Bioethanol and Furfural Production from Palm Pressed Fiber

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
Prince of Songkla University
2011

Thesis Title Bioethanol and Furfural Production from Palm Pressed Fiber

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ชื่อวิทยานิพนธ์ การผลิตไบโอเอทานอลและเฟอร์ฟูรอลจากเส้นใยปาล์ม

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ปีการศึกษา 2553

บทคัดย่อ

เส้นใยปาล์มเป็นแหล่งของเฮมิเซลลูโลสที่สำคัญและสามารถนำมาใช้เป็นวัตถุดิบเริ่มต้นเพื่อการผลิตเฟอร์ฟูรอลได้ การศึกษานี้ทำการกำจัดลิกนินออกจากเส้นใยปาล์มโดยการแช่ส่วนผสมของเส้นใยปาล์มกับสารละลายโซเดียมคลอไรท์อัตราส่วน 10:1 (โดยน้ำหนัก) ในสารละลายกรดอะซิติกเข้มข้น 0.01% ที่อุณหภูมิ 70 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ทำการสกัดซ้ำอีก 3-4 ครั้ง จนกระทั่งได้เส้นใยปาล์มที่มีสีขาว จากนั้นนำเส้นใยปาล์มที่ผ่านการกำจัดลิกนินแล้วมาศึกษาหาสภาวะที่เหมาะสมของพารามิเตอร์ 3 ประการ ในการสกัดเฮมิเซลลูโลสด้วยวิธีการหาพื้นผิวตอบสนอง (RSM) ด้วย 20 การทดลอง พบว่า สภาวะที่เหมาะสมในการสกัดเฮมิเซลลูโลสคือ การใช้สารละลายโปแตสเซียมไฮดรอกไซด์ที่ความเข้มข้น 28.8% อัตราส่วนระหว่างเส้นใยปาล์มที่ผ่านการกำจัดลิกนินต่อสารละลายโปแตสเซียมไฮดรอกไซด์คือ 1:20 (น้ำหนัก/ปริมาตร) และเวลาที่ใช้ในการสกัดคือ 20 นาที (R^2 = 98%) จากสภาวะดังกล่าวสามารถผลิตเฮมิเซลลูโลสได้ 38.67 ± 1.21% (คำนวณจากเส้นใยปาล์มที่ผ่านการกำจัดลิกนิน) คิดเป็นเปอร์เซ็นต์การสกัดได้ถึง 99.25% และมีน้ำตาลไซโลสเป็นองค์ประกอบหลักในปริมาณ 80.8% เมื่อนำเฮมิเซลลูโลสที่สกัดได้มาผลิตเฟอร์ฟูรอลแบบขั้นตอนเดียว โดยศึกษาปัจจัยที่มีผลต่อการผลิตเฟอร์ฟูรอล ได้แก่ อุณหภูมิ (120-150 องศาเซลเซียส) อัตราส่วนระหว่างสารละลายกรดซัลฟูริกต่อเฮมิเซลลูโลส (8-10 มิลลิลิตรต่อกรัมเฮมิเซลลูโลส) ความเข้มข้นของกรดซัลฟูริก (5-10% ปริมาตรต่อปริมาตร) และเวลาทำปฏิกิริยา (0-120 นาที) พบว่า อัตราส่วนระหว่างสารละลายกรดซัลฟิวริกต่อเฮมิเซลลูโลสเป็น 8 มิลลิลิตรต่อกรัมเฮมิเซลลูโลส กรดซัลฟิวริกเข้มข้น 5% อุณหภูมิที่เหมาะสม คือ 150 องศาเซลเซียส ระยะเวลาในการทำปฏิกิริยา คือ 90 นาที สามารถผลิตเฟอร์ฟูรอลได้ 0.86 กรัมต่อลิตร หรือ 3.44% โดยน้ำหนัก (กรัมเฟอร์ฟูรอลต่อ 100 กรัม เฮมิเซลลูโลส)

