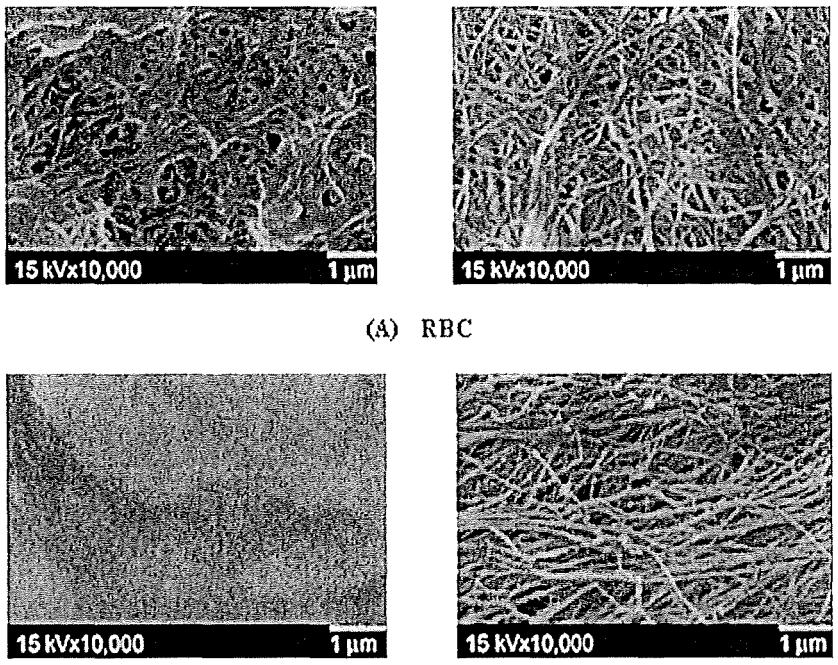

Figure 5 SEM images of the surface morphology of (A) RBC membranes and (B) 80/20 BC/Al blend membranes before (left) and after (right) reswelling in DI water and supercritical drying.

ties, and permeability of the cellulose membrane could also depend on the coagulation conditions.[20]

Surface morphology

The RBC and blend membranes exhibited dense nanoporous structures. The surface morphology of the membranes before and after reswelling in DI water at 30°C and supercritical drying is presented in Figure 5. In the case of the blend membrane composed of 80 wt % BC and 20 wt % Al, SEM displayed a homogeneous structure, which exhibited a certain level of miscibility of the blend. It was shown that the fibers of the blend membrane were more orderly nonwoven than the pure RBC membrane. Furthermore, the apparent pore size of the blend membrane decreased with the increase in the Al content. By the introduction of more than 20 wt % Al, the miscibility of the blend membrane was less orderly, and the structure became less homogeneous (figure not shown). Therefore, the optimum ratio of the BC/Al blend membrane was 80/20 wt %.

Water vapor permeability

The water vapor permeability of the dry films was determined by a water vapor permeation tester, with the test conditions following ISO 15106-1. However, with this analysis, there might be a lag time during which water solubilized in the membrane before permeation. Overall, WVTR of the RBC membrane was 0.25 g/cm^2 day, whereas that of the Al membrane was 0.56 g/cm^2 day (Fig. 6). WVTR of the blend membranes was significantly improved by the introduction of Al. WVTR of the blend membrane with 20% Al content was 0.51 g/cm^2 day or 2.1 times that of the unblended RBC membrane. The considerable improvement in WVTR of the blend membrane was

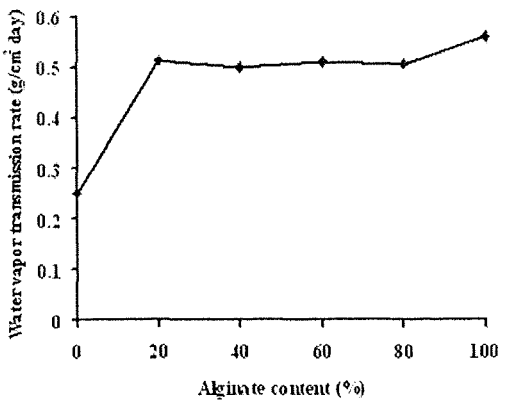

Figure 6 WVTR of the BC/Al blend membranes as a function of the Al content.

TABLE I
Characteristics of RBC and the Blend Membrane

Membrane	Tensile properties		WAC (%)	WVTR (g/cm^2 day)	Pore diameter (Å)	Surface area (m^2/g)
	Tensile strength (MPa)	Elongation at break (%)				
RBC	4.32	35.20	49.67	0.25	12.63	17.57
80/20 RBC/Al blend	3.38	31.60	52.25	0.51	10.60	19.50

due to the high hydrophilic property of Al, which formed the strong interaction between water molecules and —COO^- and —OH functional groups.[9] More water molecules binding to the surface of the blend membranes led to a greater driving force of the concentration gradient across the membrane, resulting in the enhancement of water transportation through the membrane.

Porosity

The average pore diameters of the RBC membrane and the blend membrane with 20% Al, characterized by a BET analyzer, were 12.63 and 10.60 Å with surface areas of 17.57 and 19.50 m^2/g, respectively. The pore sizes of the RBC membrane and the blend membrane were approximately 1/20 of that of the biosynthesis BC membrane (224.0 Å).[6] In comparison with the BC membrane with the total surface area of 12.62 m^2/g,[6] the surface areas of the RBC and blend membranes were increased by 39.2 and 54.5%, respectively. Table I gives a summary of the characteristics of the blend membrane versus the RBC membrane.

CONCLUSIONS

The nanoporous membranes were satisfactorily prepared with mixtures of BC/Al solutions as substrate materials. BC (3 wt %) was dissolved in a 4 wt % NaOH/3 wt % urea solution, and this was followed by a freeze–thaw process. The mixture of BC and sodium alginate solutions was spread over a Teflon plate and was then coagulated in a 5 wt % $CaCl_2$ solution and treated with a 1% HCl solution. The chemical structure, surface morphology, porosity, water vapor permeability, and mechanical properties were determined with FTIR, SEM, BET surface analysis, water vapor permeation testing, and tensile property testing. The blend membrane of 20 wt % Al and 80 wt % RBC demonstrated a homogeneous structure with a certain level of miscibility of the blend. The FTIR spectra displayed the specified interaction between the hydroxyl group of cellulose and the carboxyl group of Al. The mechanical properties of the blend membranes were slightly decreased compared to those of the pure RBC membrane; however, WVTR and the degree of swelling in water were significantly improved. The tensile strength and elongation at break of the blend membrane with a 0.09-mm thickness were 3.38 MPa and 31.6%, respectively. WAC and WVTR of the blend membrane were 52.25% and 0.51 g/cm^2 day, respectively. The membrane pore was classified as a micropore with an average diameter of 1.06 nm and a total surface area of 19.50 m^2/g. Because the developed membrane had a nanoporous structure and exhibited chemical stability, high mechanical strength, high water adsorption capacity, and high WVTR, its potential use in membrane separation processes is expected. The evaluation of the blend BC/Al film as a separation membrane in pervaporation processes is ongoing.

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γ – ALUMINA DOPED ALGINATE GEL FOR CELL IMMOBILIZATION IN FERMENTATION PROCESSES

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ABSTRACT

γ-Alumina (γ-Al_2O_3) doped alginate gel (AEC) was developed as a cell carrier in fermentation processes of *Saccharomyces cerevisiae* M.30 for ethanol production and *Clostridium butyricum* DSM 5431 for 1,3-propanediol production. In a single batch system of ethanol fermentation, the final ethanol concentration of suspended cell (SC), immobilized cell on γ-Al_2O_3 (AC) and AEC cultures were 82.4, 77.1 and 74.6 g/l, respectively. In 4-cycle repeated batch fermentation, the AEC culture demonstrated a good potential of reusability. Its ethanol production and conversion yield of the 1st, 2nd and 3rd repeated batch were comparable to those of the SC and AC cultures with the immobilization yield of 86%. AEC was also found to be effective for the cell immobilization of *C. butyricum* with the immobilization yield of 83%. However, the strong inhibition effect of cell-γ-Al_2O_3 immobilization towards 1,3-propanediol production was observed. Moreover, 1, 3-propanediol fermentation stability in the SC, AC and AEC systems tended to be lowered during the repeated batch fermentation. Interfering of positive charge of γ-Al_2O_3 on the cell membrane was thought to be the cause of the inactivity of *C. butyricum* DSM 5431 in 1,3-propanediol production.

Keywords: γ-Alumina, alginate, *Clostridium butyricum*, immobilization, *Saccharomyces cerevisiae*

INTRODUCTION

Due to the world energy crisis, the potential for renewable energy and its by-product development is experiencing an increase in attention. The production of ethanol from renewable carbohydrate materials has become interesting worldwide (Bai et al. 2008; Yu et al., 2007). Sugar cane molasses is an abundant and low cost sustainable raw material in Thailand. It is a by-product from sugar industries that can be fermented by yeast to produce ethanol under anaerobic condition (Bai et al., 2008; Nguyen, 2008). On the other hand, the demand of biodiesel has been increasing from time to time which leads to glycerol surplus in the world market since glycerol is a main by-product of biodiesel production. The production of biodiesel fuel produces glycerol about 10% by weight (Eggersdorfer et al., 1992; Meesters et al., 1996). Therefore, it is essential to develop a technology to convert glycerol into products of high value. Under anaerobic condition, *Clostridium butyricum* can ferment low grade glycerol and produce 1, 3 propanediol. 1, 3 Propanediol is a useful compound for

polymer industries, especially for producing biodegradable polymers such as polytrimethylene terephthalate (PTT).

Immobilized cell technology has been suggested as an effective mean for improved fermentation. The immobilization of cells leads to a high productivity, and good operational stability. The main advantages in the use of immobilized cells in comparison with suspended cells are the retention in the reactor of higher concentrations of cells, protection of cells against toxic substances and elimination of costly processes of cell recovery and cell recycle. However, the major problems of immobilization are mass transfer limitation, gel degradation and cell detachment (Yu et al., 2007; Verbelen et al., 2006; Kourkoutasa et al., 2004). For improving the performance of immobilized cell carrier, γ-Al_2O_3 and calcium alginate were applied as materials for constructing immobilized cell carriers. γ-Al_2O_3 has been reported as a good support for cells because of the electrostatic attraction between γ-Al_2O_3 and cells (Kanellaki et al., 1989; Koutinas et al., 1988). Calcium alginate is the most wildly used material for entrapment because of its simplicity and non-toxic (Verbelen et al., 2006; Arasaratnam, 1994).

In this study, adsorption and entrapment techniques were used together for improving the drawback of cell immobilization. γ – Alumina doped alginate gel (AEC) was developed as a new type of cell carrier. The immobilization system of *Saccharomyces cerevisiae* M.30 for ethanol production and *Clostridium butylicum* DSM 5431 for 1, 3–propanediol production from glycerol were used to evaluate the performance of the new carrier. These systems were examined by a single batch and 4-cycle repeated batch. The activities of the immobilized cells were then compared to the systems of free cells and immobilized cells by adsorption on γ-Al_2O_3.

MATERIALS AND METHODS

Microorganism

S.cerevisiae M30 strain was kindly provided by Prof. Dr Savitree Limtong, from Department of Microbiology, Kasetsart University, Bangkok. The culture was stored in Potato Dextrose Agar (PDA) slant at 4 °C. *C. butyricum* DSM 5431, obtained from American Type Culture Collection (ATCC) BAA-557^{TM}. The culture was stored in Reinforced Clostridial Medium (RCM) at 4 °C

Pre-culture and immobilization

S. cerevisiae M30 was grown in a 500 ml Erlenmeyer flask containing 100 ml pre-culture medium. The composition of the pre-culture medium per liter was: 50 g sugar from palm sugar; 0.5 g $(NH_4)_2SO_4$; 0.1 g KH_2PO_4; 0.035 g $MgSO_4.7H_2O$. The medium was adjusted to pH of 5, and sterilized at 121 °C for 15 min. Cell cultivation was carried in an Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 20 hours.The late exponential phase cells were harvested by decantation to obtain stock cell suspension.

C. butyricum DSM 5431 was grown in a 500 ml Erlenmeyer flask containing 100 ml pre-culture medium. The composition of the pre-culture medium per liter was: 3.4 g K_2HPO_4; 1.3 g KH_2PO_4; 2 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 2 g $CaCO_3$; 1 g yeast extract; 20 g glycerol; 1 ml trace element solution; 2 ml Fe solution. The Fe solution per liter consisted of: 5 g $FeSO_4.7H_2O$; 4 ml HCl (37%). The trace element solution per liter consisted of: 70 mg $ZnCl_2$; 0.1 g $MnCl_2.4H_2O$; 60 mg H_3BO_3; 0.2 g $CoCl_2.2H_2O$; 20 mg $CuCl_2.2H_2O$; 25 mg

$NiCl_2.6H_2O$; 35 mg $Na_2MoO_4.2H_2O$; 0.9 ml HCl (37%). Cell cultivation was carried in shaker at 100 rpm, 33°C for 20 hours.

Immobilization

Immobilization of cells was investigated using two general methods. The first was the adsorption of cells onto γ-Al_2O_3 powder. The second was entrapment of γ-Al_2O_3 -cells in calcium alginate matrix.

Sterilized γ-Al_2O_3 powder was immersed in pre-culture medium and incubated with cell culture for 20 hours to induce natural cells adhesion. γ-Al_2O_3 -cell mixture was added to 3% w/v sodium alginate solution to form an alginate- γ-Al_2O_3 -cell mixture with volumetric ratio of 1:1. The formation of AEC bead was initiated by adding the alginate- γ-Al_2O_3 -cell mixture drop wisely into 500ml 0.12M $CaCl_2$ using a syringe (1.2 mm diameter). AEC carriers with the diameter (∅) of 3 mm were left to harden in $CaCl_2$ solution for 30 minutes and then rinsed 3 times with 0.9% w/v NaCl. Immobilization yield (Y_I, %) was calculated as follows:

$$Y_I = \frac{X_I}{X_T} \times 100$$

X_I = immobilized cell concentration, g/L

X_T = total cell concentration, g/L

Batch fermentation

For ethanol fermentation, the composition of the fermentation medium per liter was: 220 g reducing sugar from molasses; 0.5 g $(NH_4)_2SO_4$ at pH of 5. Suspended / immobilized cells were cultured in a 500 ml Erlenmeyer flask containing

250 ml fermentation medium. Batch fermentation was performed in the incubator shaker at 150 rpm, 33°C.

For 1,3-propanediol fermentation, The composition of the fermentation medium per liter was: 1.0 g K_2HPO_4; 0.5 g KH_2PO_4; 4 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 1 g yeast extract; 80 g glycerol; 1 ml trace element solution; 1 ml Fe solution at pH of 7. Suspended / immobilized cells were cultured in a 1-L glass fermenter (Biostat Q®, B Braun Biotech International, Germany) containing 600 ml fermentation medium and the system was purged under nitrogen at a rate of 0.1 vvm in order to promote anaerobic condition. The agitation speed was controlled at 100 rpm and the pH was adjusted to 7.0 by automatic addition of 4 M NaOH. The incubation temperature was 33°C.

Analytical methods

Ethanol concentration was determined using gas chromatography (GC-7AG, Shimadzu, Japan) (Phisalaphong et al., 2005). Residual sugars were measured using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). 1, 3-Propanediol assay was measured by HPLC equipped with refractive index Detector (LC-3A, Shimadzu, Japan) using a column (Lichrocart C18) with length of 250 mm. outer diameters of 4 mm. Operating condition was: 20 mM H_3PO_4 as a mobile phase. flow rate 1.2 ml/min. column temperature at room temperature. Free and immobilized cell concentrations were measured as cell dry weight. Samples of fermentation broth were centrifuged at 2000 rpm for 15 min and the cells were re-suspended in water for free cell determination. A known mass of cell carriers was dissolved in 0.05 M sodium citrate. The suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. Biomass concentrations were measured as optical

density (UV-2450, Shimadzu, Japan) at 660 nm for *S. cerevisiae* M30 and 650 nm for *C. butyricum* DSM 5431 and converted to dry cell concentration on the basis of a corresponding standard curve.

RESULTS AND DISCUSSION

Ethanol fermentation

Ethanol fermentation was carried out with 220 g/l of initial sugar concentration from cane molasses as a carbon source for *S. cerevisiae* M30. The fermentations were performed using suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and γ-Al_2O_3 doped alginate gel (AEC) culture in a single batch for 60 hours and a 4-cycle repeated batch with the duration of each batch of 48 hours.

The time-course of the single batch of ethanol fermentation is shown in Figure 1. The final total cell concentrations of AC culture at 4.3 g/l and AEC culture at 4.2 g/l were obtained (data not shown), which were slightly higher than that of SC culture (3.9 g/l). The immobilization yields of the AC and AEC cultures were 90 % and 86% respectively. It was demonstrated that γ-Al_2O_3 has a positive effect on the growth of *S. cerevisiae* M30. This is similar to the finding of Kanellaki et al. (1989) who reported that γ-Al_2O_3 was a good supporter of ethanol fermentation because of the electrostatic attraction between alumina particles and yeast cells. *S. cerevisiae* can be adsorbed on γ-Al_2O_3 in a wide pH range from 3.0 to 6.5 owing to the opposite electric charges (Kana et al., 1989). As shown in Figure 1. sugar concentration gradually decreased while ethanol concentration increased for duration of 60 hours. The final ethanol concentration of the SC, AC and AEC systems were 82.4, 77.1 and 74.6 g/l,

respectively ($Y_{P/S}$ of 43%, 41% and 42%, respectively). During fermentation time from 12 to 36 hours, the ethanol concentration of the AEC system was lower than that of the AC system. This could be indicated that the mass transfer resistance of the AEC carrier did affect cell growth and product formation. However, the ethanol concentration of the AEC culture increased until it reached similar level of the AC culture at the end of the fermentation.

For the repeated batch mode, the results of the fermentation are summarized in Table 1. In the first batch, after 48 hours, ethanol concentration of the SC system was 77.1 g/l, whereas the final ethanol concentrations of immobilized cells (IC) in AC and AEC systems were 67.3 g/l and 69.7 g/l, respectively. In the second - the forth batch, all system exhibited ethanol production without any occurrence of the lag phase. In the fourth batch, the majority of sugar was consumed with the final ethanol concentrations of 70.9, 71.8 and 70.7 g/l for the SC, AC and AEC cultures, respectively. The final total cell concentrations of the AC culture (5.8 g/l, Y_I 90%) and the AEC culture (5.7 g/l, Y_I 86%) were higher than that of the SC culture (4.5 g/l). Increase in cell concentrations in the AC and AEC carriers was due to the growth of immobilized cells in the carriers during the fermentation. Instability of the SC culture in the repeated batch fermentation was observed from the comparison of its final ethanol concentration and $Y_{P/S}$ from batch to batch. The ethanol concentration of the SC system dropped from 77.1 g/l in the first batch to 72.9 and 70.9 g/l in the second and the fourth batch, respectively. This may be attributed to the negative effect of high ethanol concentration on cell activity and viability. The inhibition of ethanol and sugar especially at high concentrations on suspended cell activities and stability has been previously reported (Phisalaphong et al., 2007). The ethanol production of immobilized cells in the AC and AEC cultures were relatively stable;

the ethanol yield factors ($Y_{P/S}$) were quite stable in all batches. The stability of the immobilized cell cultures was higher than that of the SC culture since the matrix of the cell carriers could protect yeast by fortification from toxins and inhibitor (Verbelen et al., 2006; Kourkoutasa et al., 2004; Phisalaphong et al., 2007).

1, 3-propanediol fermentation

The fermentations of *C. butyricum* DSM 5431 were carried out in the 1 L glass fermenter with 80 g/l initial glycerol concentration. The fermentations in this study were also performed using SC, AC and AEC cultures. These three cultures were examined by a single batch for 33 h and a 4-cycle repeated batch with the duration of each batch of 24 hours.

The time-course of the single batch of 1, 3-propanediol is shown in Figure 2. At the end of the fermentation, the total cell concentration of the AC culture (4.4 g/l) and AEC culture (3.6 g/l) (data not shown) were higher than that of the SC culture (3.1 g/l) and the immobilization yield of the AC and AEC cultures were 80 % and 83 % respectively. It was demonstrated that the cell carriers might promote the growth of *C. butyricum* DSM 5431. This result agreed with the previous ethanol fermentation study. However, the final 1, 3-propanediol concentrations of the SC, AC and AEC systems were 41.4, 20.6 and 15.5 g/l, respectively. The 1, 3-propanediol yields ($Y_{P/S}$) of AC culture (26 %) and AEC culture (19 %) were significantly lower than that of the SC culture (52 %). It was found that there was an occurrence of the inhibition effect on 1, 3-propanediol production by γ-Al_2O_3 based carrier. High positive charge density on the surface of the carrier (γ-Al_2O_3) was considered to be the possible negative impact on the cell activities. The adverse effects on cell membrane could affect its enzyme activities.

In 4-cycle repeated batch fermentation, the experimental results are shown in Table 2. The 1, 3-propanediol concentrations of all systems decreased from the first to the fourth batch. Instability of the SC culture was observed from the comparison of $Y_{P/S}$ in the first to the fourth batch (46 % to 33%). This can be attributed to the effect of the product and by-products from this fermentation such as butyric acid and acetic acid, which could be directly toxic to the cell. The stability of the AC culture was slightly higher than that of the SC culture; however 1, 3-propanediol production of the AC culture was much lower than that of the SC culture. The stability of the AEC culture was significantly decreased due to the negative effect of γ-Al_2O_3 on the cell activity together with the reduced diffusivity of product and by products due to the alginate entrapment.

CONCLUSIONS

This study indicated that the ethanol fermentation by immobilized *S. cerevisiae* M30 in the AC and AEC carriers was promising. The high ethanol production from the immobilized cultures was achieved owing to the high density of cells and high stability of the cell activities for long term operation. In 4-cycles of repeated batch ethanol fermentation, the AC and AEC cultures exhibited a good potential of reusability. However, the AC and AEC carriers were found unfavorable for *C. butyricum* DSM 5431 immobilization. High positive charge density on the surface of the carrier (γ-Al_2O_3) was considered to be the possible negative impact on *C. butyricum* activities. It was found that with the use of the immobilized carriers incorporated with γ-Al_2O_3, the production of 1, 3-propanediol was much lower than that of the SC system. The 4-cycle repeated batch of 1, 3-propanediol fermentation

revealed that the instability of *C. butyricum* DSM 5431 was very high in all three systems.

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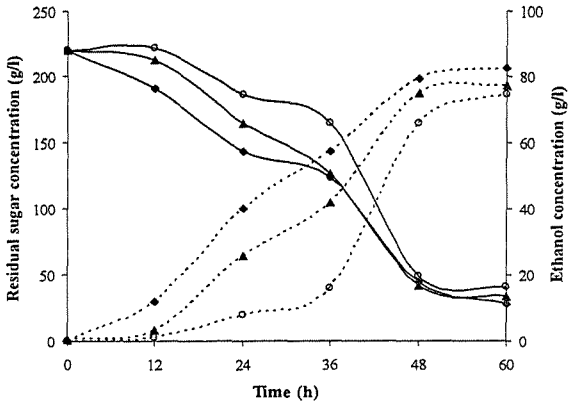


Figure 1 Time-course of single batch fermentation by *S. cerevisiae* M30 at 220 g/l of initial sugar concentration; residual sugar concentration (solid lines) and ethanol concentration (dash lines); ◆, SC; ▲, AC and ○, AEC.

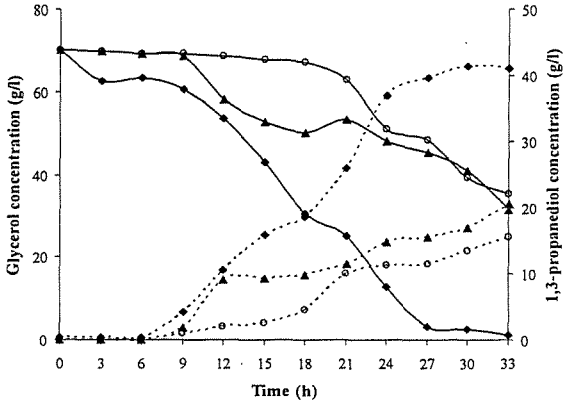


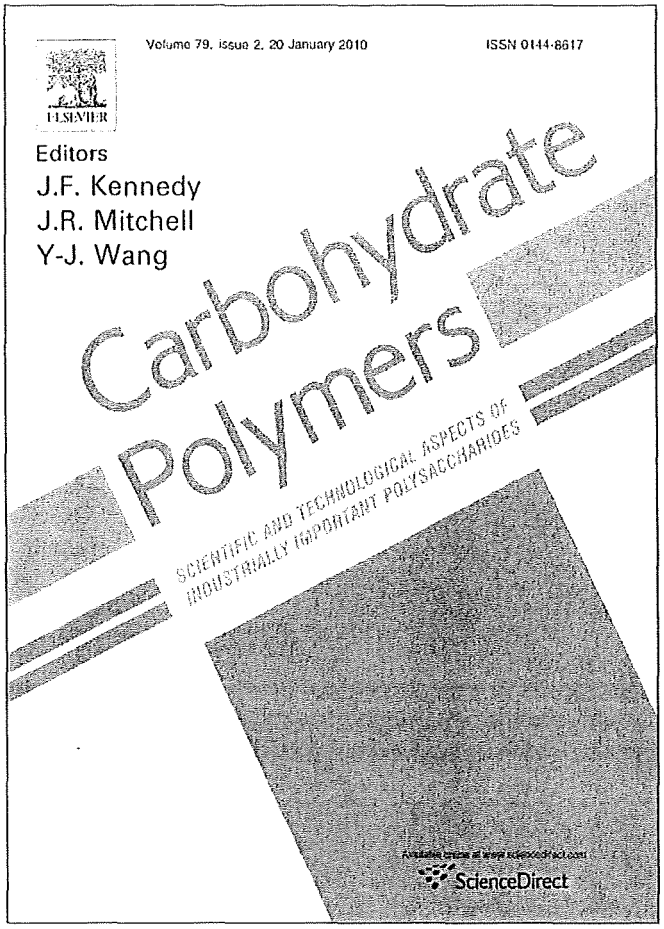
Figure 2 Time-course of single batch fermentation by *C.butyricum* DSM 5431 at 80 g/l of initial glycerol concentration; residual glycerol concentration (solid lines) and 1, 3-propanediol concentration (dash lines); ◆, SC; ▲, AC and ○, AEC

Table 1 Yields and end products of the repeated batch ethanol fermentations (each batch 48 hours), using the cultures of suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and γ-Al_2O_3 doped alginate gel (AEC) culture.

Batch	P	X (g/l)		Y_I	Y_S	$Y_{P/S}$
	(g/l)	X_E	X_I	(g/g)	(g/g)	(g/g)
I						
SC	77.1	-	-	-	0.79	0.43
AC	67.3	-	-	-	0.78	0.39
AEC	69.7	-	-	-	0.78	0.40
II						
SC	72.9	-	-	-	0.80	0.40
AC	70.3	-	-	-	0.78	0.40
AEC	67.2	-	-	-	0.76	0.39
III						
SC	72.8	-	-	-	0.79	0.40
AC	69.5	-	-	-	0.77	0.40
AEC	71.8	-	-	-	0.79	0.40
IV						
SC	70.9	4.5	-	-	0.78	0.39
AC	71.8	0.6	5.3	0.90	0.78	0.41
AEC	70.7	0.8	5.0	0.86	0.78	0.40

Table 2 Yields and end products of the repeated batch 1,3-propanediol fermentation (each batch 24 hours), using the cultures of suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and γ-Al_2O_3 doped alginate gel (AEC) culture.

Batch	P	X (g/l)		Y_I	$Y_{P/S}$
	(g/l)	X_E	X_I	(g/g)	(g/g)
I					
SC	36.9	-	-	-	0.46
AC	14.8	-	-	-	0.19
AEC	11.4	-	-	-	0.14
II					
SC	32.7	-	-	-	0.41
AC	13.9	-	-	-	0.17
AEC	6.0	-	-	-	0.08
III					
SC	33.5	-	-	-	0.42
AC	11.6	-	-	-	0.14
AEC	4.5	-	-	-	0.06
IV					
SC	26.4	4.4	-	-	0.33
AC	12.5	1.1	4.6	0.80	0.16
AEC	3.3	0.8	3.9	0.83	0.04



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Novo aloe vera–bacterial cellulose composite film from biosynthesis

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ABSTRACT

A novo bio-polymer composite film of cellulose and aloe vera gel was developed by means of adding aloe vera gel in the culture medium during biosynthesis using *Acetobacter xylinum* in static cultivation. The interaction between bacterial cellulose (BC) fibrils and aloe vera gel was illustrated via FTIR analysis. With the 30% v/v aloe gel supplement in the culture medium, a fibre-reinforced bio-polymer film displayed significantly improved properties in mechanical strength, crystallinity, water absorption capacity and water vapor permeability in comparison to those of the unmodified BC film. The average pore size of the modified film either in the dry or re-swollen form was approximately reduced to 1/5 of those of the unmodified BC films with a narrow pore size distribution.

1. Introduction

Cellulose, a linear polysaccharide, is the most abundant organic material with a variety of useful applications. It is found as a structural component, often bound to other polymers (pectin, lignin, hemicellulose, etc.) in the cell wall of plants, algae and also some lower animals. Only a few bacterial species, taxonomically closely related to the genus *Acetobacter xylinum*, produce and extracellularly secrete cellulose in form of fibre. Bacterial cellulose (BC) produced by *A. xylinum*, has unique properties including high water holding capacity, high crystallinity, hydrophilicity, high tensile strength and a highly pure and ultra fine fibre network structure (Iguchi, Yamanaka, & Budhiono, 2000; Wan et al., 2007). Recently, BC has been studied for its use as artificial skin and blood vessels (Klemm, Schumann, Udhardt, & Marsch, 2001) scaffold for tissue engineering of cartilage (Svensson et al., 2005), and wound-dressing (Czaja, Krystynowicz, & Bielecki, 2006). Still, an innovative wound-dressing has been continuously developed in a wide range of good candidate materials such as alginate, polyurethane, chitosan, and aloe vera.

Aloe vera is one of the oldest healing plants known to mankind. Aloe vera gel is the mucilaginous gel obtained from the squeezing of the clear jelly-like substance of the parenchyma tissue. Aloe vera gel has been reported to have multiple beneficial properties for wound healing, including the abilities to penetrate and anesthetize tissue, preclude bacterial, fungal, and viral growth, act as an anti-inflammatory agent and enhance blood flow (Davis, DiDonato, Hartman, & Haas, 1994; Grindlay & Reynolds, 1986; Heggers et al., 1996; Reynolds & Dweck, 1999; Yao et al., 2009). Aloe vera gel was applied to inhibit fibroplasia in wound healing, to promote both tissue growth and differentiation in tissue culture and for the treatment of burn wounds (Reynolds & Dweck, 1999). Acemannan, an ordered linear polymer of substantially acetylated mannose monomers isolated from the aloe vera gel is considered by many to be one of the major active ingredients (Femenia, García-Pascual, Simal, & Rosselló, 2003; Femenia, Sánchez, Simal, & Rosselló, 1999; Ni, Turner, Yates, & Tizard, 2004; Reynolds & Dweck, 1999; Yu, Jin, Xin, & Min, 2009). The activities of acemannan as an antiviral agent, an immunomodulator, an agent in reducing opportunistic infections and stimulating the healing processes were reported (Reynolds & Dweck, 1999; Tai-Nin Chow, Williamson, Yates, & Goux, 2005).

Based on the advantageous properties of BC and aloe vera gel, in this study, a novo nanostructure film composed of BC and aloe vera was developed by the supplementation of aloe vera gel during biosynthesis of the bacteria cellulose film. The surface morphology, pore structure, tensile strength, water absorption capacity (WAC), crystallinity, and water vapor permeability of the modified films were then examined and compared with those of the typical BC film. To the best of our knowledge, this type of blend combination was first prepared in this work.

2. Materials and methods

2.1. Microbial strains

The *A. xylinum* AGR 60 kindly supplied by the laboratory of Pramote Tammarate (The Institute of Food Research and Product Development, Kasetsart University, Bangkok) was used in this study.

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2.2. Culture media and method

The culture medium was coconut-water supplemented with 5.0% sucrose, 0.5% ammonium sulfate and 1.0% acetic acid. Preculture was prepared by a transfer of 50 mL stock culture to 1000 mL medium in a 1500 mL bottle and incubated statically at 30 °C for 7 days. After that the surface pellicle was removed and the preculture broth of 5% (v/v) was added to the main culture medium with the various aloe vera contents from 0% to 50% v/v. The 75 mL of the activated main culture medium was inoculated in a Preti-dish and kept at 30 °C for 7–10 days. After that, all sample films were purified by washing with deionized (DI) water and then were treated with 1% (w/v) sodium hydroxide solution at room temperature (30 °C) for 24 h to remove bacterial cells followed by a rinse with DI water until pH became neutral. Afterward, the films were air-dried at room temperature and stored in plastic film.

2.3. Characterization of membranes

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was used primarily to identify the chemical structure of the membrane. The FTIR spectra of the membranes were measured at wave numbers ranging from 2800 to 1200 cm^{-1} with a Nicolet (United States) SX-170 FTIR spectrometer.

2.3.2. Water absorption capacity (WAC)

To determine the WAC, the dried membranes were immersed in DI water at room temperature until equilibration. After that the membranes were removed from the water and excess water at the surface of the membranes was blotted out with Kimwipes paper. The weights of the swollen membranes were measured, and the procedure was repeated until no further weight change was observed. The water content was calculated with the following formula:

$$\mathrm{WAC}(\%) = \frac{W_h - W_d}{W_d} \times 100$$

where W_h and W_d denote the weight of hydrate and dry membrane, respectively.

2.3.3. Mechanical property

All the membranes under the study in dry and re-swollen forms were tested for tensile strength, Young's modulus and elongation at break. The film samples were cut into strip-shaped specimens 10 mm width and 10 cm long. The maximum tensile strength and break strain of the film samples were determined with a Lloyd (Southampton, UK) 2000R universal testing machine. The test conditions followed ASTM D 882. The tensile strength and break strain were the average values determined from 10 specimens.

2.3.4. Scanning electron microscopy (SEM)

The films were frozen in liquid nitrogen, immediately snapped, vacuum-dried and then sputtered with gold and photographed. Images were taken on a JOEL (Tokyo, Japan) JSM-5410LV scanning electron microscope.

2.3.5. Water vapor permeability measurement

The water vapor transmission rate (WVTR) of the dry film with area of 50 cm^2 and 0.03 mm of thickness was determined with a Lyssy (Switzerland) L80-4000 water vapor permeation tester. The test conditions followed ISO 15106-1. The determination of WVTR was done under 38 °C and 90% relative humidity. The principle of this electronic tester was similar to that of conventional method. One side of the film was exposed to water vapor. As water solubilized into the film and permeated through the sample material, on the other side of the film, nitrogen gas swept and transported the transmitted water vapor molecules to a calibrated infrared sensor. The response was reported as a transmission rate.

2.3.6. Brunauer–Emmett–Teller (BET) surface analysis

The pore size and surface area of the membranes were determined with a BET surface area analyzer. To remove moisture from the film samples, the samples were placed in sample cells, which were then heated up to 348 K for 3 h and cooled down to room temperature before the BET analysis. The BET pore size and surface area were determined with N2 adsorption at 77 K in a Micromeritics (Atlanta, GA) ASAP 2020.

2.3.7. Wide-angle X-ray diffractometry

X-ray diffraction was measured with an X-ray diffractometer (Model D8 Discover, Bruker AXS, Karlsruhe, Germany). X-ray diffraction patterns were recorded with CuKα, radiation (λ = 1.54 Å). The operating voltage and current were 40 kV and 30 mA, respectively. Samples were scanned from 10–40° 2θ at a scan speed of 3°/min. The crystallinity index (CI) was calculated from the reflected intensity data with Segal et al.'s method (Phisalaphong & Jatupaiboon, 2008).

3. Results and discussion

A bio-polymer composite of cellulose and aloe vera gel was produced in the form of pellicles that floated on the medium surface during the cultivation of *A. xylinum* using a culture medium containing aloe vera gel. A decline in the pellicle formation rate relating to the ratio of aloe vera gel in the medium culture was observed (data not shown). Since the supplement of aloe vera gel increased the viscosity of the culture medium, it resulted in a decrease in the oxygen transfer rate. *A. xylinum* is an aerobic bacterium; therefore, the reduction of oxygen in the medium could become the limiting factor for cell growth and cell activities.

After the air-drying processes, the film in dry state was obtained namely, bacterial cellulose–aloe vera (BCA) film. It was found that the BCA film formation using the culture medium with 30% (v/v) aloe vera gel addition gave a uniform structure with a slightly lower BC pellicle synthesis rate. However, the addition of more than 30% aloe vera gel caused a considerable decrease of the pellicle formation. The effects of aloe vera gel supplement on the film characteristics such as surface morphology, chemical structure, mechanical strength, water absorption capacity, crystallinity, porous structure and water vapor transmission were then investigated.

3.1. Surface morphology

SEM images in Figs. 1–3 illustrate the differences in the surface morphology of the developed films supplemented with 0%, 30% and 50% (v/v) of aloe vera gel in the culture mediums, respectively. Compared to the dried films, the re-swollen films exhibited a looser fibril network structure according to the high water absorption of the films. In Fig. 2, the film with the supplement of 30% aloe vera gel displayed good incorporation of aloe vera gel into the BC fibril network. However, in the case of the 50% aloe vera gel supplement (Fig. 3), the structure of the film became less uniform with noticeable excessive gel on the film surfaces.

3.2. FTIR analysis

Fourier transform infrared (FTIR) spectroscopy of BC and BCA films was carried out in order to detect the occurrence of new peaks or any peak shift that could be attributed to interactions between cellulose and aloe vera gel. The FTIR spectra of all samples were detected at wavenumbers ranging from 2800 to 1200 cm^{-1}

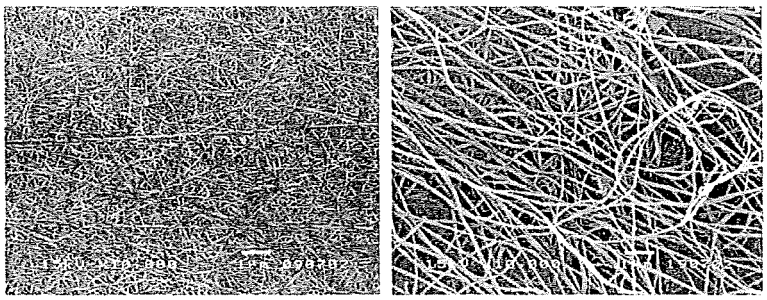

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Fig. 1. SEM images of surface morphology of BC film in dry form (left) and re-swollen form (right).

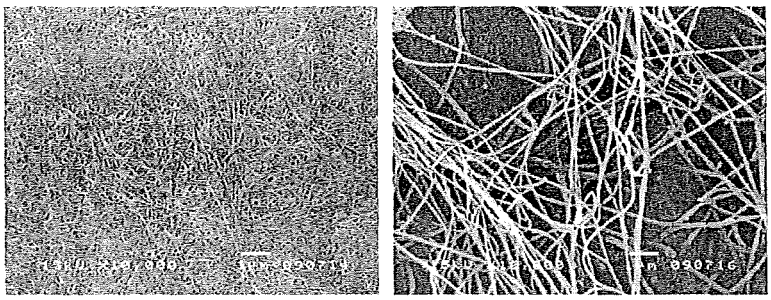

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Fig. 2. SEM images of surface morphology of BCA-30% aloe vera film in dry form (left) and re-swollen form (right).

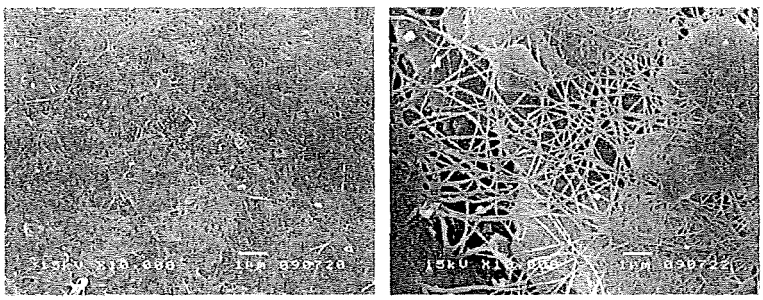

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Fig. 3. SEM images of surface morphology of BCA-50% aloe vera film in dry form (left) and re-swollen form (right).

as shown in Fig. 4. For close observation, the expansion of the FTIR spectra of the films at wave number ranging from 1800 to 1500 cm^{-1} was displayed in Fig. 5. In this region, the intense absorption in the spectrum of the cellulose was the band at 1642.9 cm^{-1}, which has been assigned to carbonyl groups (Klemm et al., 2001), while the characteristic absorption of the aloe vera was the band at 1594.1 cm^{-1}, which was assigned to amino groups as shown in Fig. 4(a) and (h), respectively. The bands at 1650–1578 cm^{-1} were assigned to C=O stretching, which overlaps with NH bending. The absorption band at 1565–1540 cm^{-1} was NH deformation. In Fig. 5(b–h), the films developed by the supplement of aloe vera gel of 5%, 10%, 20%, 30%, 40%, and 50% (v/v) in the culture medium present a peak shift and an occurrence of two new peaks from 1562.6 cm^{-1} to 1562.0, 1562.7, 1563.2, 1573.9/1541.7, 1574.3/1541.9, and 1574.6/1541.8 cm^{-1}, respectively. The presence of the two new peaks in FTIR spectra in the region of 1574 (higher frequency) and 1542 cm^{-1} (lower frequency) when the films were developed by the supplement of 30–50% (v/v) aloe vera gel implied the incident of the intermolecular interaction of BC and the amino groups of aloe vera gel. As referred to the SEM analysis, it supported that aloe vera gel could attach and intermolecular bond to the cellulose fibril.

3.3. Mechanical property

Fig. 6 shows the tensile strength of BC and BCA films as a function of aloe vera gel content observed at the average film thickness of 0.030 mm. By supplementation of the gel from 0% to 30% (v/v), it was found that the gel merged well into the fibril network resulting in improvement of the tensile strength of the films from 5.32 to 8.67 MPa in proportion to the gel content. However, with the excessive gel supplements in the range of 40–50% v/v, the pellicle formation rate was significantly decreased and the miscellaneous structure of the films was observed as previously shown in the SEM images. With the gel supplement of 50% (v/v), the tensile strength of the film was drastically reduced to 3.42 MPa.

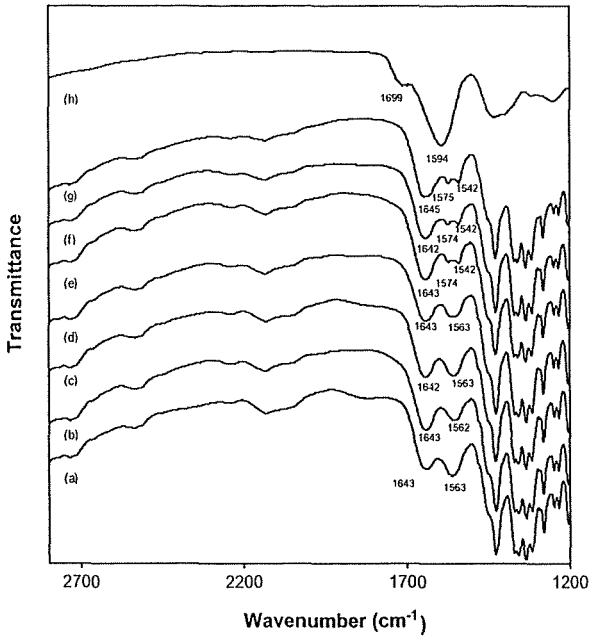

Fig. 4. The FTIR spectra of (a) BC and (b–g) BCA films and (h) aloe vera gel, in wave numbers ranging from 2800 to 1200 cm^{-1}. The supplement of aloe vera gel (% v/v): (b) 5; (c) 10; (d) 20; (e) 30; (f) 40 and (g) 50.

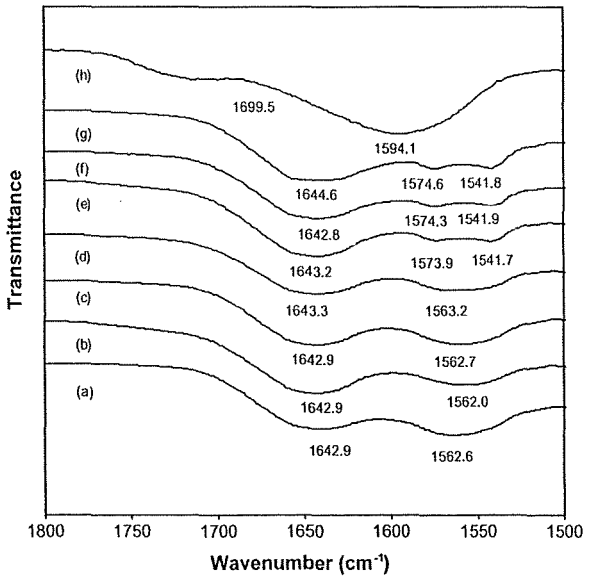

Fig. 5. The FTIR spectra of (a) BC and (b–g) BCA films and (h) aloe vera gel in wave numbers ranging from 1800 to 1500 cm^{-1}. The supplement of aloe vera gel (% v/v): (b) 5; (c) 10; (d) 20; (e) 30; (f) 40 and (g) 50.

The effect of the aloe vera gel supplement on Young's modulus (Fig. 7) and elongation at break (Fig. 8) were similar to that on the tensile strength. Supplementation of aloe vera gel from 0% to 30% (v/v) increased the Young's modulus of the film from 161.80 to 190.20 MPa. However, the Young's modulus of the film was reduced with the gel supplement of 40–50% (v/v). The elongation at break also increased with the gel supplement from 0% to 30% (v/v) and declined with the surplus supplement to 40–50% (v/v).