การผลิตเฟอร์ฟูรอลแบบสองขั้นตอนประกอบด้วยขั้นตอนแรก คือ การย่อยสลายเฮมิเซลลูโลสด้วยกรดเพื่อผลิตน้ำตาล ขั้นตอนต่อมาคือการดึงน้ำออกเพื่อให้โมเลกุลของไซโลสเปลี่ยนเป็นเฟอร์ฟูรอล เมื่อศึกษาหาสภาวะที่เหมาะสมโดยใช้เทคนิคการหาพื้นที่ตอบสนอง (RSM) ตัวแปรที่สำคัญในขั้นตอนของการย่อยสลายเฮมิเซลลูโลสเพื่อผลิตน้ำตาล ได้แก่ อุณหภูมิที่เกิดปฏิกิริยา (100-150 องศาเซลเซียส) ความเข้มข้นของกรดซัลฟิวริก (1-10%) อัตราส่วนระหว่างสารละลายกรดซัลฟิวริกต่อเฮมิเซลลูโลส (8-10 ปริมาตรต่อน้ำหนัก) และระยะเวลาที่ใช้ในการผลิตน้ำตาล (30-120 นาที) พบว่าสามารถผลิตน้ำตาลไซโลสได้สูงสุด (12.32 ± 2.42 กรัมต่อลิตร) ภายใต้สภาวะที่อุณหภูมิ 120 องศาเซลเซียส กรดซัลฟิวริกที่ความเข้มข้น 5.7% อัตราส่วนระหว่างสารละลายกรดซัลฟิวริกต่อเฮมิเซลลูโลสเท่ากับ 8.5 มิลลิลิตรต่อกรัมเฮมิเซลลูโลส ระยะเวลาทำปฏิกิริยา 31 นาที โดยมีค่าสัมประสิทธิ์ของพื้นที่ตอบสนองเท่ากับ 0.90 สำหรับตัวแปรที่มีผลในขั้นตอนที่สองในการดึงน้ำออก คือ อุณหภูมิที่เกิดปฏิกิริยา (120-160 องศาเซลเซียส) และระยะเวลาในการเกิดปฏิกิริยา (30-150 นาที) พบว่าการผลิตเฟอร์ฟูรอลสูงสุด (8.67 ± 0.62 กรัมต่อลิตร) ที่อุณหภูมิ 135.24 องสาเซลเซียสเป็นระยะเวลา 90.34 นาที โดยให้ค่าสัมประสิทธิ์ของพื้นที่ตอบสนองเท่ากับ 0.93 เฟอร์ฟูรอลที่ผลิตได้สูงกว่าการผลิตแบบขั้นตอนเดียวถึง 10 เท่า

เซลลูโลสที่ได้หลังจากการสกัดเฮมิเซลลูโลสและลิกนินมีลักษณะเป็นเส้นใยสีขาว สามารถใช้เป็นแหล่งในการผลิตกลูโคส โดยการใช้เอนไซม์และการย่อยด้วยกรด เมื่อศึกษาปัจจัยที่มีผลต่อการทำงานของเอนไซม์เซลลูเลส (เอนไซม์จากเชื้อ *Trichoderma reesei*) คือ พีเอช อุณหภูมิ ความเข้มข้นของสารตั้งต้นและปริมาณเอนไซม์ รวมทั้งระยะเวลาในการบ่ม พบว่า ได้ปริมาณน้ำตาลกลูโคส (ซึ่งรายงานในรูปน้ำตาลรีดิวซ์) สูงสุด (7.9 กรัมต่อลิตร) (60% saccharification) เมื่อใช้ความเข้มข้นของเซลลูโลส 12 กรัมต่อลิตร ความเข้มข้นของเอนไซม์ 80 ยูนิตต่อกรัมสับสเตรท ภายใต้สภาวะเหมาะสมที่พีเอช 4.8 อุณหภูมิ 50 องศาเซลเซียส เป็นระยะเวลา 15 ชั่วโมง สำหรับการผลิตกลูโคสด้วยกรดโดยกระบวนการผลิตแบบสองขั้นตอน คือ เริ่มต้นย่อยสลายเซลลูโลสด้วยกรดซัลฟิวริกเข้มข้น 72% เป็นเวลานาน 90 นาที จากนั้นทำการย่อยต่อด้วยสารละลายกรดซัลฟิวริกเจือจาง 4% โดยใช้อัตราส่วนระหว่างเซลลูโลสต่อสารละลายกรดซัลฟิวริกที่ 1 ต่อ 16 (น้ำหนักต่อปริมาตร) ที่อุณหภูมิ 120 องศาเซลเซียส เป็นเวลา 86 นาที สามารถผลิตน้ำตาลกลูโคสได้เพียง 0.54 กรัมต่อลิตร ซึ่งมีค่าน้อยกว่าการผลิตน้ำตาลกลูโคสโดยการใช้เอนไซม์ถึง 14.6 เท่า