The supplement of aloe vera gel at 30% (v/v) in the culture medium yielded about 1.6 times of tensile strength, 1.2 times of

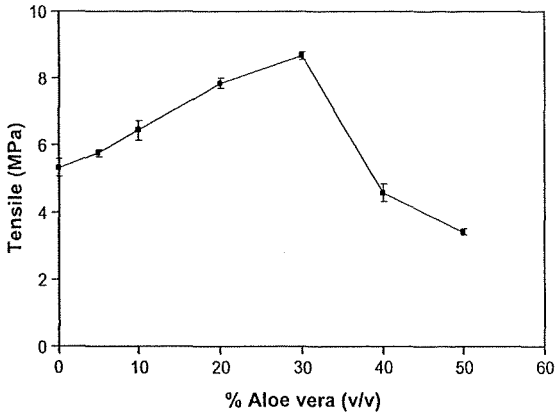

Fig. 6. Tensile strength of the BCA films as a function of aloe vera content (% v/v) in culture medium.

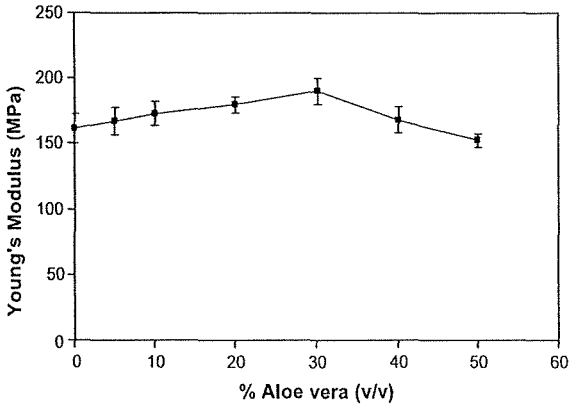

Fig. 7. Young's modulus of the BCA films as a function of aloe vera content (% v/v) in culture medium.

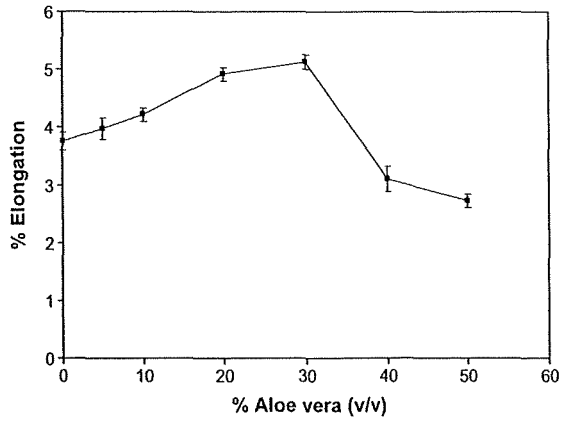

Fig. 8. The elongation at break of the BCA films as a function of aloe vera content (% v/v) in culture medium.

Young's modulus and 1.4 times of elongation at break higher than those of the film synthesized in the normal culture medium. By addition of 30% (v/v) aloe vera gel in the culture medium, the developed BCA film was reinforced to withstand stronger pull force than the typical BC film as a result of the formation of the aloe vera gel–cellulose composite film. The combination of crystalline cellulose and amorphous gel at a suitable ratio could form a material

with the advantageous properties of strength and stiffness. In addition, the improved mechanical strength of the BCA film could be as a result of the denser structure with smaller pore diameter and narrow pore size distribution of the film.

3.4. Water absorption capacity (WAC)

The effect of the aloe vera gel supplement on the water absorption capacity (WAC) of the films was analogous to that of the mechanical strength (Fig. 9). The aloe vera gel supplementation at 30% v/v improved the WAC of the film to 735% or about 1.5 times of that of the unmodified BC film. Degree of water swelling and WAC of the blend films could be increased due to the introduction of hydrophilic component. On the previous study (Phisalaphong, Suwanmajo, & Tammarate, 2008), the blending of bacteria cellulose with alginate gel resulted in the increase in the water absorption ability of the film. Aloe vera gel is very hydrophilic and could conjugate well into the cellulose network structure; therefore, the WAC of the BCA film was enhanced. On the other hand, the WAC of the film dropped when the aloe vera gel supplement was greater than 30% (v/v). At the excessive gel addition, the developed film structure became miscellaneous and weak; consequently, the water holding capacity of the film decreased.

3.5. XRD (X-ray diffraction)

The X-ray diffraction (XRD) patterns of BC and BCA films are shown in Fig. 10. The XRD pattern of the BC film developed from the normal culture medium demonstrated the peaks observed at 14.56°, 16.87° and 22.74° in the comparable intensities as previously reported for the typical BC cultured in static circumstances (Hong et al., 2006; Phisalaphong & Jatupaiboon, 2008; Phisalaphong et al., 2008). Because BC is not a completely crystalline material, the diffraction patterns from the films show broad peaks. The percentages of aloe vera gel supplement in the range of 0–50% v/v in the culture medium showed no differences in the reflective-angle and *d*-spacing from those of the typical BC film (Table 1). However, with the aloe gel supplement from 0% to 30% (v/v), the crystalline indices (CI) of the films gradually increased from 78.45 to 82.77, respectively. The higher peak intensity could be as a result of the relatively denser structure of the BCA films. In general, the controlling parameters such as the composition of culture media, pH, temperature and oxygen tension could have an effect on bacterial growth and the cellulose biosynthesis. The higher CI of the film could be because of a relatively higher degree of order form of the polymer chains owning to the different culture medium composition. Similar to the mechanical properties, the CI dropped considerably to 69.75 and 68.52 with the aloe vera gel supplement at 40% and 50% (v/v), respectively. The result implied that with the addition of aloe vera gel in the range of 0–30% (v/v), the developed film of BCA was more orderly arranged in uniform and firmly fibre network than those in the range of 40–50% (v/v).

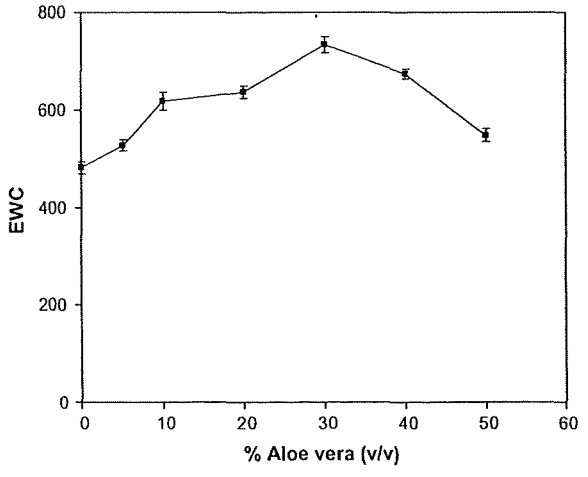

Fig. 9. The equilibrium water content (EWC) of the BCA films as a function of aloe vera content (% v/v) in culture medium.

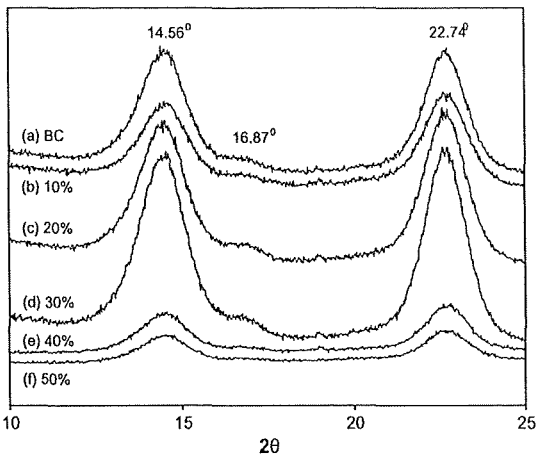

Fig. 10. X-ray patterns of the BC and BCA films: (a) BC; (b) BCA-10% aloe vera; (c) BCA-20% aloe vera; (d) BCA-30% aloe vera; (e) BCA-40% aloe vera; and (f) BCA-50% aloe vera.

Table 1
CI, reflective-angle, *d*-spacing values of the BC and BCA films.

Film sample	CI	2θ [d(1 $\bar{1}$ 0)]	2θ [d(1 1 0)]	2θ [d(0 2 0)]
BC	78.45	14.56° (6.08)	16.87° (5.25)	22.74° (3.91)
BCA-10% aloe vera	79.16	14.56° (6.08)	16.87° (5.25)	22.74° (3.91)
BCA-20% aloe vera	80.25	14.56° (6.08)	16.87° (5.25)	22.74° (3.91)
BCA-30% aloe vera	82.77	14.56° (6.08)	16.87° (5.25)	22.74° (3.91)
BCA-40% aloe vera	69.75	14.56° (6.08)	16.87° (5.25)	22.74° (3.91)
BCA-50% aloe vera	68.52	14.56° (6.08)	16.87° (5.25)	22.74° (3.91)

3.6. Porosity

The total surface area and average pore size determined by BET of the BC film were 12.6 m^2/g and 224 Å in dry form and 55.2 m^2/g and 612 Å in re-swollen form, respectively. As shown in Table 2, Figs. 11 and 12, the BCA films had average pore sizes smaller than that of the BC, while the surface area slightly increased. The pore sizes slightly decreased with the increase of aloe vera gel content. Overall, the average pore sizes of the modified films either in dry or re-swollen form were approximately reduced to approximately 1/5

Table 2
Pore diameter and surface area of the BC and BCA films analyzed by BET analyzer.

Film sample	Average pore diameter (Å)	Surface area (m^2/g)
BC (dry form)	224	12.6
BCA-10% aloe vera (dry form)	53	14.2
BCA-30% aloe vera (dry form)	41	15.7
BCA-50% aloe vera (dry form)	38	19.5
BC (re-swollen form)	612	55.2
BCA-10% aloe vera (re-swollen form)	154	59.1
BCA-30% aloe vera (re-swollen form)	150	62.4
BCA-50% aloe vera (re-swollen form)	138	65.2

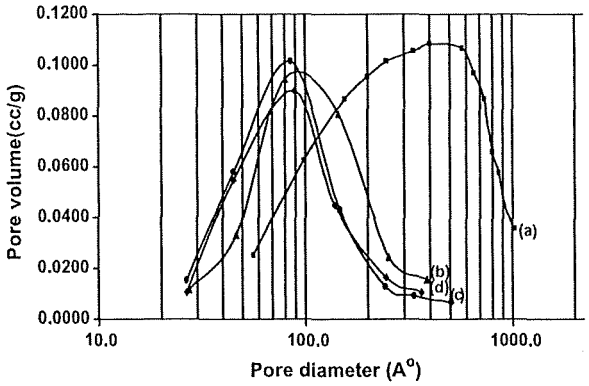

Fig. 11. The pore size distribution of the BC and BCA films in re-swollen form: (a) BC; (b) BCA-10% aloe vera; (c) BCA-30% aloe vera; (d) BCA-50% aloe vera.

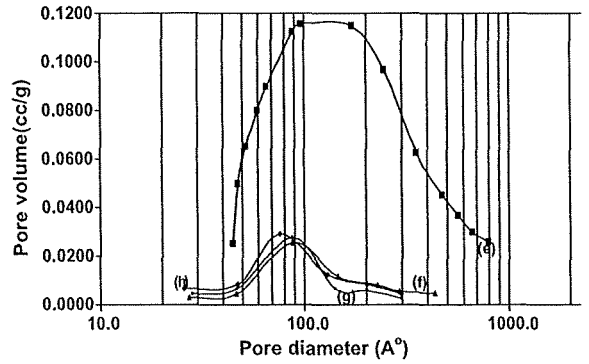

Fig. 12. The pore size distribution of the BC and BCA films in dry form: (e) BC; (f) BC-10% aloe vera; (g) BC-30% aloe vera; (h) BC-50% aloe vera.

of those of the unmodified BC films. The results indicated the occurrence of gel coating and filling pores of the films. The BET results were in accord with the observation from the SEM micrographs.

3.7. Water vapor transmission test

Corresponding to the result regarding water absorption capacity (WAC), the WVTR increased with the addition of aloe vera gel in the range of 0–30% (v/v), and then it decreased with the addition of aloe vera gel at 50% (v/v). The water vapor transmission rate (WVTR) of the BCA film at the aloe vera gel supplement of 0%, 10%, 30%, and 50% (v/v) were at 1616.5, 1821, 2029.5, and 1066 g/m^2 day, respectively. The improved water vapor permeability could be according to the increase of swelling degree and equilibrium water content of the films. A similar result was previously observed in the blended cellulose–alginate membrane (Phisalaphong et al., 2008).

4. Conclusions

The modification of BC film by means of adding aloe vera gel in the culture medium during biosynthesis using *A. xylinum* provided many advantageous properties. The FTIR spectra of the modified films revealed the intermolecular interaction of BC and the amino groups of aloe vera gel. The significant improvement in terms of mechanical properties, water absorption capacity (WAC), water vapor transmission rate (WVTR) and crystalline index were apparently obtained by the addition of up to 30% (v/v) of aloe vera gel in the culture medium. However, the introduction of more than 30% (v/v) aloe vera gel in the culture medium inhibited the film formation and the developed films exhibited miscellaneous structures with inferior film properties. On the nature of biocompatibility of BC and aloe vera gel and on the excellent physical properties of the BCA film, a wide range of applications of the BCA film in medical areas is expected. Investigation of the BCA as materials for tissue engineering is on going.

Acknowledgments

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Biocompatibility and Growth of Human Keratinocytes and Fibroblasts on Biosynthesized Cellulose–Chitosan Film

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Abstract

Bacterial cellulose (BC)–chitosan (BCC) films made *via* bio-co-polymerization by *Acetobacter xylinum* were developed and characterized for physical and biological properties. With the incorporation of chitosan MW 3×10^4 and 8×10^4 into bacterial cellulose, the modified films (BCC-MW 30 000 and BCC-MW 80 000, respectively) became denser, with a smaller average pore size of 13.1–15.3 nm in dry form. The BCC films have no toxicity against L929 mouse fibroblast cells. Tissue compatibility was then evaluated by growth and spreading of human skin keratinocytes and fibroblasts. The results revealed that the growth of human skin keratinocytes and fibroblasts on the BCC films was comparable to that on the BC film; however, improvement of cell adhesion and spreading on the BCC films was observed in human skin keratinocytes. The results of the biological response experiments showed no significant difference between BCC-MW 30 000 and BCC-MW 80 000.

Keywords

Bacterial cellulose, chitosan, *Acetobacter xylinum*, biocompatibility, film

1. Introduction

Nearly-purified cellulose film of nano-sized fibrils can be biosynthesized by the bacteria *Acetobacter xylinum*, using glucose as a common substrate. With its ultra-fine network structure, bacterial cellulose (BC) displays advantages superior to its counterpart from plants with its physical and chemical properties, such as mechanical strength, crystallinity and hydrophilicity. Several applications of BC in medical

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fields have been reported, such as artificial skin for humans with extensive burns [1], artificial blood vessels for microsurgery [2], scaffolds for tissue engineering of cartilage [3] and wound-dressing [4]. BC dressing adheres to the wound sites very well, displaying a high degree of adherence, even to the moving parts, and maintains a proper moist environment and good sorption of the wound's exudates. It is non-allergenic [4–6], and can be safely sterilized without any change to its characteristics. Our previous work [7] reported that BC film supported the growth of human keratinocytes and fibroblasts and promoted the re-epithelialization process to facilitate wound healing.

Chitosan has also been widely studied as a wound-dressing material, and is known in many medical applications; for instance, implantation, topical ocular, transparent membrane, tissue reconstruction and wound healing. The use of chitosan and its derivatives as a supporting material for tissue-engineering applications has been reviewed [8]. Application of chitosan wound dressing made chronic ulcers heal faster. Chitosan could be used to inhibit fibroplasia in wound healing, as well as to promote tissue growth and differentiation [9]. Chitin, chitosan and water-soluble chitin are useful in wound management as a wound-healing accelerator [10]. Chitosan has also been found to be a good supportive material for gene delivery, cell culture, tissue engineering, drug delivery, anti-microbial agents and adsorption agents [11, 12]. Chitosan with a relatively high degree of deacetylation modulated human skin cell mitogenesis and the stimulatory effect was described as a result from the interaction of chitosan with the growth factors presented in the serum [13].

Based on the unique physical and biological properties together with the similar backbone structure of chitosan and cellulose, the bio-co-polymerization of cellulose and chitosan using *A. xylinum* was developed and the nanostructure and mechanical properties of the modified films were compared to the general BC film [14]. Furthermore, in this work, the biological properties of the bacteria cellulose–chitosan (BCC) film were evaluated by using the mouse fibroblast cell line L929, human-transformed skin keratinocyte (HaCat) and primary human skin fibroblast (CRL-2211) in order to explore the developed BCC film for application in skin wounds therapy.

2. Materials and Methods

2.1. Microbial Strains

The *A. xylinum* AGR 60 was isolated from nata de coco. The stock culture was kindly supplied by Pramote Tammarate, the Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand.

2.2. Culture Media and Method

The medium for the inoculums was coconut water containing 5.0% sucrose, 0.5% ammonium sulfate and 1.0% acetic acid [7]. The medium was sterilized at 121°C for 15 min. Pre-cultures were prepared by transferring 50 ml of a stock culture to

1000 ml medium in 1500-ml bottles and incubating statically at 30°C for 5 days. After the surface pellicle was removed, the 5% (v/v) pre-culture broth was added to the main culture medium supplementation with 0.75% chitosan content (MW 3×10^4 and 8×10^4 with a degree of deacetylation (DAC) of 0.85). Chitosan was purchased from Seafresh Chitosan (Lab) (Bangkok, Thailand) in the form of dried powder. From our previous study, chitosan of higher MW ($\geqslant 2 \times 10^5$) significantly inhibited the formation of BC biosynthesis (data not shown). Activated medium with the pre-culture broth (75 ml) was inoculated in a Petri dish and kept at 30°C for 7–10 days. The developed gel-like cellulose pellicle was first purified by washing with deionized (DI) water and then was treated with 1% (w/v) NaOH at 35°C for 24 h to remove bacterial cells and rinsed with DI water until the pH was 7. Afterwards, the purified sheets were air-dried at room temperature (30°C) and stored in plastic film before use.

2.3. *Characterization of Membranes*

For surface morphology, scanning electron micrographs (SEM) were taken using a Jeol (Tokyo, Japan) JSM-5410LV scanning electron microscope. The BC films were frozen in liquid nitrogen, immediately snapped and vacuum-dried. Then, the films were sputtered with gold and photographed.

The pore size, porosity and pore size distribution of the BC film were determined by a BET (Brunauer, Emmett and Teller) model Micromeritics (Atlanta, GA, USA) ASAP 2020. The BET pore size and surface area were determined with N_2 adsorption at 77 K.

2.4. *Mechanical Analysis*

BC film was cut into strips of 10 mm in width and 10 cm in length. The average thickness of the dry samples was 0.04 mm. The maximum tensile strength and break strain of BC film in dry and wet states were determined using a Lloyd (Southampton, UK) 2000R universal testing machine. The test conditions followed ASTM D882. The tensile strength and break strain were the average values of 10 specimens.

2.5. *Cytotoxicity Test*

The cytotoxicity was evaluated based on a procedure modified from the ISO10993-5 standard test method using the mouse fibroblast cell line L929 purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The experiments were conducted in triplicate. The cell culture was cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Biochrom, Cambridge, UK), 100 units/ml penicillin (Gibco, Brooklyn, NY, USA), 100 μg/ml streptomycin (Gibco), 1% L-glutamine and 2 mg/l lactalbumin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. A single-cell suspension of L929 cells was obtained after trypsinization, and the cells were counted in a hemocytometer (Hausser Scientific, Horsham, PA, USA).

The films were sterilized by autoclaving at 121°C for 20 min [7], transferred aseptically to 24-well culture plates (Nunc, Rockford, IL, USA) and incubated at 37°C in serum-free culture medium (SFM) for 24 h. The film/medium ratio was 10 mg/ml. The extraction medium from the film incubation was used to culture L929 mouse fibroblast cells. SFM was used as control. The cells were cultured in 24-well plates at concentrations of 5×10^4 cells in 500 µl culture medium per well, and the cells were allowed to attach to the plates for 16 h in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Then, the cell-culture medium was replaced with 500 µl of the 100% (v/v) extraction medium, the 50% (v/v) extraction medium or the control medium. The number of living cells was finally quantified using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphynyl-tetrazoluim bromide (MTT) assay.

2.6. Cell Study

For the application in the therapy of skin wounds, two types of cells were used in cell study: human-transformed skin keratinocyte (HaCat) and primary human skin fibroblast (CRL-2211, ATCC). Keratinocytes and fibroblasts were grown in DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% FBS. When the cells reached 80% confluence, they were serially subcultured. For fibroblasts, cells from the 4th to the 7th subculture passages were used in the described experiments. Both cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Air-dried BC and BCC films were punched into round samples of 14 mm in diameter. The samples were sterilized by autoclaving at 121°C for 20 min and transferred aseptically to 24-well culture plates. The experiments were conducted in triplicate. Culture medium (500 µl) was added to each well to equilibrate the samples for 30 min before cell seeding. Proliferation of skin keratinocytes and fibroblasts on BC films was determined by MTT assay as described previously [12]. Briefly, cells were seeded into 24-well culture plates (Nunc) at an initial density of 3×10^4 cells per well on BC film, BCC film or polystyrene plate control. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 16 h. Then, the culture medium was removed and replaced for another 48 h by SFM for cultures of both keratinocytes and fibroblasts. The number of living cells was determined using the MTT assay. Cell morphological imaging was performed using a Jeol JSM-5410LV SEM.

3. Results and Discussion

3.1. Characterizations

BCC film was developed by means of adding chitosan to the culture medium during biosynthesis by *A. xylinum*. It was previously reported that water-soluble and lysozyme-susceptible phosphoryl chitin (P-chitin) could be de-polymerized in a culture medium and then incorporated into the metabolic pathway for biosynthesis of BC film [15]. From our previous study [14], the FT-IR spectra of the BCC

films indicated intermolecular interaction between the hydroxyl groups of cellulose fiber and the amino groups of chitosan.

In static condition, gel-like cellulose–chitosan pellicles were developed in the form of thin films. To avoid the inhibition of growth and BC formation, the maximum amount of the chitosan addition in the culture medium was limited at 0.75% (w/v). BCC-MW 30 000 and 80 000 refer to the samples of BCC films supplemented with chitosan of MW 3×10^4 and 8×10^4, respectively. The films were purified and air-dried at room temperature. The average thickness of the films was about 0.04 mm in dry form and about 0.25 mm in re-swollen form. Both of the developed BC and BCC films displayed an ultrafine fiber network structure of microfibrils; however, the BCC film structure was found to be relatively denser than the BC film. The pore size distribution of the dried and re-swollen films of BC, BCC-MW 30 000 and BCC-MW 80 000 are shown in Fig. 1. The average pore sizes of the re-swollen films in water of BC, BCC-MW 30 000 and BCC-MW 80 000 were at 60.80 ± 0.40, 48.95 ± 0.35 and 39.70 ± 0.40 nm, respectively. Our previous FT-IR analysis showed intermolecular connections between BC and chitosan; consequently, the improved mechanical properties and water absorption capability, which are the required properties of a wound care dressing material, were obtained [14]. However, the small amount of chitosan supplement in this work barely affected the water vapor transmission rate and crystallinity index of the films and the anti-microbial ability of the BCC films was only slightly enhanced. More information of those characteristics of the BC and BCC films has been reported elsewhere [7, 14]. The BCC films did not dissolve in water or ethanol and could be safely sterilized in several ways, for instance, by heat sterilization in an autoclave, gas chemical sterilization and 70% ethanol disinfection [7]. Table 1 gives a summary

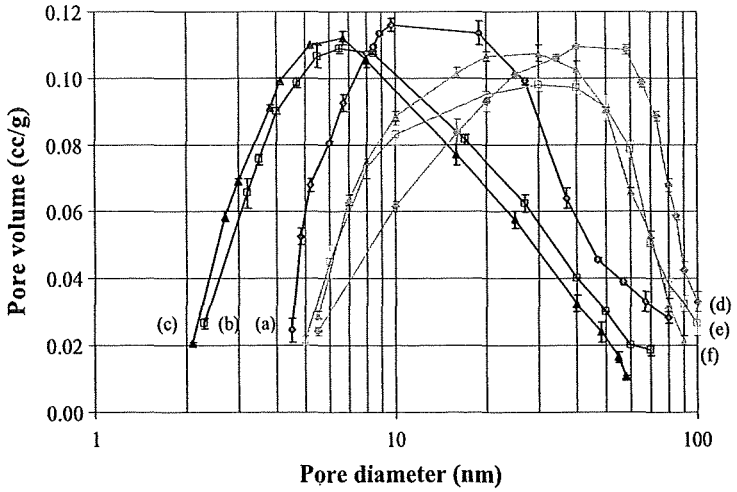

Figure 1. The pore size distribution of BC and BCC films: in dry form of (a) BC, (b) BCC-MW 30 000 and (c) BCC-MW 80 000; in re-swollen form of (d) BC, (e) BCC-MW 30 000 and (f) BCC-MW 80 000.

Table 1.
Characteristics of the BC and BCC films

Property	BC		BCC-MW 30 000		BCC-MW 80 000	
	Dry	Re-swollen	Dry	Re-swollen	Dry	Re-swollen
Tensile strength (MPa)	5.32 ± 0.26	1.45 ± 0.17	7.66 ± 0.15*	2.07 ± 0.17*	8.26 ± 0.33*	2.72 ± 0.21*
Young's modulus (MPa)	161.80 ± 11.08	21.79 ± 2.36	195.00 ± 7.84*	33.64 ± 1.67*	221.80 ± 15.56*	36.13 ± 1.23*
Break strain (%)	3.75 ± 0.16	8.17 ± 0.32	1.91 ± 0.14*	5.76 ± 0.26*	1.44 ± 0.09*	4.36 ± 0.21*
Average pore diameter (nm)	22.15 ± 0.25	60.80 ± 0.40	15.25 ± 0.15*	48.95 ± 0.35*	13.05 ± 0.15*	39.70 ± 0.40*
Surface area (m^2/g)	12.8 ± 0.1	56.0 ± 0.8	14.1 ± 0.1*	80.5 ± 1.9*	15.0 ± 0.2*	99.2 ± 1.1*
Water absorption capacity (%)	482 ± 24	–	606 ± 15*	–	652 ± 18*	–
Water vapour transmission (g/m^2day)	1593 ± 18	–	1578 ± 23	–	1564 ± 15	–

* $P < 0.05$, significant difference in mean value relative to the control BC film.

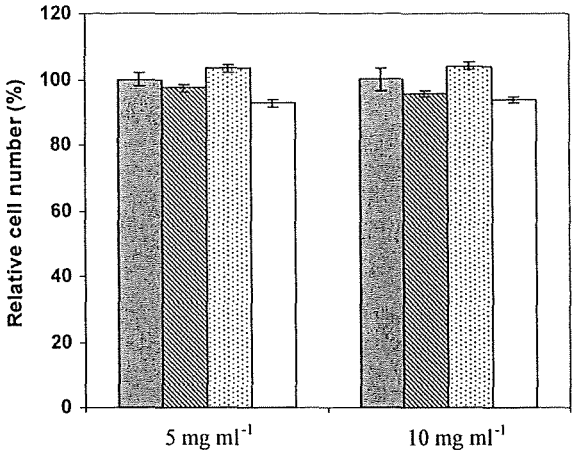

Figure 2. Toxicity test against L929 mouse fibroblast cells at the extraction medium concentrations of 5 and 10 mg/ml on the culture-treated polystyrene plate (■), BC film (▨), BCC-MW 30 000 film (▤) and BCC-MW 80 000 films (□).

of the overall characteristics of the BCC films compared to the BC film. For biomedical applications, the cytotoxicity test and the *in vitro* skin cell study of the developed BCC films were further examined.

3.2. Cytotoxicity Test

The cytotoxicity of the BC and BCC films was examined against L929 mouse fibroblast cells using the MTT assay (Fig. 2). The percentages of living cells cultured with the extraction medium of BC, BCC-MW 30 000 and BCC-MW 80 000 for 24 h, in comparison with those cultured with fresh SFM were found to be $97 \pm 2.25\%$, $103 \pm 2.57\%$ and $93 \pm 3.44\%$ with the extraction medium concentration of 5 mg/l and $96 \pm 2.62\%$, $104 \pm 2.42\%$ and $94 \pm 4.83\%$ with the extraction medium concentration of 10 mg/l, respectively. The results indicated that the BC and BCC films had no toxicity against L929 mouse fibroblast cells with no statistically significant difference, calculated using a two-tailed t-test for a 95% confidence limit ($P > 0.05$). The high biocompatibility of BC *in vitro* and *in vivo* evaluated on a variety of cells has been documented previously [1–7].

3.3. Cell Study

The effects of BC and BCC on the proliferation and morphology of human skin cells were studied. The major human skin cells, human-transformed keratinocytes (HaCat), and human normal skin fibroblasts (CRL-2211) were used, and the percentage of the living cells after seeding on BC and BCC films from 0 to 24 and 48 h was gradually increased, as shown in Figs 3 and 4, respectively. Cultures of both cell types exhibited no significant difference in proliferation on BC and BCC films calculated using a two-tailed t-test for a 95% confidence limit ($P > 0.05$).

In the culture of keratinocytes, the percentage of cell number increased from $100 \pm 15\%$ to $238 \pm 14\%$ and $310 \pm 8\%$ on the BC, $100 \pm 8\%$ to $262 \pm 13\%$ and

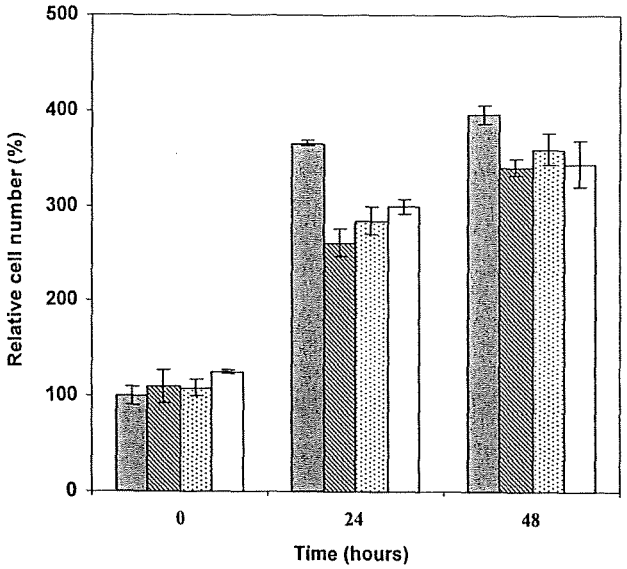

Figure 3. Proliferation of human skin keratinocytes on the culture-treated polystyrene plate (■), BC film (▨), BCC-MW 30 000 film (▤) and BCC-MW 80 000 films (□). The percentage of living cells was assessed at 0, 24 and 48 h of culture by MTT assay.

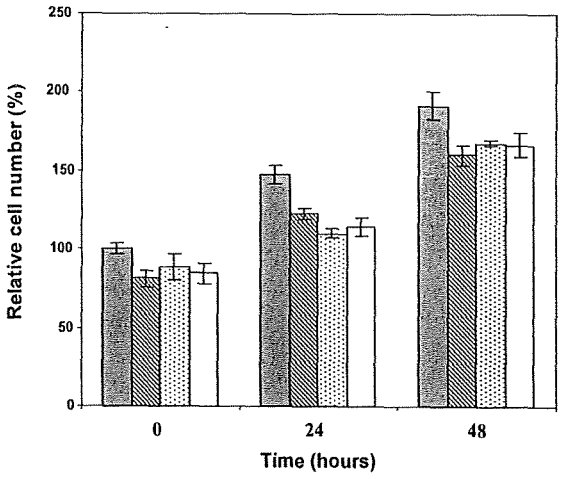

Figure 4. Proliferation of human fibroblasts on the culture-treated polystyrene plate (■), BC film (▨), BCC-MW 30 000 film (▤) and BCC-MW 80 000 films (□). The percentage of living cells was assessed at 0, 24 and 48 h of culture by MTT assay.

331 ± 15% on the BCC-MW 30 000, and 100 ± 1% to 239 ± 6% and 274 ± 20% on the BCC-MW 80 000 at 24 and 48 h, respectively, while the percentage of the number of fibroblasts increased from 100 ± 6% to 151 ± 4% and 198 ± 8% on the BC, 100 ± 6% to 124 ± 3% and 190 ± 2% on the BCC-MW 30 000, and 100 ± 8% to 135 ± 7% and 197 ± 9% on the BCC-MW 80 000 at 24 and 48 h, respectively.

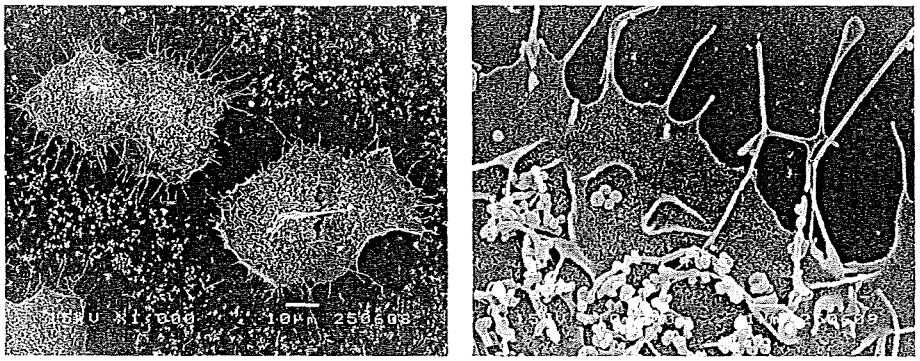

Figure 5. SEM images of human skin keratinocytes on the culture-treated polystyrene plate at 24 h.

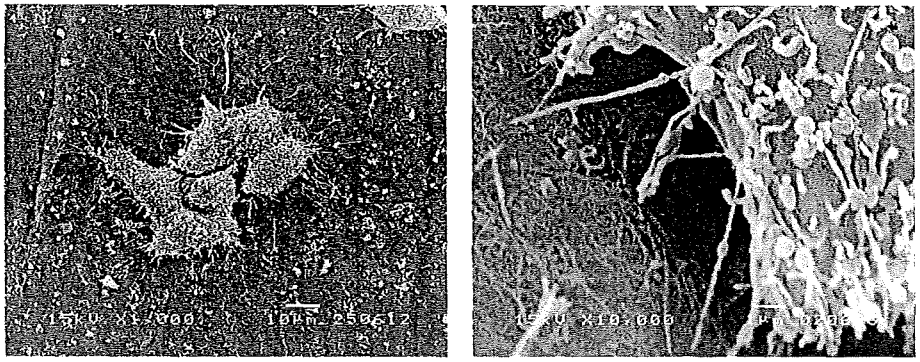

Figure 6. SEM images of human skin keratinocytes on the BC film at 24 h.

A comparison of cell number showed that the number of both cell types on the BC and BCC films was relatively lower than that on the polystyrene culture plate control; however, the rate of cell proliferation was similar (Figs 3 and 4). This proliferation result did not only support the biocompatibility of these kinds of materials, but also showed the potentiation to increase the cell number which would enhance the wound-healing process.

SEM images of keratinocytes at a low magnification of 1000× on the control culture plate at 24 h demonstrated well-spread keratinocytes over the surface of the plate, which was seen as a flattened epithelial-like shape with slight trace of filopodia at a high magnification of $1 \times 10^4\times$ (Fig. 5), whereas a lower degree of spreading and looser cell adhesion was observed in keratinocytes cultured on the BC surface, which were seen as more rounded cells, indicating fewer cell–material interactions (Fig. 6). However, other normal cell surface appearances, such as filopodia, were found.

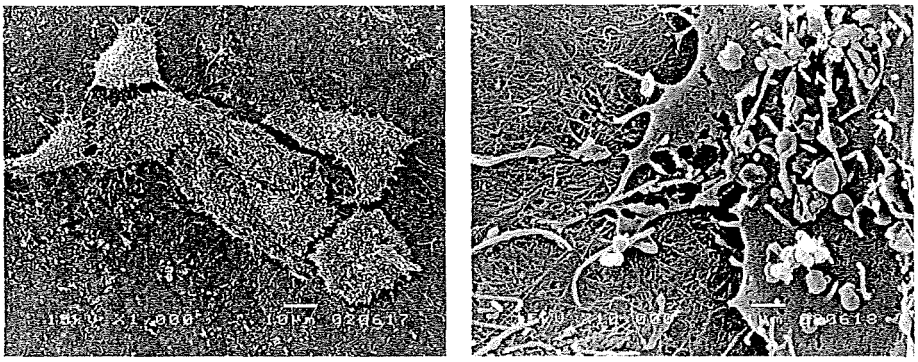

Figure 7. SEM images of human skin keratinocytes on the BCC-MW 30 000 film at 24 h.

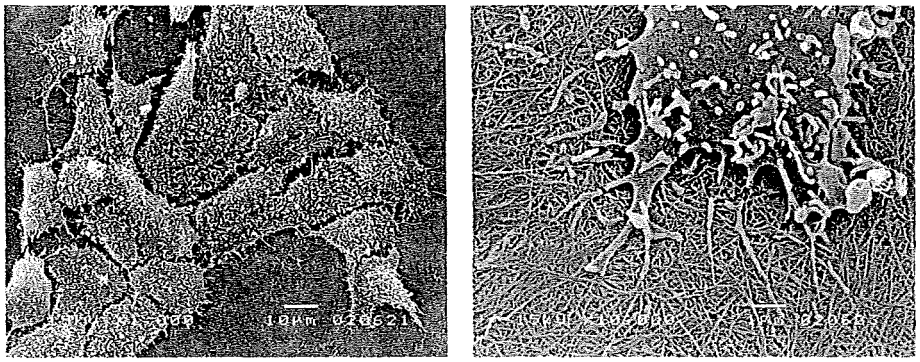

Figure 8. SEM images of human skin keratinocytes on the BCC-MW 80 000 film at 24 h.

Regarding the BC surface, the patterns of cell distribution and attachment of keratinocytes on the BCC films were different. The extent of cell spreading and adhesion on the BCC-MW 30 000 and BCC-MW 80 000 surfaces was higher, as shown in Figs 7 and 8, respectively. A possible explanation for this phenomenon was that the adhesion of keratinocytes to the BC film was less than that of the cells to the BCC films. The influence of cell attachment from the interaction of positively charged amino groups of chitosan chains and negatively charged cell membranes has been widely reported [18]. Therefore, the improved keratinocyte attachment on the BCC films could be due to the presence of chitosan on the film surface. In addition to our previous report which demonstrated that BC film could maintain the normal characteristics of keratinocytes [7], the present study showed that the incorporation of chitosan to the BC film not only promoted keratinocyte proliferation, but also improved cell spreading and adhesion on the surface.

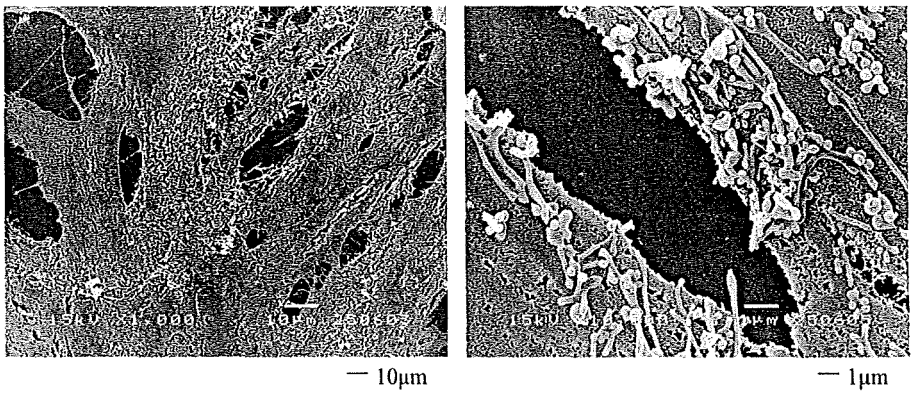

Figure 9. SEM images of human skin fibroblasts on the culture-treated polystyrene plate at 24 h.

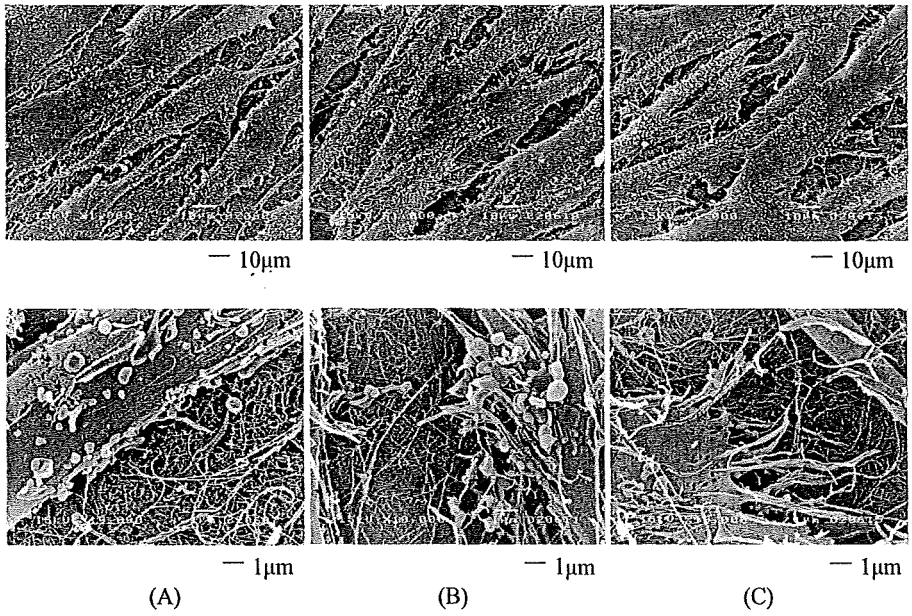

Figure 10. SEM images of human skin fibroblasts on the (A) BC film, (B) BCC-MW 30 000 film and (C) BCC-MW 80 000 film at 24 h.

The SEM images of fibroblasts showed the typical elongated fibroblast pattern with good spreading on the control culture plate and all types of tested materials with no significant difference in morphology (Figs 9 and 10). At a high magnification of $1 \times 10^4\times$, several of the thread-like filopodia were seen showing good interaction with the material underneath (Fig. 10). This normal phenotypic shape suggested that the cells function biologically on these materials.

According to previous works [13, 16], chitosan does not directly enhance the rate of fibroblast cell proliferation. Chitin and its derivatives showed no direct acceler-

atory effect on the proliferation of fibroblasts *in vitro*. In addition, chitosan at high concentration could reduce the proliferation rate. However, the indirect stimulatory effect on fibroblast proliferation was suggested to be a result from the interaction of chitosan with the growth factors present in the serum, thus potentiating their effect [13]. Proliferation of fibroblasts *in vivo* was suggested to be accelerated indirectly by chitin derivatives, possibly due to the formation of a polyelectrolyte complex between chitosan and heparin [13, 16, 17]. In this study, the supplementation of chitosan of MW 3×10^4 and 8×10^4 in the bacterial culture medium showed no significant influence on the cell proliferation profile of the modified films compared to that of the BC film.

4. Conclusion

For the improvement of physical and biological properties, the modification of the bacterial cellulose (BC) film by supplementing chitosan in culture medium during biosynthesis by *A. xylinum* was performed. The bacterial cellulose–chitosan (BCC) film had remarkable water holding capacity and improved tensile strength. Our results indicate that BCC film has no toxicity against L929 mouse fibroblast cells. The examination of the growth of human skin keratinocytes and fibroblasts on the BCC films demonstrates that the modified films supported proliferation for both cell lines almost comparable to those on the BC film. Cultures of human keratinocytes showed no differences between on BCC-MW 30 000 and BCC-MW 80 000 films, but were slightly better than on the BC film, as they exhibited relatively better cell adhesion and spreading on the surface. Our results indicate that chitosan supplemented bacterial cellulose may be a good candidate for biomedical applications, such as wound dressing.

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Biosynthesis and Characterization of Bacteria Cellulose–Alginate Film

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ABSTRACT: A novel polysaccharide membrane containing alginate in bacterial cellulose matrix was synthesized by *Acetobacter xylinum* under static conditions using a culture medium supplementation with sodium alginate. By increasing alginate content, the bacterial cellulose–alginate (BCA) membrane was more hydrophilic and the film structure became denser with the smaller average pore size. Scanning electron microscope images displayed the deposits of alginate gel on the surfaces of the multilayer cellulose film. The declines in the tensile strength, the Young's modulus, and the elongation at break of the BCA membrane were dependent on the degree of alginate supplement. The BCA membrane showed higher water absorption capacity. The addition of alginate slightly affected the water vapor transmission rate but remarkably decreased the oxygen transmission rate of the membrane. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 115: 1581–1588, 2010

Key words: bacteria cellulose; alginate; *Acetobacter xylinum*; characterization; film

INTRODUCTION

Cellulose is the most abundant natural polysaccharide produced by plants and microorganisms. It was formed out of glucose-based repeat units, connected by 1,4-β-glucosidic linkages.[1] Cellulose fibrils are highly inelastic and insoluble.[2] The β-1,4 linkage results in a ribbon-like molecule, which is suited for forming fibril via hydrogen bonding.[3] The bacterial cellulose (BC) film with its highly pure nanofibril cellulose network can be formed by the *Acetobacter species*. It is distinguished by its mechanical strength, water absorption capacity (WAC), and crystallinity. Its unique structural features and properties facilitate diverse applications, ranging from food matrix, dietary fiber, wound-dressing, carrier for mammalian cell culture, immobilization of enzymes, and other biomolecules, as well as diaphragms in speakers for acoustic and separation membranes.[4–8] The pellicle which is flat can be easily processed into a uniform porous membrane, whereas plant cellulose is often interspersed with lignin, hemicellulose, and pectin leading to nonuniformity in porosity and inconsistent permeability. Furthermore, the production yield and the structure of BC can be changed from the control conditions such as composition of the culture media, pH, temperature, and oxygen tension as well as drying procedure.[1,9] The specific application of BC as a dialysis membrane was examined by Shibazaki et al. (1993).[10] The BC film showed a significantly higher permeation rate and a greater molecular weight cut-off when compared with regenerated cellulose membrane. The BC membrane was investigated for the pervaporation of aqueous-organic mixtures.[4,11] It was found that the permeate flux was incredibly high but the selectivity was fairly low.