เมื่อศึกษาการเพิ่มผลผลิตน้ำตาลไซโลสจากเส้นใยปาล์มที่ผ่านการกำจัดลิกนินให้ได้ค่าสูงสุดด้วยสารละลายกรดซัลฟูริก และลดการเปลี่ยนน้ำตาลไซโลสเป็นเฟอร์ฟูรอล รวมถึงลดการเกิดกรดอะซิติกให้เกิดน้อยที่สุด เนื่องจากทั้งเฟอร์ฟูรอลและกรดอะซิติกเป็นสารยับยั้งการเจริญ

ของจุลินทรีย์ในขั้นตอนการผลิตเอทานอล ตัวแปรที่มีผลต่อการผลิตน้ำตาลไซโลสที่ศึกษาโดยวิธีการหาพื้นที่ผิวตอบสนอง (RSM) คือ ความเข้มข้นสารละลายกรดซัลฟูริกเจือจาง (0-10%) อุณหภูมิที่เกิดปฏิกิริยา (75-148 องศาเซลเซียส) และระยะเวลาในการเกิดปฏิกิริยา (0-180 นาที) พบว่าสภาวะที่เหมาะสมในการผลิตน้ำตาลไซโลส คือ กรดซัลฟิวริกที่ความเข้มข้น 2% (ปริมาตร/ปริมาตร) อัตราส่วนระหว่างเฮมิเซลลูโลสต่อสารละลายกรดซัลฟิวริก เป็น 1 ต่อ 10 (น้ำหนัก/ปริมาตร) ที่อุณหภูมิ 120 องศาเซลเซียส เป็นเวลา 30 นาที ให้การผลิตน้ำตาลไซโลสและกลูโคสได้สูงสุดเท่ากับ 28.7 และ 3.5 กรัมต่อลิตร ตามลำดับ สำหรับกรดอะซิติกและเฟอร์ฟูรอลภายใต้สภาวะดังกล่าวมีค่า 10.56 และ 0.55 กรัมต่อลิตร ตามลำดับ นอกจากนี้ พบว่า สภาวะการผลิตไซโลสดังกล่าวนี้ ยังเหมาะกับการผลิตไซโลสจากเส้นใยปาล์มที่ยังไม่ผ่านกระบวนการกำจัดลิกนินอีกด้วย ได้ไซโลสและกลูโคสเท่ากับ 27.23 และ 2.3 กรัมต่อลิตร ตามลำดับ อีกทั้งยังผลิตกรดอะซิติก (5.99 กรัมต่อลิตร) และ เฟอร์ฟูรอล (0.42 กรัมต่อลิตร) ต่ำกว่าเมื่อเทียบกับค่าที่ได้จากการใช้เส้นใยปาล์มที่ผ่านการกำจัดลิกนิน ดังนั้นจึงได้สภาวะที่เหมาะสมในการผลิตไซโลสจากเส้นใยปาล์มโดยตรง

การศึกษาการผลิตเอทานอลด้วยเชื้อ *Candida shehatae* TISTR5843 ในอาหารสังเคราะห์ พบว่า เมื่อเลี้ยงเชื้อในอาหารสังเคราะห์ที่มีกลูโคสเป็นแหล่งคาร์บอน ความเข้มข้นกลูโคสที่เหมาะสมเท่ากับ 24 กรัมต่อลิตร และเมื่อเลี้ยงเชื้อในอาหารสังเคราะห์ที่มีไซโลสเป็นแหล่งคาร์บอน ความเข้มข้นที่เหมาะสมเท่ากับ 20 กรัมต่อลิตร นอกจากนี้อัตราส่วนที่เหมาะสมระหว่างกลูโคสและไซโลสเท่ากับ 2:8 กรัมต่อกรัม ที่พีเอชเริ่มต้น 5.0 อุณหภูมิห้อง (30 องศาเซลเซียส) และการเขย่าที่อัตราเร็วเท่ากับ 180 รอบต่อนาที ให้ค่าผลผลิตเอทานอลเท่ากับ 0.42-0.45 กรัมเอทานอลต่อกรัมน้ำตาล อัตราการผลิตเอทานอลเท่ากับ 0.103-0.343 กรัมเอทานอลต่อลิตรต่อวัน และ ความเข้มข้นเอทานอลเท่ากับ 0.37-1.30%