Among the hydrophilic polysaccharide membranes, alginate film has gained special interest for its high flux and separation factor.[12–15] Alginate is naturally polysaccharide of linear copolymers of (1–4)-linked β-D mannuronic acid and α-L-guluronic acid, derived primarily from brown seaweed. Alginate can be transformed into the most widely used water-soluble form, sodium alginate, through the incorporation of sodium salt. However, alginate membrane has poor stability in aqueous solutions because of its highly hydrophilic character due to both of its carboxyl and hydroxyl groups, resulting in significant reduction of membrane selectivity and mechanical strength.[15]

The present research aimed to develop a composite film of bacterial cellulose–alginate for the application in membrane separation. To increase the hydrophilic property and generate the dense film structure, the biosynthesis of bacterial cellulose–alginate film was developed by the supplement of sodium alginate into the medium under static

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cultivation of *Acetobacter xylinum*. For further application in membrane systems, the effects of alginate content on the membrane properties, for instance, its structure, pore morphology, chemical structure, mechanical strength, WAC, and permeability of water vapor and oxygen were investigated.

MATERIALS AND METHODS

Culture, culture media, and method

The *Acetobacter xylinum* AGR 60 was kindly supplied by the laboratory of Pramote Tammarate (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. The medium was sterilized at 121°C for 15 min. Precultures were prepared by transferring 50 mL of a stock culture to 1000 mL of medium in 1500 mL bottles and incubated statically at 30°C for 5 days. After the surface pellicle was removed, the 5% (v/v) preculture broth was added to the main culture medium supplementation with different sodium alginate content. The 75 mL of activated medium was inoculated in a pretidish and kept at 30°C for 7 days. The developed gel-like cellulose pellicle was first purified by washing with deionized (DI) water and then was treated with 1% (w/v) NaOH at 35°C for 24 h to remove bacterial cells and rinsed with DI water until the pH was 7. Afterward, the purified sheets were air-dried at room temperature) (30°C) and stored in plastic film before use.

Characterization of membranes

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectra of the membranes were measured at wave numbers ranging from 2000 cm^{-1} to 800 cm^{-1} at a resolution of 4 cm^{-1} with a Nicolet (US) SX-170 FTIR spectrometer.

Water absorption capacity

To determine the WAC, the dried membranes were immersed in DI water at room temperature) (30°C) until equilibration. After that the membranes were removed from the water and excess water at the surface of the membranes was blotted out with Kimwipes paper. The weights of the swollen membranes were measured, and the procedure was repeated until no further weight change was observed. The water content was calculated with the following formula:

$$\text{WAC}\ (\%) = \frac{W_h - W_d}{W_d} \times 100$$

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

Mechanical strength

The air-dried membranes were cut into strip-shaped specimens 10 mm width and 10 cm long. The maximum tensile strength, Young's modulus, and break strain of BC and BCA films were determined with a Lloyd (Southampton, UK) 2000R universal testing machine. The test conditions followed ASTM D 882. The tensile strength, Young's modulus, and break strain were the average values determined from 10 specimens.

Scanning electron microscopy

The films were frozen in liquid nitrogen, immediately snapped, vacuum-dried, and then sputtered with gold and photographed. Images were taken on a JOEL (Tokyo, Japan) JSM-5410LV scanning electron microscope (SEM). The accelerating voltage was adjusted to 15 kV. The specimen was examined at magnifications from 2000× to 10,000×.

Brunauer-Emmett-Teller (BET) surface analysis

The pore size and surface area of the membranes were determined with a BET surface area analyzer. To remove moisture from the film samples, the samples were placed in sample cells, which were then heated upto 348 K for 5 h and cooled down to room temperature before the BET analysis. The BET pore size and surface area were determined with N_2 adsorption at 77 K in a Micromeritics (Atlanta, GA) ASAP 2020.

Water vapor permeability measurement

The water vapor transmission rate (WVTR) of the dry membranes with area of 50 cm^2 was determined with a Lyssy (Zollikon, Switzerland) L80-4000 water vapor permeation tester. The test conditions followed ISO 15106-1. The determination of WVTR was done at 38°C and 90% relative humidity. One side of the membrane was exposed to a humid air atmosphere and the other side was exposed to a nitrogen atmosphere. As water solubilized into the membrane and permeated through the sample film, nitrogen gas swept and transported the transmitted water vapor molecules to a calibrated infrared sensor. The response was reported as a transmission rate.

The oxygen permeability measurement

Oxygen transmission rate (OTR) of the dry membranes was determined with a oxygen permeation

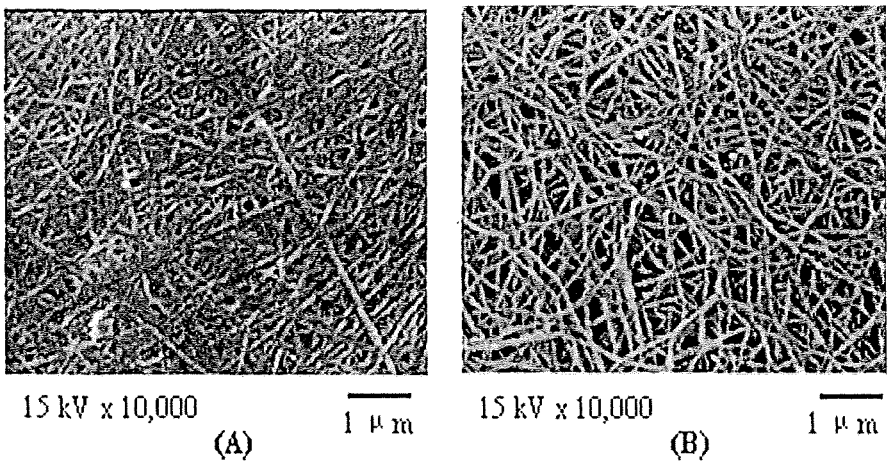

Figure 1 SEM images of surface morphology of BC film in: (A) dry form and (B) reswollen form.

analyzer: Illinois Instruments (Johnsburg, IL) Model 8000. The test conditions followed ASTM D3985. The determination of OTR was done at 23°C and 0% relative humidity. The membrane was held in such a manner that it separated two side of a test chamber. One side was exposed to an oxygen atmosphere and the other side was exposed to a nitrogen atmosphere. Testing was completed when the concentration of oxygen in the nitrogen side was constant.

RESULTS AND DISCUSSION

In case of cultivation in a shake flask or in a stirred-tank reactor, the addition of 0.04% (w/v) sodium alginate into culture medium enhanced yields and changed the morphology of cellulose.[16] Our preliminary test demonstrated that under static cultivation, to avoid the inhibition of cell and film production, the maximum amount of sodium alginate addition in the culture medium was limited at 1.0% (w/v) (data not shown). Therefore, a study of the alginate supplement in culture medium was performed in the concentration range of 0–1.0% (w/v). The structure, pore morphology, mechanical strength, chemical structure, WAC, as well as well as WVTR and OTR of the developed films were then examined to investigate the effect of alginate supplement.

Membrane morphology

The SEM images for surface morphology of BC and BCA membranes are shown in Figures 1 and 2, whereas the SEM images for the cross section are shown in Figures 3 and 4. Regarding the definitions, the BC and BCA membranes refer to the BC

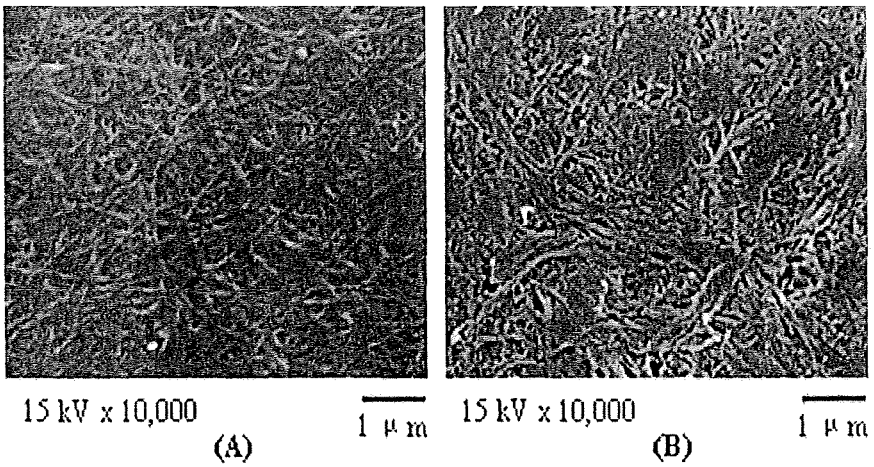

Figure 2 SEM images of surface morphology of 1% Al-BCA film in: (A) dry form and (B) reswollen form.

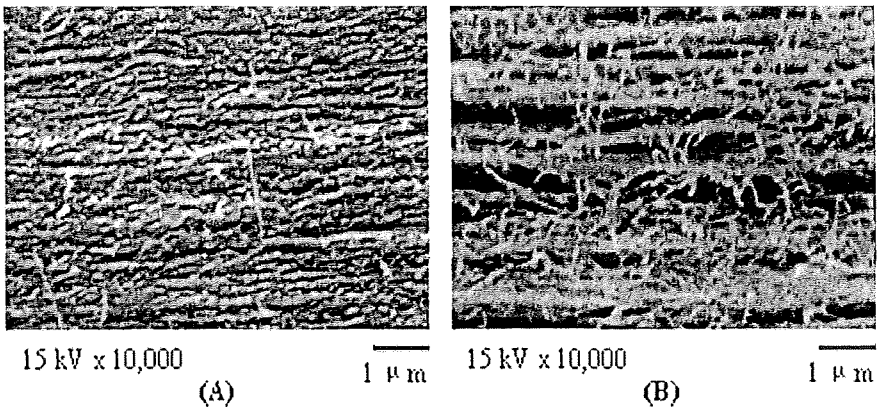

Figure 3 SEM images of cross section of BC film in: (A) dry form and (B) reswollen form.

membranes without and with the addition of alginate in culture medium, respectively, whereas, *x*% Al-BCA refers to the BCA membrane with *x*% (w/v) sodium alginate supplement in culture medium. The SEM images of surface morphology present the porous film composed of a continuous network of cellulose nanofibrils of 50–100 nm. According to high WAC of the membranes, the reswollen membranes exhibited a looser structure and contained larger pores than those of the dried ones. From the surface morphology images of the BCA membrane (Fig. 2), with the supplement of sodium alginate into the culture medium, the deposits of alginate gel on the film surfaces were observed and the apparent pore sizes of BCA films decreased with increasing the percent of alginate. Since alginate gel was well incorporated into the cellulose fibril network and filled the pores, the structure of the BCA membrane was denser with the smaller pore size than that of the BC membrane. As shown from the cross section images of BC and BCA membranes (Figs. 3 and 4), the membranes possessed numerous sheets composed of a network of nanofibrils. The membranes in dry form were made of thin sheets that were tightly packed, whereas the reswollen ones had looser packed structures.

FTIR analysis

FTIR spectroscopy is utilized in determining the specific functional groups or chemical bonds in a material. The FTIR spectra of the BC and BCA membranes were measured with the wave number ranging from 2000 cm^{-1} to 800 cm^{-1} as shown in Figure 5. All the characteristic bands of the BC film were present in the spectra of the BCA films without any occurrence of new peaks. The BC membrane showed a band at 1647.0 cm^{-1}, which was attributed to the glucose carbonyl of the cellulose. The carbonyl group band for BCA films slightly shifted from 1647.0 cm^{-1} to lower wave numbers, 1645.4–1646.6 cm^{-1} and the band became broader, which might indicate some weak physical interactions between the carbonyl groups of cellulose and sodium alginate. No evidence of peak shift that would indicate a chemical interaction. However,

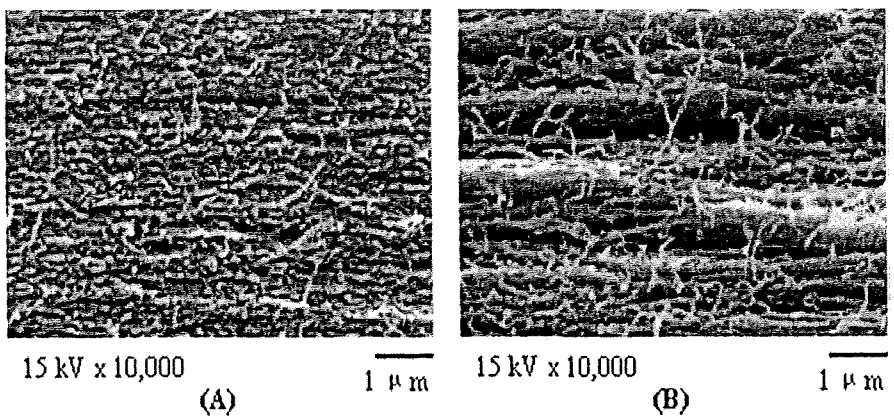

Figure 4 SEM images of cross section of 1% Al-BCA film in: (A) dry form and (B) reswollen form.

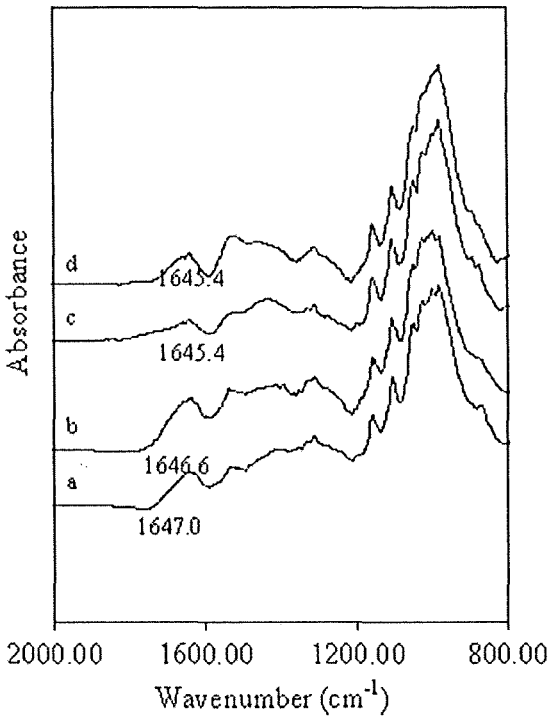

Figure 5 The FTIR spectra of the BC and BCA membranes in wave numbers ranging from 2000 cm^{-1} to 800 cm^{-1}: (a) BC; (b) 0.5% Al-BCA; (c) 0.75% Al-BCA; and (d) 1.0% Al-BCA.

under agitated culture, Zhou et al.[16] reported the interaction from the shift of —OH and C—O—C bands in the presence of sodium alginate in the culture medium. From our previous work on the alginate–bacteria cellulose blend membrane,[17] changes in the region of 1640–1600 cm^{-1} were reported, which indicated some interactions between the hydroxyl group of cellulose and the carboxyl group of alginate. As the membrane composition and structure were different, the interaction of alginate and cellulose in the BCA membrane might not be the same as that of the blend membranes.[16,17]

Mechanical properties

End-use applications usually involve some degree of mechanical loading, therefore, the effects of alginate content on the mechanical properties such as the tensile strength, Young's modulus, and elongation at break were examined. As shown in Figure 6, the tensile strength of the BC membrane at the average thickness of 40 μm was 5.30 MPa, whereas those of the 0.5–1.0% Al-BCA membranes varied from 4.71 MPa to 3.80 MPa. The decreasing of the tensile strength of the BCA membranes was in a linear manner ($R^2 = 0.9854$) with the amount of alginate content.

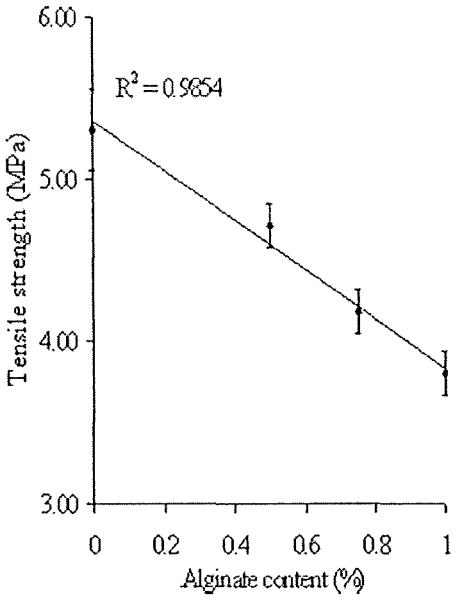

Figure 6 The tensile strength of the BC and BCA membranes as a function of alginate content (% w/v) in culture medium.

The decrease of Young's modulus of the BCA membranes as a function of alginate content was similar to that of the tensile strength (Fig. 7). The Young's modulus of the BC and the 1.0% Al-BCA membranes were 172.8 MPa and 144.4 MPa, respectively. The elongation at break also decreased when

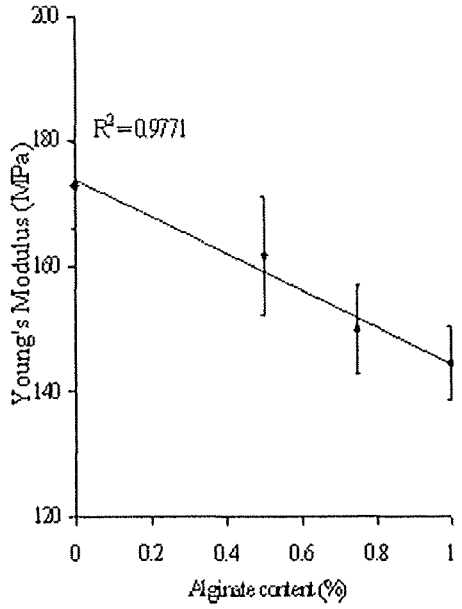

Figure 7 The Young's modulus of the BC and BCA membranes as a function of alginate content (% w/v) in culture medium.

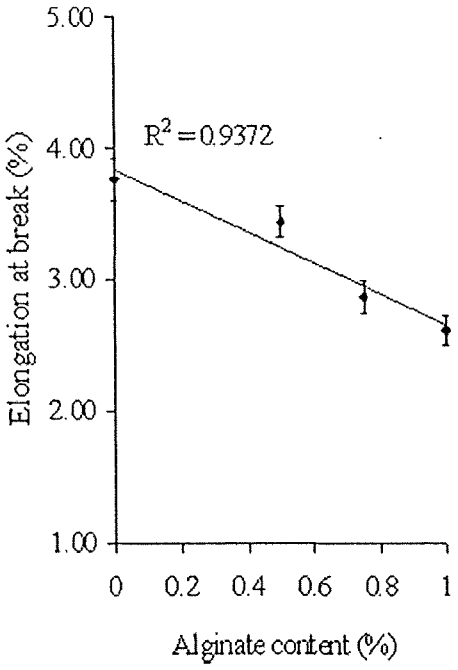

Figure 8 The elongation at break of the BC and BCA membranes as a function of alginate content (% w/v) in culture medium.

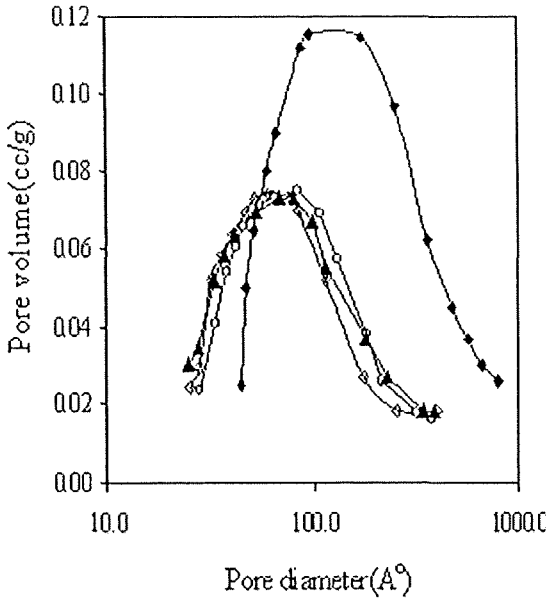

Figure 9 The pore size distribution of the BC and BCA membranes: (a) BC [◆]; (b) 0.5% Al-BCA [○]; (c) 0.75% Al-BCA [▲]; and (d) 1.0% Al-BCA [□].

increasing the alginate content as shown in Figure 8. The elongation at break of the BC and the 1.0% Al-BCA membranes were 3.76% and 2.61%, respectively.

With the supplement of alginate, alginate was incorporated into the bulk of cellulose fibril networks of the BCA membrane. A comparison of mechanical properties of the BCA film with 0.5–1.0% Al and the BC film indicated statistically significant decreases for tensile strength ($P < 0.005$), elongation at break ($P < 0.005$), and Young's modulus ($P < 0.05$) for a one-tailed test. The effect of alginate content on the mechanical properties of the BCA membranes was similar to those previously observed in the blend membranes of BC and alginate blend in NaOH/urea,[17] cellulose cuoxam with alginate,[18] and cotton cellulose and alginate blend in NaOH/urea aqueous solution.[19] Although the cellulose/alginate blend membranes were mechanically weaker than cellulose membranes, they were mechanically stronger than the alginate membranes with a promising performance for pervaporation dehydration.[20] The sodium alginate film was mechanically weak and had poor stability in aqueous solution.[15] The presence of alginate in the cellulose–alginate composite could 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.[17–19]

Porosity

The results of the pore size distribution of the BC and BCA membranes from BET analysis are shown in Figure 9. Corresponding to the results from the SEM images, the BCA membranes had an average pore size much smaller than that of the BC membrane and the average pore diameter decreased with increasing alginate content, whereas the surface area slightly decreased (Table I). A possible explanation was that by means of adding sodium alginate in the culture medium, as in this work, the alginate gel deposited on the film sheet, the alginate molecules diffused through the pores and partially filled the pores of the films. The SEM observations of cross sections of the reswollen BC and BCA membranes (Figs. 3 and 4) revealed that the film sheets united together to form the bulk membrane. Therefore, the presence of alginate incorporated into the cellulose fibril networks and filled pores resulted in a significant reduction of the membrane pore size. From our

TABLE I
Surface Areas and Pore Diameters of the Dry BC and BCA Membranes Analyzed by BET Analyze

Alginate content (% w/v)	Average pore diameter (Å)	Surface area (m^2/g)
0	224	12.6
0.5	97	12.1
0.75	64	11.8
1	39	11.2

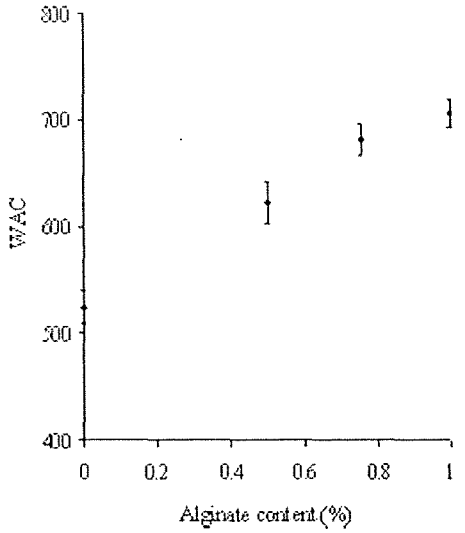

Figure 10 The water absorption capacity (WAC) of the BC and BCA membranes as a function of alginate content (% w/v) in culture medium.

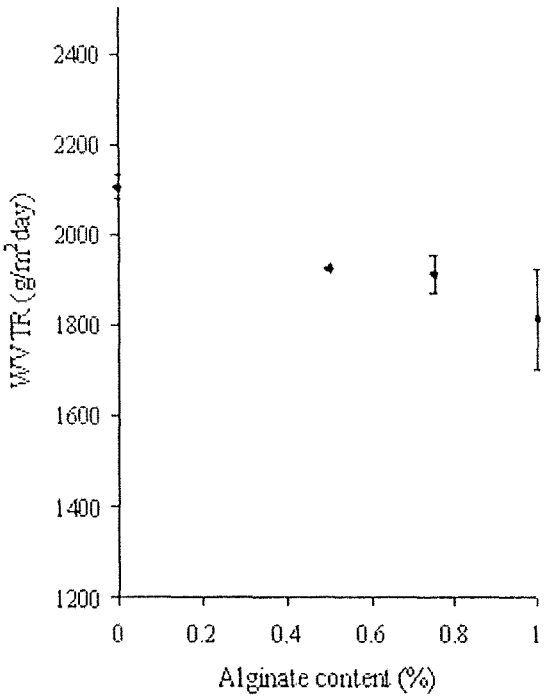

Figure 11 The water vapor transmission rate (WVTR) of the BC and BCA membranes as a function of alginate content (% w/v) in culture medium.

previous observation in the alginate–bacteria cellulose blend membrane, the apparent pore size of the blend membrane to some extent also decreased with the increase in the alginate content.[17]

Water absorption capacity

From Figure 10, the WAC of the BC and the 1.0% Al-BCA membranes were 542% and 706%, respectively. The WAC increased with the alginate content. Alginate is very hydrophilic; the water molecule is easily absorbed into the alginate membrane.[14] As alginate was incorporated into the BCA membrane, the film was more hydrophilic. Therefore, the BCA film was enabled to adsorb and retain more water than the BC film.

Water vapor permeability test (WVTR)

The BC and BCA films are highly hydrophilic. During the WVTR analysis, a feed of water vapor (90% relative humidity) could make the membranes in a swollen state. The solubilization of water into the membrane has a strong influence on the morphology and porous structure of the membrane. A major change of porosity and pore size in form of the reswollen film was previously demonstrated (Figs. 1–4). Figure 11 demonstrates the water vapor permeability of the films at an average thickness of 40 μm. The water vapor transmission rate (WVTR) slightly decreased when increasing alginate content. Although the hydrophilic property and the WAC of the BCA membranes were improved, as a result of the denser structure and decrease in pore size from the supplement of alginate, the WVTR decreased relatively. In comparison with the BC film, the reduction in the WVTR of the 0.5, 0.75, and 1.0% Al-BCA membranes were at 8.5%, 9.1%, and 13.8 %, respectively. Compared with the WVTR of the BC membrane, the supplement of alginate (0.5–1.0%) causes a statistically significant decrease in WVTR calculated using a one-tailed *t*-test for a 95% confidence limit ($P < 0.05$).

Oxygen permeability test (OTR)

Figure 12 shows the oxygen transmission rate (OTR) of the membranes at an average thickness of 40 μm. As alginate content increased, the OTR decreased considerably due to the denser structure and smaller pore size of the film. It was noted that the effect of alginate supplement on the reduction in the OTR of the BCA membranes was much greater than that in the WVTR. The alginate supplement at 0.75 and 1.0 % (w/v) in culture medium caused a remarkable decline in the OTR to 101 and 57 cc/m^2 day or 0.28 and 0.16% of those of the BC membrane, respectively, however, only minor influence on WVTR was observed. The approximate diameter of water molecule is 0.28 nm,[21] which is only slightly smaller than that of oxygen molecule (0.36 nm).[22] Nonetheless, the decrease in pore size of the BCA film did not have a strong effect on WVTR results. This could

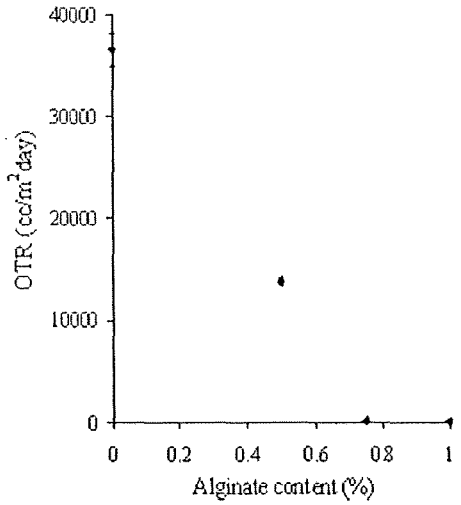

Figure 12 The oxygen transmission rate (OTR) of the BC and BCA membranes as a function of alginate content (% w/v) in culture medium.

possibly be explained from the hydrophilic characteristics of the films. The BC and BCA films could be swollen by water vapor contained in a feed gas. Solubilization of water into the films enlarged pore diameter and caused a looser fibrous structure of the films. Consequently, it exhibited high-water vapor permeability. This phenomenon has been reported in the other hydrophilic membranes.[23] On the other hand, the OTR analysis was preformed at a relative humidity of 0%; therefore, the fibrous structure during the test should be tighter and less porosity. As a result, the presence of alginate-filled pores caused a considerable decrease in oxygen permeability. It was found that for the BC membrane, the WVTR was less than 1/15 of the OTR but for the 1.0% Al-BCA membrane, the WVTR was more than 30 times the OTR. The results revealed that at 0% relative humidity, the oxygen transfer resistance of the BCA film could be significantly increased by adding sodium alginate into the culture medium during biosynthesis.

CONCLUSIONS

The bacterial cellulose membrane was modified by means of 0.5–1.0% (w/v) alginate supplements in culture medium during its biosynthesis by *Acetobacter xylinum* under static conditions. The cellulose–alginate composite was produced in the form of pellicle that floats on the culture medium surface. The bulk membrane was composed of thin films united together. With the supplement of alginate in the culture medium, the alginate was incorporated into the cellulose fibril network and filled the pores. The presence of alginate-filled pores resulted in the significant reduction of the membrane pore size, denser structure, and improved hydrophilicity. The results from the SEM micrograph and BET revealed that the average pore size significantly decreased when increasing alginate content. The 1% Al-BCA membrane showed 30% higher water absorption and slight reduction in WVTR. The significant decrease of the mechanical strengths dependent on the degree of alginate supplement was observed. The remarkable reduction of OTR of the 0.75–1.0% Al-BCA membranes to 57–101 cc/m^2 day or 0.28%–0.16% of the BC film was achieved. These modified characteristics make the BCA membrane a good candidate for the applications in membrane separation. The further investigation of the BCA membrane for use in pervaporation is ongoing.

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Continuous ethanol production using immobilized yeast cells entrapped in loofa reinforced alginate carriers

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Abstract

Culture of *Saccharomyces cerevisiae* M30 entrapped in loofa reinforced alginate was used for continuous ethanol fermentation in a packed bed reactor with initial sugar concentration of 200 to 248 g/L. The maximum ethanol productivity was obtained at 11.5 g/(L·h) with ethanol concentration of 57.4 g/L at 220 g/L of initial sugar concentration and a dilution rate (D) of 0.2 h^{-1}, whereas, the maximum of ethanol concentration of 82.1 g/L (productivity of 9.0 g/(L·h)) was obtained at a D of 0.11 h^{-1}. The ethanol productivity in the continuous culture was 6-8 fold higher than that of the batch culture. Due to the high biocompatibility, high porosity and good mechanical strength of the developed carrier, advantages such as cell regeneration, reusability,

altered mechanical strength and high capacity to trap active cells in the reactor were achieved in this study. The immobilized cell reactor was successfully operated over a period of 30 days, without loss in ethanol productivity. The average conversion yield was 0.43-0.45 throughout the entire operation with an immobilization yield of 47.5%. The final total cell concentration in the reactor was 37.3 g/L (17.7 g/L immobilized cells and 19.6 g/L suspended cells). The concentration of the suspended cells in the effluent was 0.8 g/L.

Keywords: ethanol; loofa; alginate; immobilization; continuous

Introduction

The demand for ethanol has continued to grow as an alternative fuel and a fuel extender for petroleum fuel due to gasoline shortages. Production of ethanol from renewable carbohydrate materials has been attracting worldwide interest and much research has focused on the production of ethanol by immobilized viable microbial cells using continuous systems. Continuous fermentation using immobilized cell (IC) carriers offers many advantages such as higher productivity, relative ease of product separation, reuse of biocatalyst and high productivity.

Fermentative ethanol production by *S. cerevisiae* immobilized within alginate beads was found to have a higher productivity than a batch system (4). However, some limitations such as gel degradation, low physical strength, and severe mass transfer restrictions were often observed in the use of alginate-based carriers. Despite these shortcomings, the loofa sponge was demonstrated to be an excellent cell carrier for

ethanol fermentation by flocculating the cells in a bubble column with external loop for recirculation of the fermentation broth (7). Its strength, abundance, low price, biodegradability, and natural origin have become the main sources of interest. However, a low shear environment and a large aggregate of cells were required in the application of the loofa sponge in order to prevent excessive cell sloughing from the carrier (5, 6). In our previous study, immobilized yeast cells entrapped in loofa reinforced alginate (ALM) carriers were successful developed for repeated batch ethanol fermentations in a 500-mL shake flask system (9). The carriers were simply fabricated by the gelation of the peripheral loofa sponge which was previously dipped in alginate-cell mixture. The ALM with a size of 9×9×3 mm^3 was found to be effective for yeast immobilization, which was comparable to a 2-mm-diameter alginate bead. Moreover, after storage for 4 months, the ALM-immobilized cell culture was still active, and the stability of IC cultures in the ALM was higher than that of the suspended culture (9). The ALM structure was proved to be more porous and less dense than a typical alginate bead allowing for better internal mass transfer diffusion. The aim of present investigation is to apply the ALM carrier with a size of 20×20×3 mm^3 for continuous ethanol fermentation in a packed bed reactor (PBR) and also evaluate the performance for long-term operation.

Materials and methods

Microorganism and media

Saccharomyces cerevisiae M30 was selected for this study on the basis of its high efficiency in ethanol production from molasses at high temperature. Starter

cultures were prepared by transferring cells from stock PDA slants to 150 ml of sterilized medium followed by incubation at 33°C, 150 rpm for 20 h. The medium for the starter culture contained 0.05% ammonium sulfate and 5% inverse sugar from palm sugar at pH 5.0. Subsequently, the obtained cell suspension was concentrated by decantation and then transferred to the main culture.

Cells immobilization

Sodium alginate (3% w/v) solution was formulated by dissolving Na-alginate powder in 0.9% (w/v) NaCl solution. It was autoclaved for 5 min at 121°C and kept overnight at 4°C to facilitate deaeration. Cell suspension of 5 ml was then added to 50 ml of 3% (w/v) alginate solution to form an alginate-cell mixture. Loofa sponges were cut into small thin square pieces of 19 x 19 x 2 mm^3 using scissors. To form alginate-loofa matrices (ALM), 2 g of sterilized thin square sponges of loofa was dipped into the alginate-cell mixture. The gel carriers were transferred to 1.5 % (w/v) $CaCl_2$ solution and left to harden in this solution with mild stirring for 15 min. The carriers were then rinsed 3 times with 0.9% (w/v) NaCl solution. Carriers were prepared under aseptic conditions and the average size of ALM was 20 x 20 x 3 mm^3.

Fermentations

A 1-L (Ø= 5.7 cm; height= 43.4 cm) reactor column containing immobilized cell bed of ALM carriers was used for the study. The experimental set-up for the packed bed reactor (PBR) with a packed volume of 32 % (v/v) of the total bed volume is shown

in Fig.1. 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 200, 220 and 240 g/L were continuously fed into the bottom of the reactor with dilution rates of 0.11, 0.16, 0.20 and 0.30 h^{-1}, respectively. Sampling was done regularly with volume of 5 ml every 8 hours. The samples were frozen before determining the sugar, ethanol, and cell concentrations in order to enable all samples to be analyzed at the same time.

Analytical methods

Free cell dry weight was determined from the absorbance at 660 nm with a UV-2450 UV-visible spectrophotometer and converted to dry cell concentration on the basis of a corresponding standard curve. For immobilized cells, a known mass of cell carriers was dissolved in 0.05 M sodium citrate. After the sponge was removed, immobilized cell concentrations were determined similarly as for the free cells. Yeast cell viability was determined by the use of methylene blue staining procedure (10). The concentration of ethanol was determined by gas chromatography (model GC-7AG; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. To measure reducing sugar concentration, the sample solution was hydrolyzed in 33% HCl at 100°C for 10 min and neutralized with NaOH solution. Reducing sugar content was then determined by the dinitrosalicylic acid method (11).

Results

Batch fermentation

The purpose of this work was to expand upon our previous work (9) in an attempt to develop an efficient continuous process for ethanol production from sugar-cane molasses by using immobilized *S. cerevisiae* M.30 culture. The loofa reinforced alginate (ALM) was chosen based on its high potential as a cell carrier. The ALM carrier has many advantages including high regeneration ability, reusability, altered mechanical strength and high ethanol productivity. Inspection of the cross-section of the ALM carrier in Fig. 2 exposes the carrier's structure as being composed of loofa fiber, a hollow space between the loofa fiber-alginate gel and a layer of alginate gel. The hollow section between the alginate and loofa fiber was found to be an ideal space for cell growth (9). Fig.3 reveals that yeasts can also grow well in the alginate layer and in the hollow space of the core fiber. The cells were of normal oval shape as confirmed by microscopic images and filaments connecting cells to cells and to cellulose fibers were observed. This filamentous structure should promote both firm attachments of the cells to the carrier and cell aggregations.

For more convenient preparation, the ALM carrier used in the packed bed reactor was modified in the square form of 20 x 20 x 3 mm^3. The pre-examination was performed in 500 ml Erlenmeyer flasks containing 250 sterilized medium under the following controlled conditions: 220 g/L initial sugar concentration, initial pH of 5.0, 150 rpm and 33°C. The ethanol production, growth rate and immobilized yield (Y_I) using the square forms of 20 x 20 x 3 mm^3 and 9 x 9 x 3 mm^3 were comparable (Fig. 4). Ethanol concentration at 89-90 g/L was produced within 60 hours of the batch fermentation, whereas the immobilized cell and free cell concentrations were about 4.0

g/L and 1.0 g/L, respectively. No significant differences in data obtained from the ALM carriers of the two different sizes.

Continuous fermentation

Effect of dilution rate and initial sugar concentration

Continuous ethanol fermentations using cane-molasses in a 1-L packed-bed reactor were performed under the following conditions: temperature 32 ± 1°C, initial pH of 5.0. The initial reducing sugar concentrations were 202, 222 and 248 g/L under various dilution rates from 0.11-0.30 h^{-1}. The effects of dilution rate on ethanol concentration and ethanol productivity are presented in Fig. 5(A) and Fig.5 (B), respectively. The ethanol concentration decreased as the dilution rate was increased, as commonly observed. With the initial sugar concentration (S_0) at 200–220 g/L, the ethanol productivity increased linearly with the dilution rate, from 0.1 to 0.2 h^{-1} and then remained nearly constant. However, at the very high initial sugar concentration of 248 g/L, the ethanol productivity was limited at 8.0 g/(L·h). The optimal S_0 for ethanol productivity was 220 g/L. Under steady state conditions and at varying dilution rates of 0.11, 0.16, 0.20 and 0.30 h^{-1}, the average ethanol concentrations in the effluent were 82.1, 66.1, 57.4 and 37.2 g/L, respectively, corresponding to the ethanol productivity (P_E) of 9.0, 10.6, 11.5 and 11.2 g/(L·h), respectively. The ethanol conversion yield ($Y_{E/S}$) was almost constant at 0.45±0.02. The optimal ethanol productivity at 11.5 g/(L·h) was obtained with the ethanol concentration of 57.4 g/L at the initial sugar concentration of 220 g/L and the dilution rate (D) of 0.2 h^{-1}, whereas the maximum of ethanol concentration of 82.1 g/L (P_E = 9.0 g/(L·h) was obtained at D of 0.11 h^{-1}. At the end of

the operation (360 h), the concentrations of immobilized cells, suspended cells in the reactor and suspended cells in the effluence were 16.0±0.4, 12.3±0.5 and 0.6±0.1 g/L, respectively with an average immobilized yield of 56.5%.

Effect of superficial velocity

The design of packing materials to achieve ideal conditions corresponding to a plug flow and uniform distribution of the fluid across the cross-section throughout the column is important for a packed bed reactor. The results of ethanol and reducing sugar concentrations examined from the five sampling ports on both sides of the packed bed reactor after the system reached steady state revealed satisfactory uniform distribution of the fluid. The ethanol concentration profiles in a plug flow reactor packed with immobilized cells entrapped in ALM are shown in Fig.6. Usually for anaerobic ethanol fermentation with high substrate concentrations, it is likely that the external mass transfer is not limiting, at least for a large portion of the packed bed (12). External mass transfer coefficient can be enhanced by an increase in the liquid superficial velocity. In this study, the effect of liquid superficial velocity was examined. Liquid superficial velocity (V_S, cm/h)) was calculated from: $V_S = \frac{Q}{\varphi \ A}$

Where Q is the volumetric flow rate of the fluid (cm^3/h), A is the cross-sectional area of the bed (cm^2) and φ is the packed bed porosity (the volume of voids per volume of reactor).

From the results, the effects of superficial velocity on ethanol concentration profiles can be divided into two groups, one with S_0 of 200-220 g/L and the other with S_0 of 248 g/L. Fig.6 (A) and Fig. 6(B) represent the results with S_0 at 200 and 220 g/L,

respectively. The results indicated the increase of ethanol concentration as the liquid superficial velocity was increased from 4.8 cm/h to 6.9 cm/h. However, no significant enhancement in the ethanol production was observed when the liquid superficial velocity was further increased to 8.7-13.0 cm/h. According to the result with S_0 at 200-220 g/L, the external mass transfer resistance showed some negative effects on the overall fermentation rate of the packed bed reactor when the liquid velocity creeping over the static solid particles was less than about 7 cm/h. However, at high initial sugar concentration at S_0 of 248 g/L, a decrease in sugar consumption and ethanol production as the liquid superficial velocity increased was observed (Fig. 6(C)). The inhibition effect of high initial sugar and high ethanol concentrations has been previously reported (8). Therefore, the mass transfer resistance showed a positive effect on ethanol fermentation under very high sugar concentration owing to the reduction of the sugar-induced inhibition.

Stability test

The long-term stability test of the immobilized yeast cells entrapped in loofa reinforced alginate carriers in continuous ethanol fermentation was examined using the initial sugar concentration of 220 g/L at a constant dilution rate of 0.11 h^{-1}. After 30 days of operation, the degradation of alginate films due to cell growth and production of CO_2 was observed resulting in cell leakage. Such leaks could also be observed from the concentrations of suspended cells in the reactor and in the effluent. Fig. 7 (A) and Fig. 7 (B) show the images of the suspended yeasts in the reactor and in the effluent, respectively. The suspended cells in the reactor appeared healthy and retained their

normal oval shape. In addition, the aggregation of yeast cells with filament connecting was observed. These cell filaments were hardly observed in alginate gel layer. Therefore, it is possible that the formation of these filaments would be activated by the cellulose fibers of the loofa sponge. It was found that almost all of the aggregated cells were trapped in the packed bed. On the other hand, the suspended cells in the effluent were thin and much smaller with no cell aggregation. From the dead/alive cell discrimination system using a hemacytometer based on methylene blue, almost all of the cells (>95%) in the reactor were alive but more than 70% of cells in the effluent were dead.

The high performance of the immobilized cells in the alginate-loofa cube for continuous ethanol fermentation in the packed bed reactor was confirmed by satisfactory operational stability during the 30-day fermentation at the dilution rate of 0.11 h^{-1}. There was no significant decline in the productivity during the continuous operation and the average ethanol productivity was achieved at 8.7 g/ (L·h) with an average ethanol concentration of 79.3 g/L. The average conversion yield was 0.43-0.45 throughout the entire operation with an immobilization yield of 47.5%. The final total cell concentration in the reactor was 37.3 g/L (17.7 g/L immobilized cells and 19.6 g/L suspended cells). The concentration of the suspended cells in the effluent was 0.8 g/L.

Discussion

The data presented in this work demonstrates that the continuous ethanol production from molasses using immobilized cell of *S. cerevisiae* M30 entrapped in loofa reinforced alginate (20 x 20 x 3 mm^3) is promising. Compared to the batch fermentation, higher ethanol productivity (6-8 folds) was obtained with the continuous

fermentation in the packed-bed reactor. The maximum productivity of 11.5 g/L h with an ethanol concentration of 57.4 g/L was obtained by using a 220 g/L initial sugar concentration at a 0.20 h^{-1} dilution rate, whereas the maximum ethanol concentration at 82.1 g/L was obtained at a 0.11 h^{-1} dilution rate. The steady state ethanol concentration in the effluent of the packed-bed column obtained from this work was reasonably high in comparison with those of previous reports (1-4, 13 and 14). The experimental results revealed that the alginate-loofa matrix was suitable for yeast immobilization in a continuous packed bed reactor. With the favorable mechanical properties, high biocompatibility and superior porous structure of the ALM, the result is a system with a high cell density, high ethanol production and high stability. Furthermore, the ALM should also be applied as a cell carrier for efficient production in other fermentation systems.

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Acknowledgements

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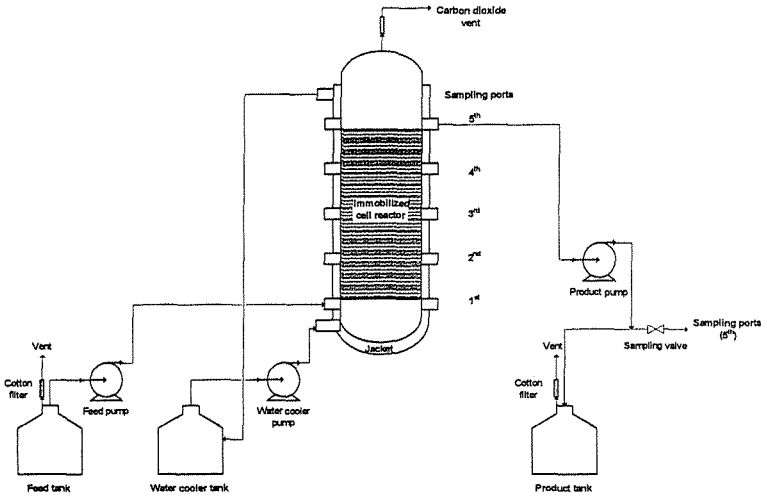

Fig. 1. Schematic diagram of immobilized cell packed bed reactor.