เมื่อศึกษาการผลิตเอทานอลจากเซลลูโลสไฮโดรไลเสตโดยเชื้อยีสต์สองสายพันธุ์ (*Candida shehatae* TISTR5843 และ *Saccharomyces cerevisiae* TISTR5017) และเชื้อแบคทีเรีย (*Zymomonas mobilis* TISTR405) พบว่า *S. cerevisiae* TISTR5017 สามารถผลิตเอทานอลได้สูงสุดเท่ากับ 2.82 กรัมต่อลิตร (0.34 กรัมเอทานอลต่อกรัมน้ำตาล) จากการศึกษาสามปัจจัยที่มีผลต่อการผลิตเอทานอลด้วยวิธีการหาพื้นที่การตอบสนอง (RSM) ได้แก่ พีเอชเริ่มต้น (4-6) อัตราการเขย่า (120-240 รอบต่อนาที) และ ความเข้มข้นของเชื้อเริ่มต้น (0.4-1.0 กรัมต่อลิตร) พบว่า สภาวะที่สามารถผลิตเอทานอลได้สูงสุดที่พีเอชเริ่มต้นเท่ากับ 5.40 ที่อัตราการเขย่าเท่ากับ 137 รอบต่อนาที

และความเข้มข้นของเชื้อเริ่มต้นที่ 0.56 กรัมต่อลิตร หลังการหมัก 24 ชั่วโมง ได้เอทานอลเข้มข้น 3.98 ± 0.42 กรัมต่อลิตร (0.48 กรัมเอทานอลต่อกรัมน้ำตาล) (R^2=0.97)

เมื่อศึกษาผลของแหล่งไนโตรเจน ความเข้มข้นของไนโตรเจน อัตราส่วนระหว่างคาร์บอนต่อไนโตรเจนและสารยับยั้งต่อการผลิตเอทานอลโดยเชื้อ *C. shehatae* TISTR5843 ในอาหารสังเคราะห์ที่มีไซโลสเป็นแหล่งคาร์บอน พบว่า เปปโตนที่ความเข้มข้นเท่ากับ 3 กรัมต่อลิตร และอัตราส่วนระหว่างคาร์บอนกับไนโตรเจนเท่ากับ 9.3 เป็นสภาวะที่เหมาะสม สามารถผลิตเอทานอลได้ 4.75 กรัมต่อลิตร อีกทั้งสารยับยั้งต่างๆ ที่มีอยู่ในไฮโดรไลเสตของเส้นใยปาล์ม ได้แก่ อะซิเตท เฟอร์ฟูรอลและวานิลินต้องมีค่าน้อยกว่า 2.5 0.5 และ 0.5 กรัมต่อลิตร ตามลำดับ เพื่อให้ได้อัตราการผลิตเอทานอลที่ดี อีกปัจจัยที่มีผลต่อการผลิตเอทานอลจากไฮโดรไลเสตของเส้นใยปาล์มโดยเชื้อ *C. shehatae* TISTR5843 คือ อัตราการเจือจางของไฮโดรไลเสต พบว่า อัตราการเจือจางที่ 1/2 เป็นอัตราการเจือจางที่เหมาะสม ซึ่งสามารถผลิตเอทานอลได้สูงสุดเท่ากับ 0.32 กรัม เอทานอลต่อกรัมน้ำตาล และอัตราการผลิตเอทานอลเท่ากับ 0.125 กรัมต่อลิตรต่อชั่วโมง

ไฮโดรไลเสตของเส้นใยปาล์มประกอบด้วยไซโลสและกลูโคสเท่ากับ 27.23 และ 2.3 กรัมต่อลิตร ตามลำดับ มีการเติมสารอาหารต่างๆ และปรับพีเอชเท่ากับ 5.0 จากนั้นนำมาหมักเพื่อผลิตเอทานอลโดยเชื้อ *C. shehatae* TISTR5843 เมื่อหาสภาวะที่เหมาะสมของปัจจัยสิ่งแวดล้อมต่อการผลิตเอทานอล ได้แก่ พีเอชเริ่มต้น อัตราการเขย่า และความเข้มข้นของเชื้อเริ่มต้น ด้วยวิธีการหาพื้นที่การตอบสนอง (RSM) พบว่า สภาวะที่เหมาะสมคือ พีเอชเริ่มต้นเท่ากับ 5.25 อัตราการเขย่าเท่ากับ 135 รอบต่อนาที ร่วมกับความเข้มข้นของเชื้อเริ่มต้นเท่ากับ 1.08 กรัมต่อลิตร สามารถผลิตเอธานอลได้สูงสุดเท่ากับ 5.25 ± 0.72 กรัมต่อลิตร และได้ผลผลิตเท่ากับ 0.40 กรัมเอทานอลต่อกรัมไซโลส (R^2=0.98) ความเข้มข้นของกรดอะซิติกและเฟอร์ฟูรอลในอาหารไฮโดรไลเสตเท่ากับ 0.30 ± 0.01 และ 0.08 ± 0.02 กรัมต่อลิตร ตามลำดับ