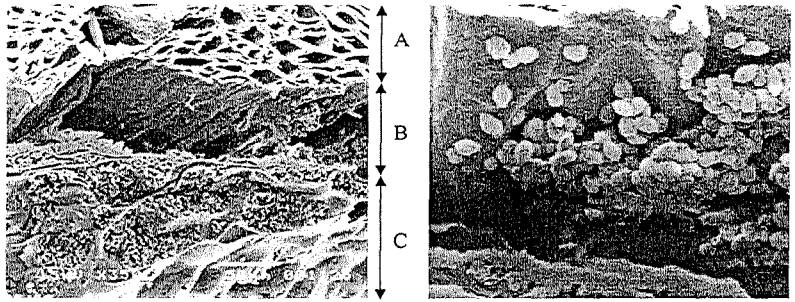

Fig. 2. Cross-section of the ALM (after 72 h of ethanol fermentation) consisting of loofa fiber (A), hollow space between loofa fiber and alginate gel (B) and alginate gel (C) (left) and a closer look at the hollow space (right).

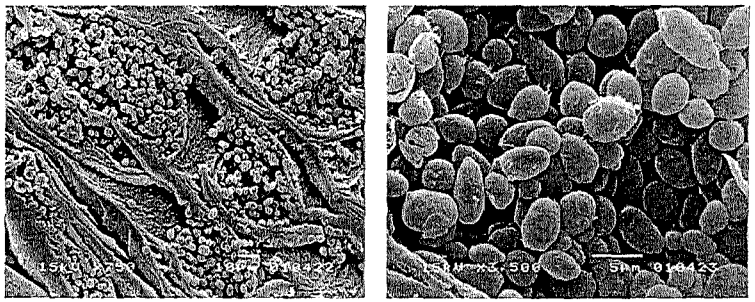

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Fig. 3. Yeast cells in alginate gel layer (top) and in a hollow core fiber (bottom) of the ALM after 72 h of ethanol fermentation.

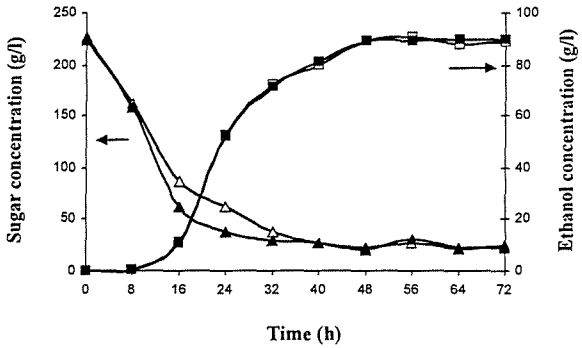


Fig. 4. Batch fermentations using the alginate-loofa (ALM) carriers of 9 x 9 x 3 mm^3 (open symbols) and 20 x 20 x 3 mm^3 (filled symbols); Δ,▲= sugar: □,■= ethanol.

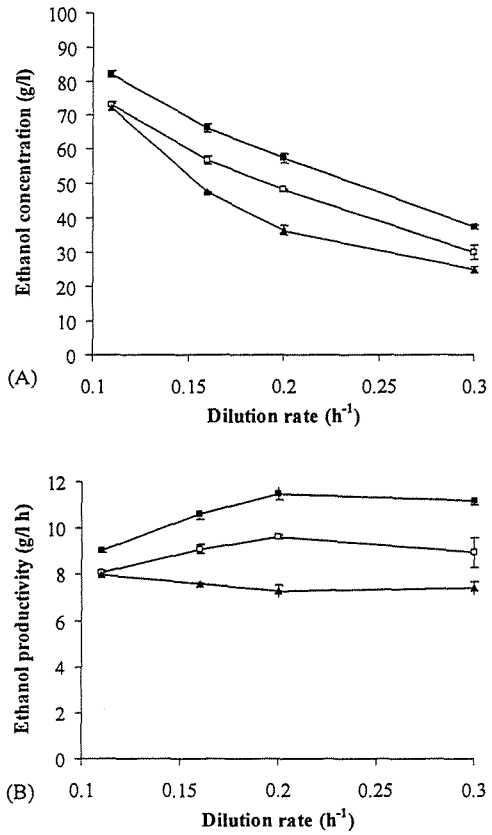


Fig. 5. Ethanol concentration (A) and productivity (B) at dilution rates of 0.1-0.3 h^{-1} for initial sugar concentration of 200 g/L (□), 220 g/L (■), and 248 g/L (▲).

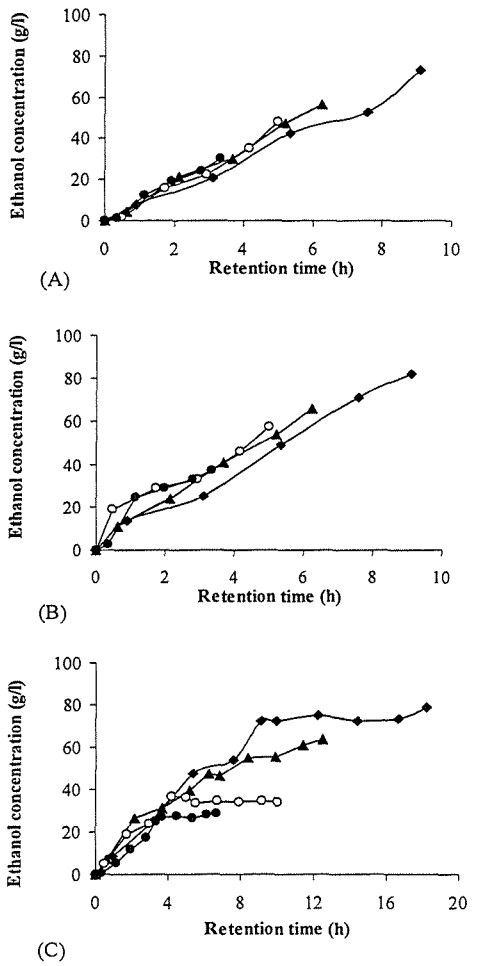
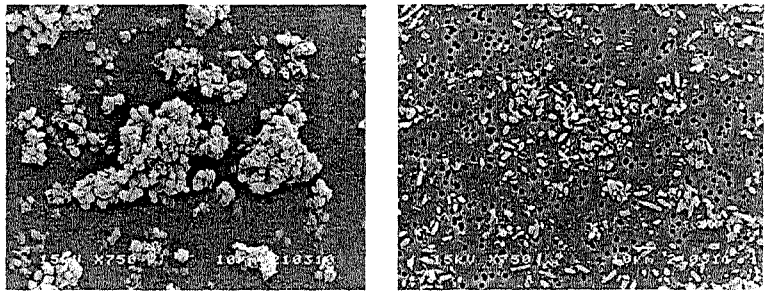


Fig. 6. The steady state ethanol concentration profiles with initial sugar concentration of 200 g/L (A), 220 g/L (B), and 248 g/L (C): at superficial velocities of 4.8 cm/h (♦), 6.9 cm/h (▲), 8.7 cm/h (○), and 13.0 m/h (●).



(A) X 750 (B) X 750

Fig. 7. Suspended cells in the packed bed reactor (A) and in the effluent (B) (after 30 days of continuous fermentation).

TITLE:

Alumina Oxide Doped Alginate Gel as a Cell Carrier for Ethanol Production

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Abstract

Background: Alginate is one of the most wildly used materials for cell entrapment because of its simplicity and non-toxicity. However, this technique has received less attention in the fermentation industry because of the diffusion limitations of nutrients and oxygen due to the dense structure of the gel beads and the problems in stability of the gel in long term operation. In the current work, Alumina oxide doped alginate gel (AEC) was developed as a new type of the cell carriers for ethanol fermentation.

Results: It was shown that the attachment of alumina oxide particles and yeast cells by electrostatic attraction could promote cell growth as well as ethanol productivity. The AEC carrier was found to be more effective for the immobilization of *Saccharomyces cerevisiae* M30 than that of the conventional calcium alginate bead. The ethanol productivities of 1.4 and 7.9–12.6 g/(L·h) were obtained by using the AEC cultures in batch and continuous modes of operation, respectively with the production yield ($Y_{P/S}$) of 0.44-0.47 and the immobilized yield (Y_I) of 80-85 %. The ethanol fermentation in a continuous packed-bed reactor using the AEC carrier worked efficiently and was stable over 30 days.

Conclusion: The presence of alumina oxide particles in alginate gel not only improved the porous structure of the carrier, but also provided many advantage characteristics such as, good mechanical strength, high stability and high immobilization yield.

Background

The recent petroleum crisis has increased the worldwide interest in green energy production from sustainable resources. Using renewable energy such as ethanol is expected to become a major contribution to the reduction of the net CO_2 emissions. Low-cost agricultural by-products or wastes can be, as well, turned into fuel which reduces the reliance on fossil fuel. Sugar cane molasses, a by-product from sugar industries is an abundant and low cost material in Thailand. It can be fermented by yeast to produce ethanol under anaerobic condition.

The ethanol production by a conventional batch fermentation suffers from various constrains such as low cell density, nutritional limitation and rather time consuming. An important characteristic of continuous fermentation with immobilized cells is high volumetric efficiency, which is usually obtained by increased yeast cell concentration in the reactor compared to traditional batch systems. The use of immobilized cells could also protect cells against toxic substances and eliminate costly processes of cell recovery and cell recycles [1-6]. Alginate is one of the most wildly used materials for cell entrapment because of its simplicity and non-toxicity [4]. Although high biomass loadings can be obtained by immobilization of *S. cerevisiae* cells using gel entrapment with various metal alginates, this technique has received less attention in the fermentation industry because of several drawbacks. One drawback is the diffusion limitations of nutrients and oxygen due to the dense structure of the gel beads and the high cell densities in the gel beads [3, 4]. Practical application of alginate beads in continuous culture has been limited by the problems in stability of the gel in long term operation.

The positive effect of Al_2O_3 on cell growth and ethanol fermentation was previously reported [7]. *S. cerevisiae* cells can attach to alumina oxide particles by electrostatic attraction in a wide pH range of 3.0-6.5 [8]. In the present study,

incorporation of alumina oxide (Al_2O_3) into alginate gel matrix to improve the mechanical strength and porosity of cell carrier has been studied. The cell carrier of alumina oxide doped alginate gel (AEC) was performed by the adsorption of yeast cells on alumina particles before the entrapment of alumina-cells within calcium alginate gel. The activities of the immobilized cells by the AEC carrier were compared to those of the suspended cells (SC) and the typical immobilized cells entrapped within calcium alginate gel (EC). The effects of bead diameter, alumina and alginate concentrations on ethanol fermentation were examined and the physical characteristics of the AEC carrier such as, morphology, porosity and mechanical properties were investigated. Afterward, the experimental studies were carried out in a vertical packed bed reactor for continuous ethanol fermentation.

Methods

Yeast strains, culture media and cell preparation

Saccharomyces cerevisiae M30 was selected for this study on the basis of its high efficiency in ethanol production from molasses. It was kindly provided by the laboratory of Dr. Savitree Limtong (Department of Microbiology, Kasetsart University, Bangkok, Thailand). 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 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 at 5.0. The cell cultivation was carried out in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm, 33°C for about 20 h. The late exponential phase cells were harvested and used as the stock cell suspension.

Cells immobilization

The cell carrier, alumina doped alginate gel (AEC) was prepared by a two-step method. The first step was the adsorption of cells onto alumina oxide particles (commercial grade, purchased from S.R. LAB Co., Ltd., Bangkok, Thailand). The second step was the entrapment of the alumina-cells in Ca-alginate matrix. Sodium alginate was purchased from Carlo Erba Reagenti (Rodano-MI, Italy) in form of flakes (molecular weight (MW) ≥200,000 g; bulk density = 0.5-0.6 kg/m^3). The cell inoculum of 5% (v/v) was transferred into the pre-culture medium supplemented with alumina oxide powders and was incubated at 33°C for 20 h to induce natural cell adhesion. The alumina-cell mixture was then added in a ratio of 1:1 (v/v) to Na-alginate solution. The mixture was cross-linked by dropping wisely by using an auto-pipette into sterile 0.12 M $CaCl_2$ solution. The cell carrier beads were left to harden in the $CaCl_2$ solution for 30 minutes and then washed with sterile 0.9% w/v NaCl solution to remove excess Ca^{2+} and untrapped cells.

Fermentations

The fermentation medium contained 0.05% w/v $(NH_4)_2SO_4$ and 220 g/L reducing sugar from sugar cane molasses with the initial pH of 5.0. It was sterilized at 121°C for 15 min. Experiments were initiated by transferring the prepared cell suspension or immobilized cells into 500 ml Erlenmeyer flasks containing 250 ml of the fermentation medium. The batch fermentation experiments were performed in duplicate in the incubator shaker at 150 rpm, 33°C for 72 h. The effects of bead diameter, alumina oxide and Na-alginate concentrations on the ethanol fermentation were investigated. A suitable condition from the batch system was, then, applied to the continuous fermentation in a 1-

L (Ø= 5.7 cm; height= 43.4 cm) packed bed column. The experimental set-up for the packed bed reactor (PBR) is shown in Figure 1. The temperature of the PBR was controlled at 32 ± 1 °C by passing cooling water inside the reactor jacket. For good liquid flow distribution, the bed was divided into 9 stages by thin trays packed with porous plastic materials made from polyvinyl acetate (PVA). A magnetic bar was placed in the bottom of the reactor for feed mixing. Fermentation performance of the system was expressed as follows:

Immobilization yield (Y_I, %) : $$Y_I = \frac{X_I}{X_T} \times 100$$

Yield of ethanol production ($Y_{P/S}$, %): $$Y_{P/S} = \frac{P_F - P_0}{S_0 - S_F} \times 100$$

Ethanol productivity (Q_P, g/(L·h)): $$Q_P = \frac{P_F}{fermentation\ time} = P_F \times D$$

Analytical methods

Ethanol concentration was determined using gas chromatography (GC-7AG, Shimadzu, Japan). To measure total reducing sugar concentration, the sample solution was hydrolyzed in 33% HCl at 100 °C for 10 min and neutralized with NaOH solution. Reducing sugar content was then determined by the 3,5-dinitrosalicylic acid (DNS) method. Free cell dry weight was determined from the absorbance at 660 nm with a UV-2450 UV-visible spectrophotometer (Shimadzu Scientific Instruments, Inc., Japan) and converted to dry cell concentration on the basis of a corresponding standard curve. For immobilized cells, a known mass of cell carriers was dissolved in 0.05 M sodium citrate. Afterward, the immobilized cell concentrations were determined similarly as for the free cells.

Characterization

Scanning Electron Microscope (SEM)

The immobilized cell carriers in fermentation broth were dehydrated in ascending grades of ethanol and dried in a Tousimis Samdri-780 critical point dryer (Maryland, USA) using liquid carbon dioxide as transitional fluid. The dried samples were sputtered with gold in a Balzers-SCD 040 sputter coater (Balzers, Liechtenstein). SEM images were taken on a JOEL JSM-5410LV (Tokyo, Japan) scanning electron microscope.

Tensile property testing

The tensile strength and elongation at break of the dry carrier were measured by Instron Testing Instron (5567, NY, USA). The test conditions follow ASTM D882. The tensile strength and break strain were the average values determined from five specimens.

Brunauer-Emmett-Teller (BET) surface analysis

The pore size and surface area of the carrier were determined with a BET surface area analyzer. To remove moisture from the film samples, the samples were placed in sample cells, which were then heated up to 348 K for 12 h and cooled down to room temperature before the BET analysis. The BET pore size and surface area were determined with N2 adsorption at 77 K in a Micromeritics (Atlanta, GA) ASAP 2020.The information on the porous structure was evaluated through the nitrogen adsorption isotherms using BET (Brunauer-Emmett-Teller) and BJH (Barrett-Joiner-Halenda) methods (model ASAP2000, Micrometrics Corp., Atlanta, G.A, USA).

Results and Discussion

Batch fermentation

The results of the batch fermentations using the AEC culture were compared to those using the culture of cells entrapped in calcium alginate (EC) and the suspended cell culture (SC) as shown in Figure 2 and Table 1. In the preparation of the immobilized cells, 1.5% (w/v) of Na-alginate was used for the EC carrier; 1.5% (w/v) of Na-alginate with 5% (w/v) of alumina oxide was used for the AEC carrier. The effect of bead diameter was studied at bead diameters (Ø) of 2, 4 and 6 mm, which were prepared by dropping the mixture wisely through different sizes of auto pipette tips into sterile 0.12 M $CaCl_2$ solution.

As shown in Figure 2, the ethanol concentration profiles of the immobilized cell systems (EC and AEC) were initially lower than that of the SC system for duration of 24 h. This is due to the effect of mass transfer restrictions of the immobilized supports. The final ethanol concentration of the SC system was relatively lower than those obtained from the immobilized systems using Ø 2-4 mm beads. This might be a result of the negative effect of high ethanol concentration on cell activity and viability in the SC system. The ability of cells to grow in an immobilized cell carrier made it possible for cell regeneration and product formation under hostile conditions such as high ethanol concentration [9]. It has been previously suggested that the matrix of immobilized cell carriers protected cells by fortification from toxins and inhibitors [3, 4]. Osmotic stress caused by the immobilization techniques was found to lead to an intracellular production of pressure regulating compounds, which lead to decreased water activity and consequently higher tolerance to toxic compounds [10]. Immobilization of yeast using Ca-alginate bead showed lower substrate inhibition and higher tolerance to ethanol [11].

The structure and size of cell carrier are important factors for the cell activities. The highest ethanol concentration of 91.4 g/L was obtained from the AEC system using the smallest diameter bead (Ø 2 mm). Higher ethanol production from smaller diameter beads was previously reported [2, 12-13]. Larger beads resulted in less surface area available for the mass transfer of substrate into the beads and the increase of diffusion path length. In the AEC carrier system, the increase in bead diameter (Ø) from 2 mm to 4 and 6 mm resulted in slight decreases about 1.0% and 7.6% in ethanol concentration, respectively. Decreases at 1.0% and 8.8% were observed in the EC carrier system. The immobilization yield (Y_I) of the AEC remained nearly constant at 81.4-82.9 %, while the Y_I of the EC dropped from 81.4 % to 76.5% with the increase of the diameter from 2 to 6 mm. The decrease in ethanol production and immobilization yield (Y_I) could be due to the decline of cell activities inside the carriers. The difficulty of access of the nutrients to inner portion of the carrier could become the limiting factor for cell activities, especially for the carrier with the dense structure. Overall, the maximal ethanol concentrations using the AEC culture were higher than those of the EC culture. The results indicated that the AEC carrier was more effective for the immobilization of S. *cerevisiae* M30 than the EC carrier. The supplement of alumina oxide in Ca-alginate bead could promote the growth and activity of *S. cerevisiae* M30. γ-Alumina oxide has been previously reported for positive effect on growth and ethanol efficiency of yeast cells [7, 8].

Characteristics

Morphology

A series of SEM images were taken to provide a visual description and information about the immobilized cell systems. Figure 3 presents the morphology of *S. cerevisiae* M30, alumina oxide particles, the adhesion of *S. cerevisiae* to alumina oxide

particles and the cells in alumina oxide doped alginate gel (AEC). The yeast cells had a round or oval shape with 3-6 μm in diameter (Figure 3 (A). The crystallite size of alumina oxide particles was 1-5 μm (Figure 3 (B)). *S. cerevisiae* M30 could attach to alumina oxide particles in the form of a three-dimensional (3-D) porous network structure as shown in Figure 3 (C) owing to the electrostatic attraction between alumina particles and yeast cells. The cross sectional view of the AEC in Figure 3(D) shows that yeast cells were well distributed throughout the carrier. This indicated that the cells could gain enough substrate for growth even though they were located deep inside the AEC carrier.

Porosity

The structure parameters such as pore diameter, surface area, porosity, and pore volume of the AEC carrier at the initial stage were investigated and compared to those of the EC carrier (Table 2 and Figure 4). It was found that the BET specific surface area, pore volume and porosity of the AEC carrier were 2.0, 1.8 and 1.7 times the values in those of the EC carrier, respectively. The result indicated that the AEC carrier exhibited superior pore structure than that of the EC carrier. Since the pore volume of alumina oxide particles was very low, the increase of the surface area and porosity of the AEC carrier should be generated from the micro porous structure of the alumina-cell network within the carrier. Better porosity of the AEC carrier could improve intra-particle mass transfer of the substrates and products.

Mechanical strength

The mechanical properties of AEC and EC carriers were examined in terms of tensile strength and percentage elongation at break. Tensile strength measures material strength, whereas elongation at break is an indicator of toughness and stretch-ability prior

to breakage. These parameters dictate the end-use handling properties and mechanical performance of the materials. The mechanical properties of the AEC and the EC carriers are shown in Figure 5. The tensile strength and elongation at break of the AEC carrier were 0.24 MPa and 80.8%, respectively, which were significantly higher than those of the EC carrier (0.17 MPa and 57.1%, respectively). Greater strength and flexibility of the AEC carrier were due to the presence of alumina oxide particles in alginate gel. The incorporation of small particles in gel could support and hold the components strongly together. The AEC carrier has approximately 1.4 times the tensile strength and elongation at break of the typical alginate bead (EC).

Effect of alginate and alumina oxide concentration

Cell growth in the porous matrix depends upon diffusion limitations imposed by the porosity of the material and later by the impact of accumulating biomass [3]. It was reported that for yeast cell entrapment with alginate gel, ethanol production decreased with increasing alginate concentration [14]. In this study, the effects of alginate and alumina oxide concentrations for the formation of the AEC carrier for ethanol fermentation were investigated.

The result of 72 h batch fermentation by the immobilized yeast cells in 6 mm AEC beads using different concentrations of Na-alginate is shown in Figure 6 (A). The AEC bead prepared from the solution of low Na-alginate concentration at 1.5% (w/v) was very soft and easily broken. With the use of higher Na-alginate concentrations at 2.0-3.0% w/v, the beads became more stable and the immobilized yield was increased. The decrease in ethanol concentration was observed when using the AEC carriers prepared from 3.0% (w/v) Na-alginate solution, possibly due to the mass transfer limitation of the dense gel beads. It was found that the suitable Na-alginate concentration for the AEC

formation was at 2.5% (w/v). For the conventional alginate bead (EC), the optimal alginate concentration that gave maximum ethanol production was around 2.0% (w/v) [2, 13, 15]. Göksungur et al. [2] reported that the suitable condition for the bead formation was at the diameter of 2.0-2.4 mm using 2.0% (w/v) Na-alginate solution. When the bead diameter and Na-alginate concentration were increased, ethanol production was decreased due to the diffusion limitation problem.

The concentration of alumina oxide in the gel bead also had an influence on the cell activities and immobilized yield as shown in Figure 6 (B). The maximum ethanol concentration and immobilized yield were obtained from the system supplement with 5.0-6.7 % (w/v) of alumina oxide. At the suitable concentration, alumina oxide could promote cell growth and ethanol fermentation. The ethanol production and immobilized yield (Y_I) were significantly decreased with the supplement of alumina oxide at 8.3% w/v. It could be explained by the fact that the supplement of alumina oxide particles at too high concentration might generate high positive charge density that could cause negatively effect on the cell activities. This result demonstrated that the optimal concentration of alumina oxide in the AEC carrier was at 5.0% (w/v).