เมื่อศึกษาเพื่อเพิ่มผลผลิตการผลิตเอทานอลจากไฮโดรไลเสตของเส้นใยปาล์มโดยเชื้อ *C. shehatae* TISTR5843 ในกระบวนการแบบกึ่งกะ (fed batch process) และแบบกึ่งต่อเนื่อง (semi-continuous process) โดยทดลองในถังหมักขนาด 3 ลิตร ใช้ไฮโดรไลเสตของเส้นใยปาล์มที่ผ่านการเจือจางที่ระดับ 1/2 ซึ่งจะมีการป้อนไฮโดรไลเสตเข้าสู่ระบบทั้งสิ้น 3 ครั้ง พบว่า การผลิตเอทานอลสูงสุดของแบบกึ่งกะและแบบกึ่งต่อเนื่องเท่ากับ 3.92 และ 4.02 กรัมต่อลิตร ตามลำดับ ซึ่งเป็นค่าที่ใกล้เคียงกับการผลิตเอทานอลแบบกะ (batch process) (4.07 กรัมต่อลิตร) อย่างไรก็ตาม การผลิต เอทานอลแบบกึ่งกะและแบบกึ่งต่อเนื่องจะมีค่าลดลงเรื่อยๆ เมื่อทำการหมักในรอบต่อไป เนื่องจากมีการสะสมของสารยับยั้งเพิ่มขึ้นเรื่อยๆ

การศึกษาความเป็นไปได้ในการใช้เส้นใยปาล์มและเส้นใยปาล์มที่ปราศจากลิกนินเป็นตัวตรึงเซลล์ *C. shehatae* TISTR5843 พบว่า ยีสต์ที่ตรึงด้วยเส้นใยปาล์มปราศจากลิกนินที่ผ่านการบดให้มีขนาดเส้นผ่านศูนย์กลาง 5.0 มิลลิเมตร สามารถผลิตเอทานอลได้สูงสุดถึง 11.5 กรัมต่อลิตร (0.47 กรัมเอทานอลต่อกรัมน้ำตาล) เมื่อเลี้ยงเชื้อ 36 ชั่วโมง สำหรับการเลี้ยงแบบกะ พบว่า ความเข้มข้นของเอทานอลและผลผลิตเอทานอลที่ได้โดยยีสต์ที่ตรึงด้วยเส้นใยปาล์มมีค่าสูงกว่าการใช้เชื้อยีสต์อิสระคิดเป็น 6.20% และ 6.82% ตามลำดับ เชื้อยีสต์ที่ตรึงด้วยเส้นใยปาล์มและเส้นใยปาล์มที่ปราศจากลิกนินที่ผ่านการบดขนาดเส้นผ่านศูนย์กลาง 5.0 มิลลิเมตร มีความสามารถในการผลิตเอทานอลไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ (P>0.05) ดังนั้น เมื่อใช้เชื้อยีสต์ที่ตรึงด้วยเส้นใยปาล์มที่ผ่านการบดขนาดเส้นผ่านศูนย์กลาง 5.0 มิลลิเมตร ผลิตเอทานอลด้วยการเลี้ยงแบบกะหมุนเวียน (Repeated batches process) พบว่า ประสิทธิผลของปริมาณเอทานอลที่ผลิตได้ ผลผลิตเอทานอล และอัตราการผลิตเอทานอลจากเชื้อยีสต์ที่ตรึงด้วยเส้นใยปาล์มที่ผ่านการบดขนาดเส้นผ่านศูนย์กลาง 5.0 มิลลิเมตร มีค่าสูงกว่าการเลี้ยงแบบใช้เซลล์อิสระ มีค่าเท่ากับ 10.78-30.12%, 9.52-22.22% และ 11.90-32.35% ตามลำดับ โดยเชื้อยีสต์ที่ตรึงด้วยเส้นใยปาล์มที่ผ่านการบดขนาดเส้นผ่านศูนย์กลาง 5.0 มิลลิเมตรสามารถนำกลับมาใช้ใหม่ได้ถึง 4 รอบโดยคงประสิทธิภาพการผลิตไว้ได้ถึง 93%

Thesis Title Bioethanol and Furfural Production from Palm Pressed Fiber
Author Mr. Wiboon Riansa-ngawong
Major Program Biotechnology
Academic Year 2010

ABSTRACT

Palm pressed fiber (PPF) is a potential source for production hemicellulose which is a starting material for furfural production. In this study, PPF was delignified by soaking the mixture of PPF to sodium chlorite ($NaClO_2$) in the ratio of 10:1 (w/w) in 0.01% acetic acid solution at 70°C for 1 h and repeated 3-4 times until PPF became white. Three parameters affecting extraction of hemicellulose from the delignified PPF (dPPF) were optimized using response surface methodology (RSM) with 20 batch experiments. The optimum condition was found to be 28.8% (w/v) KOH, the dPPF: KOH ratio of 1:20 (w/v) and reaction time of 20 min (R^2 = 98%). The highest hemicellulose yield of 38.67 ± 1.21% (based on dPPF) with 99.25% extraction and the xylose content of 80.8% was achieved. The extracted hemicellulose was used in one-stage process for furfural production. Optimization studies were conducted on the reaction temperature (120-150°C), liquid (sulfuric acid)/solid (hemicellulose) ratio (LSR) (8-10 ml/g hemicellulose), sulfuric acid concentration (5-10 % v/v) and reaction time (0-120 min). The best condition was reaction temperature of 150°C, LSR of 8 ml/g hemicellulose, sulfuric acid concentration of 5% (v/v) and reaction time of 90 min. This gave the highest furfural concentration of 0.86 g/l or 3.44 wt% (g furfural/100 g hemicellulose).

Two-stage process was consisted of acid hydrolysis stage followed by dehydration stage was conducted to improve furfural production from extracted hemicellulose. The optimum conditions in both steps were achieved by RSM. In the acid hydrolysis step to convert hemicellulose to xylose, there were four important parameters consisted of reaction temperature (100-150°C), sulfuric acid concentration

(1-10 % v/v), ratio of sulfuric acid to hemicellulose (LSR) (8-10 v/w), and reaction time (30-120 min). The maximum xylose production (12.32 ± 2.42 g/l) was achieved at 120°C, 5.7% sulfuric acid, L/S ratio of 8.5 ml/g for 31 min with the determination coefficient (R^2) value of 0.90. For dehydration process, two parameters; reaction temperature (120-160°C) and reaction time (30-150 min), were optimized. The maximum furfural production (8.67 ± 0.62 g/l) was achieved at the reaction temperature of 135.24°C for 90.34 min with the determination coefficient (R^2) value of 0.93. The furfural obtained was 10 times higher than that from one stage process.

The white residual solid after hemicelluloses extraction, cellulose, was used as a source for glucose production by enzymatic and concentrated acid hydrolysis. An experimental design to get the maximum of glucose production by cellulase (*Trichoderma reesei*) was conducted, in which the effect of pH, temperature, substrate concentration, enzyme dosage, and incubation time were evaluated. The maximum reducing sugar (7.9 g/l) mainly glucose was achieved when incubated 12 g/l of the extracted cellulose with cellulase of 80 U/g substrate under the optimum condition pH of 4.8 at 50 °C for 15 h, giving 60% saccarification. For acid hydrolysis, the glucose production was conducted by two-stage process, firstly it was treated by 72% sulfuric acid for 90 min and followed by 4% sulfuric acid hydrolysis using solid/liquid ratio (SLR) of 1:16 (w/v) at 120 °C for 86 min. The 0.54 g/l glucose was produced under those conditions. The glucose obtained was 14.6 fold lower than that achieved from enzymatic method.

To increase xylose production from dPPF by dilute sulfuric acid (H_2SO_4) hydrolysis to obtain solutions with a maximum xylose concentration and minimum concentration of furfural and acetic acid (inhibitors for growth and ethanol production). The effect of H_2SO_4 concentration (0-10% v/v), reaction temperature (75-148ºC), and reaction time (0