Continuous ethanol fermentation in packed bed reactor

In order to enhance the ethanol productivity, the continuous ethanol fermentations in a 1-L packed bed column were carried out based on the optimal results in the batch fermentations. The AEC carrier was formed by the use of 2.5% (w/v) Na-alginate solution supplemented with 5.0% (w/v) alumina oxide. For more convenient preparation, the AEC carrier was modified into a thin square shape. The cross-linked AEC gel was simply developed on a plastic tray under aseptic conditions and then was cut into multiple pieces of 20 x 20 x 4 mm^3. From our previous examination, the ethanol production and

immobilized yield (Y_I) using thin AEC squares of 20 x 20 x 4 mm^3 were comparable to those of the spheres of Ø 4 mm (data not shown).

The continuous ethanol fermentation was carried out at 32±1 °C in a vertical packed-bed reactor with continuous feeding of the medium at 220 g/L reducing sugar from cane molasses, pH 5.0. The dilution rate was varied at 0.09, 0.16, 0.22 and 0.28 h^{-1}, which corresponded to the hydraulic retention time (HRT) of 11.1, 6.3, 4.6 and 3.6 h, respectively. Ethanol concentration and productivity after the steady state of each dilution rate are shown in Table 3. The highest ethanol concentration of 87.8 g/L at the residual sugar concentration of 19.9 g/L was obtained at the dilution rate (D) of 0.09 h^{-1}, which was almost comparable to that obtained from the batch operation. As the dilution rates increased, the ethanol productivity increased while the ethanol concentration decreased due to the decrease in residence time. The ethanol yields ($Y_{P/S}$) was slightly increased from 0.44 to 0.47. The maximum productivity of 12.6 g/(L·h) was obtained at the dilution rate of 0.28 h^{-1}, which was approximately 9 times that of the batch operation (1.4 g/(L·h)).

The most important characteristic of a PBR is that the material should flow through the reactor as a plug. The results from the sample analysis at five different positions of the bed are depicted in Figures 7 (A) and (B). The graphs show that the ethanol concentration and the sugar consumption were continually increased with the hydraulic retention time (HRT). At the same HRT, the ethanol concentration was found slightly enhanced with the increase of the liquid velocity. The increase of the liquid velocity creeping over the static solid particles from 3.9 to 12.2 cm/h could alleviate the film resistance to mass transfer. Consequently, the increase in the ethanol production rate to some extent was achieved.

The continuous fermentation at a dilution rate of 0.09 $h^{-1}$was performed at steady state for 30 days in order to examine the fermentation consistency and the stability of the immobilized cell. It was found that the immobilized *S. cerevisiae* M30 in the AEC carrier retained their metabolic activity (97.5%) with the immobilized yield of 84.5%. The cells were mostly confined by the bed causing only a few free cells leaving from the reactor. During the 30 days of the operation, the free cell concentration in the effluent remained nearly constant at 0.6 g/L. The immobilized cell concentration was increased up to 19.3 g/L after 30 days of the continuous fermentation. The result demonstrated that the yeast cells could grow and regenerate within the AEC carrier in the PBR. No occurrence of contamination by other microorganisms throughout the entire experimental period.

Conclusions

The results demonstrated that the AEC carrier was a promising material for yeast immobilization. The incorporation of alumina oxide into the alginate gel of the AEC carrier improved the cell carrier characteristics and also showed positive influence on the yeast cell activities on ethanol fermentation. The ethanol productivities of 1.4 and 7.9–12.6 g/(L·h) were obtained by using the AEC cultures in batch and continuous modes of operation, respectively with the production yield ($Y_{P/S}$) of 0.44-0.47 and the immobilized yield (Y_I) of 80-85 %. The nearly complete conversion of total sugar to ethanol was achieved at a dilution rate of 0.09 h^{-1}, where 87.8 g/L ethanol was obtained. The results demonstrated that the AEC carrier has many favorable characteristics for long-term use including good mechanical strength, chemical stability and high immobilization yield.

List of abbreviations

X_I	immobilized cell concentration, g/L
X_E	free cell concentration, g/L
X_T	total cell concentration, g/L
S_0	initial sugar concentration, g/L
S_F	final sugar concentration, g/L
P_0	initial ethanol concentration, g/L
P_F	final ethanol concentration, g/L
D	dilution rate, h^{-1}

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JM performed the experimental work and the analysis and helped to draft of the manuscript. MP participated in the design of the study and wrote the manuscript.

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Figures legends

Figure 1. Schematic diagram of the immobilized cell packed bed reactor.

Figure 2. Concentrations of ethanol (dash lines) and reducing sugar (solid lines) in batch fermentations by immobilized *S. cerevisiae* M30 using (A) Ø 2 mm, (B) Ø 4 mm and (C) Ø 6 mm beads of EC (▲) and AEC (◇) carriers in comparison to those using suspension culture (◆).

Figure 3. SEM images of *S. cerevisiae* M30 cells (A), alumina oxide particles (B), *S. cerevisiae* M30 adhesion to alumina oxide particles (C) and *S. cerevisiae* M30 in alumina oxide doped alginate gel (AEC) after 72 h of batch fermentation.

Figure 4. Pore volume versus average pore diameter of the immobilized cell carriers: AEC (♦) and EC (×).

Figure 5. Tensile strength (A) and elongation at break (B) of the AEC and EC carriers.

Figure 6. Effects of (A) Na-alginate and (B) alumina oxide concentrations for the AEC formation on ethanol concentration (bar) and immobilized yield, Y_I (solid line); the data obtained at 72 h of the batch fermentations using Ø 6 mm beads.

Figure 7. The steady state concentrations of ethanol (A) and reducing sugar (B) in the packed bed reactor (PBR) at the liquid velocity of: 3.9(♦), 6.9(○), 9.5(+) and 12.2 (▲) cm h^{-1}.

Table 1 Yield and end products of batch fermentations at 72 h using the cultures of SC, EC and AEC.

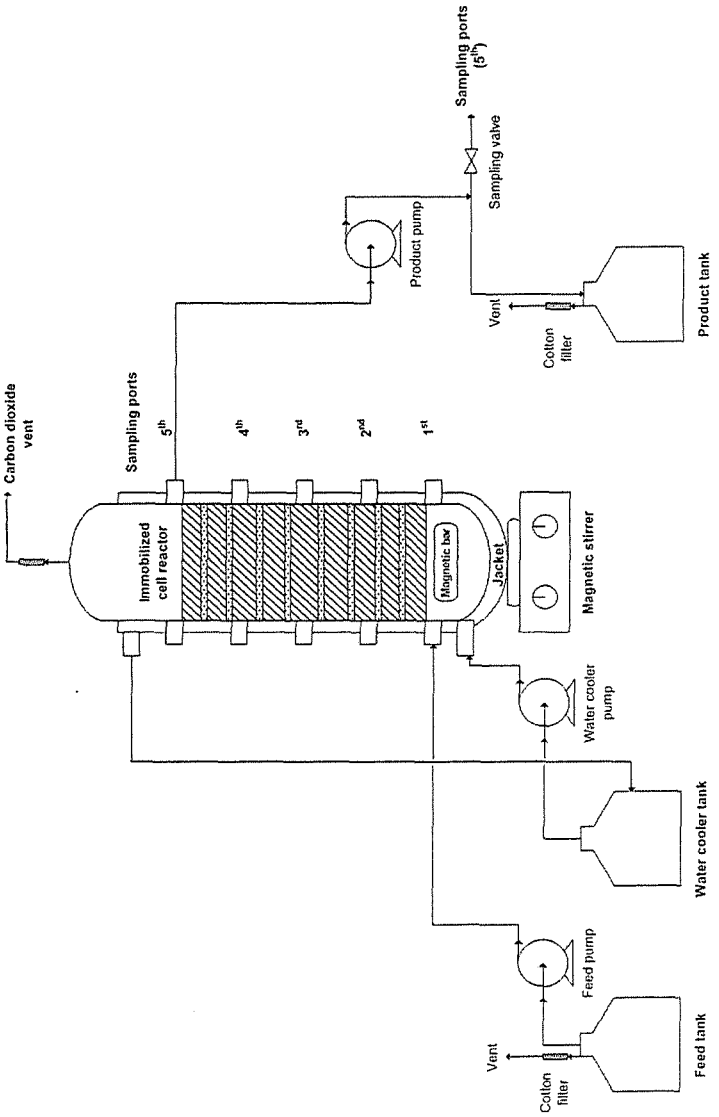
System		Ethanol (g/L)	Residual sugar (g/L)	Free cell, X_F (g/L)	Immobilized cell, X_I (g/L)	Y_I (%)	$Y_{P/S}$ (%)
SC		85.1	33.6	3.7	-	-	44.5
Ø 2 mm	EC	89.9	29.3	0.8	3.5	81.4	45.9
	AEC	91.4	27.6	0.9	4.2	82.9	46.6
Ø 4 mm	EC	89.0	21.4	0.8	3.3	81.1	44.0
	AEC	90.5	20.3	0.8	3.5	81.4	44.2
Ø 6 mm	EC	81.9	35.8	0.9	3.0	76.5	43.3
	AEC	84.4	29.4	0.8	3.4	81.6	43.2

Table 2 Pore characteristics of the EC carrier, the AEC carrier and Al_2O_3 powder.

System	BET Specific surface area (m^2/g)	Pore diameter (nm)	Pore volume (cm^3/g)	Porosity (%)
EC	60.24	14.20	0.27	24.62
AEC	119.08	15.33	0.49	41.81
Al_2O_3 powder	0.72	16.03	0.004	4.31

Table 3. Steady state operation of continuous ethanol fermentation in the packed bed reactor at different dilution rates with the feed containing reducing sugar (RS) of 220 g/L.

Parameters	Dilution Rate (h^{-1})			
	0.09	0.16	0.22	0.28
Effluent RS concentration (g/L)	19.9±1.3	63.2±1.4	101.7±3.5	123.6±0.7
Effluent ethanol concentration (g/L)	87.8±1.1	68.8±0.8	53.2±1.7	45.0±0.4
Ethanol productivity (g/(L·h))	7.9±0.1	11.0±0.1	11.7±0.3	12.6±0.1
Yield of ethanol production ($Y_{P/S,\%}$)	43.9±0.3	43.9±0.8	45.0±0.7	46.7±0.3



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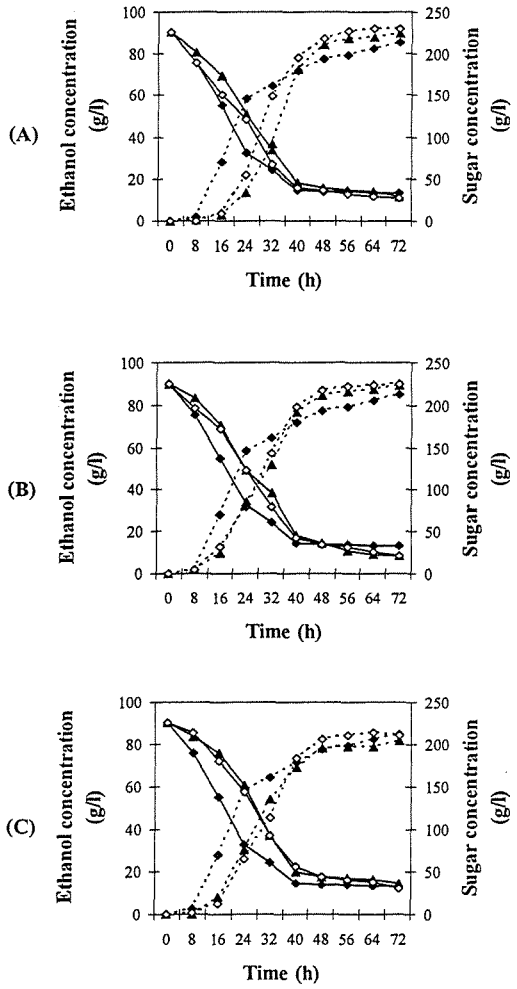

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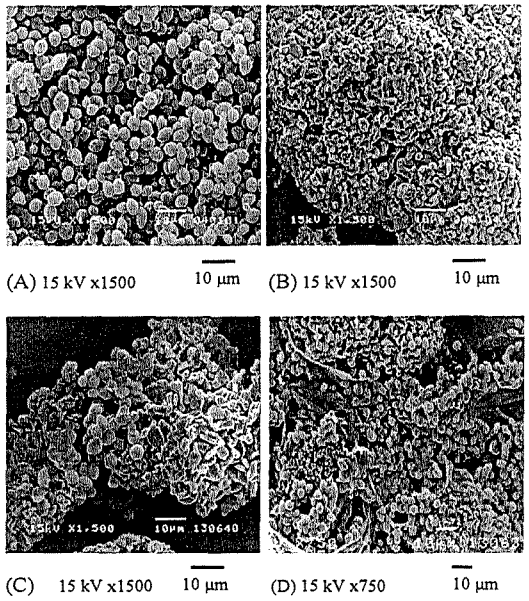

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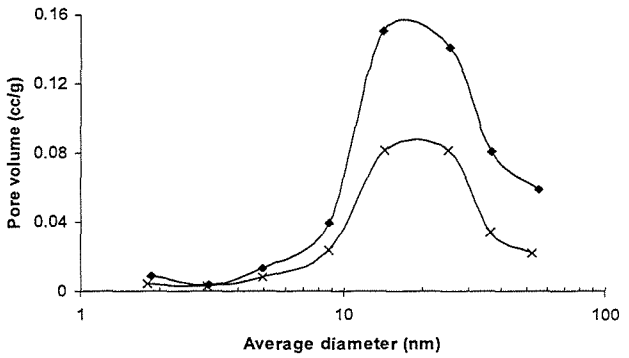

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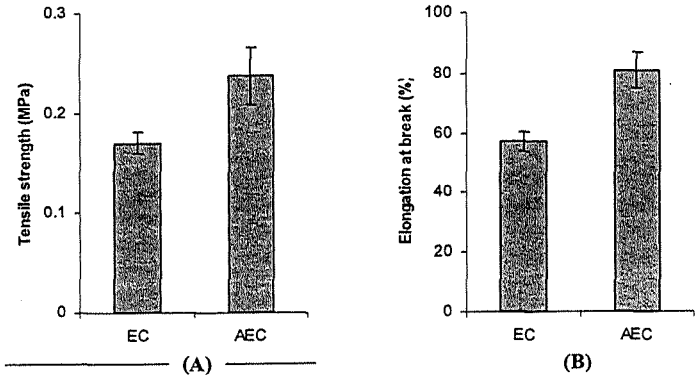
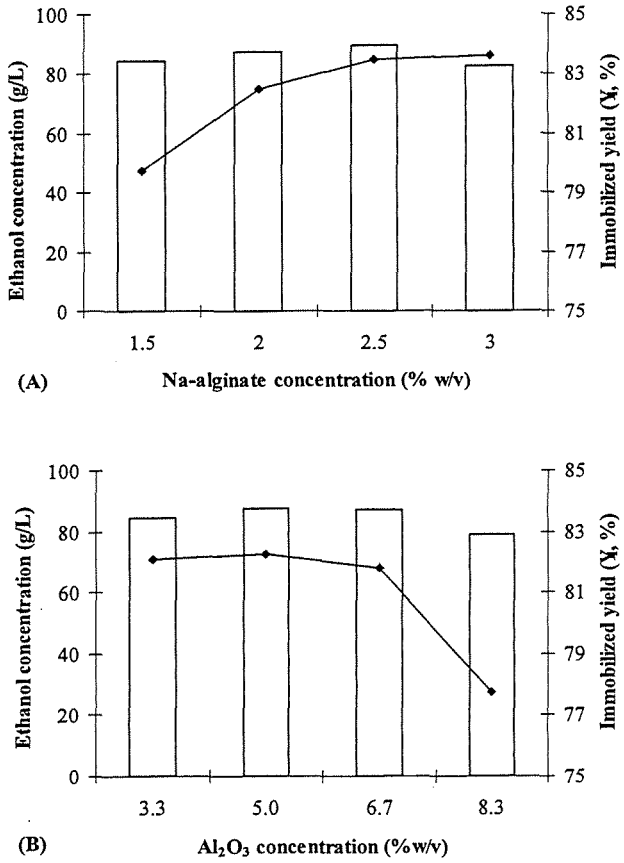


Figure 5. Tensile strength (A) and elongation at break (B) of the AEC and EC carriers.



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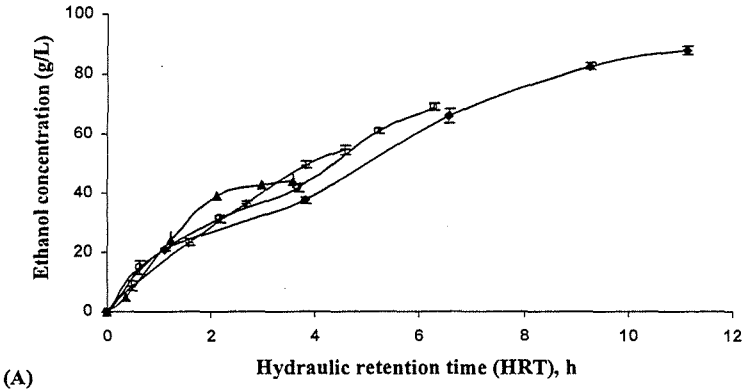
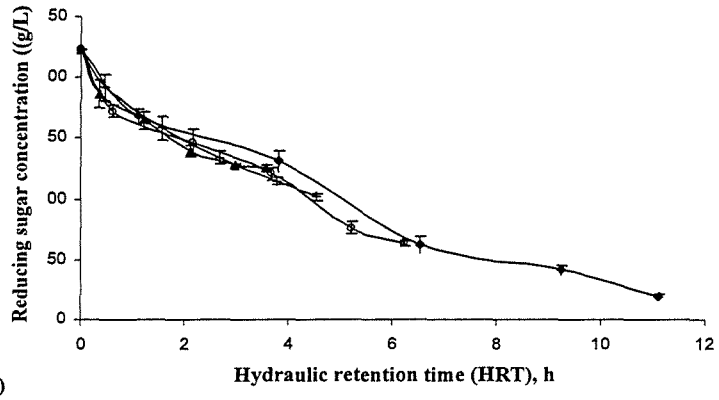


(A)



(B)

Figure7. The steady state concentrations of ethanol (A) and reducing sugar (B) in the packed bed reactor (PBR) at the liquid velocity of: 3.9(♦), 6.9(○), 9.5(+) and 12.2 (▲) cm h^{-1}.

ภาคผนวก B รางวัลที่ได้รับ

จุฬาลงกรณ์มหาวิทยาลัย

มอบกิตติบัตรฉบับนี้เพื่อประกาศเกียรติคุณของ

รองศาสตราจารย์ ดร.เหมือนเดือน พิศาลพงศ์

ในโอกาสที่ได้รับรางวัลผลงานวิจัยดีมาก กองทุนรัชดาภิเษกสมโภช ประจำปี ๒๕๕๐

ผลงานวิจัยเรื่อง "แบบจำลองเพื่อการพัฒนากระบวนการหมักเอทานอล
เพื่อใช้เป็นเชื้อเพลิงทดแทน"

ให้ไว้ ณ วันที่ ๒๘ มีนาคม พุทธศักราช ๒๕๕๑

(ศาสตราจารย์ ดร. คุณหญิงสุชาดา กีระนันทน์)

อธิการบดี

ภาควิชาเคมีอุตสาหกรรม คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

ร่วมกับ

สมาคมวิศวกรรมเคมีและเคมีประยุกต์แห่งประเทศไทย

ขอมอบเกียรติบัตรฉบับนี้ เพื่อแสดงว่า

นาย ภูวิศ บางรักษ์

ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ผลงานวิจัยเรื่อง "Development of entrapment-loofa matrix carrier for ethanol production"

ได้รับรางวัล ดี

ในการนำเสนอผลงานประเภทโปสเตอร์

การประชุมวิชาการวิศวกรรมเคมีและเคมีประยุกต์แห่งประเทศไทย ครั้งที่ 17

ระหว่างวันที่ 29-30 ตุลาคม พ.ศ. 2550

รองศาสตราจารย์ ดร. ธรณินทร์ ไชยเรืองศรี	รองศาสตราจารย์ ดร. อนันต์เสวก เก่งชีวเดชวิญ	นายชลณัฐ ญาณารณพ
หัวหน้าภาควิชาเคมีอุตสาหกรรม	ประธานฝ่ายวิชาการ	นายกสมาคมวิศวกรรมเคมีและเคมีประยุกต์แห่งประเทศไทย

ภาคผนวก C สำเนาคำขอรับสิทธิบัตร

สำเนา

แบบ สป/สผ/อสป/001-ก

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☑ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☐ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ 2535 และพระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ 2542

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วันรับคำขอ 2 ก.พ. 2552	เลขที่คำขอ 0901000435
วันยื่นคำขอ	
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

1.ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
การผลิตเอทานอลแบบต่อเนื่องโดยใช้ยีสต์ที่ถูกตรึงในตัวพยุงเจลเสริมใยบวบ

2.คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)
ดูที่หน้า 3

3.1 สัญชาติ
3.2 โทรศัพท์
3.3 โทรสาร
3.4 อีเมล์

4.สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5.ตัวแทน(ถ้ามี)/ที่อยู่ (เลขที่ ถนน จังหวัด รหัสไปรษณีย์)
นายมงคล แก้วมหา
สถาบันทรัพย์สินทางปัญญาแห่งจุฬาลงกรณ์มหาวิทยาลัย ชั้น 9 ห้อง 904
อาคารเทพทวาราวดี คณะนิติศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ 10330

5.1 ตัวแทนเลขที่ 1453
5.2 โทรศัพท์ 0-2218-2895-6
5.3 โทรสาร 0-2218-2871
5.4 อีเมล์

6.ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)
รองศาสตราจารย์ ดร.เหมือนเดือน พิศาลพงศ์ และ นายภูวิศ บางรักษ์
อยู่ที่ ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ 10330

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

สารบัญ

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☑ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☐ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ 2535 และพระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ 2542

สำหรับเจ้าหน้าที่	
วันรับคำขอ - 2 ก.ค. 2552	เลขที่คำขอ 0901002997
วันยื่นคำขอ	
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

1.ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
กระบวนการสำหรับการหมักเอทานอลด้วยยีสต์ที่ถูกตรึงด้วยเปลือกรังไหมบาง

2.คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและยื่นเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)	
ดูที่หน้า 3	3.1 สัญชาติ
	3.2 โทรศัพท์
	3.3 โทรสาร
	3.4 อีเมล์

4.สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5.ตัวแทน(ถ้ามี)/ที่อยู่ (เลขที่ ถนน จังหวัด รหัสไปรษณีย์)	
นายมงคล แก้วมหา สถาบันทรัพย์สินทางปัญญาแห่งจุฬาลงกรณ์มหาวิทยาลัย ชั้น 9 ห้อง 904 อาคารเทพทวาราวดี คณะนิติศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ 10330	5.1 ตัวแทนเลขที่ 1453 5.2 โทรศัพท์ 0-2218-2895-6 5.3 โทรสาร 0-2218-2871 5.4 อีเมล์

6.ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)
รองศาสตราจารย์ ดร.เหมือนเดือน พิศาลพงศ์ และ นายอนุชิต รัตนพันธุ์
อยู่ที่ ภาควิชาวิศวกรรมเคมี คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ 10330

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร
เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย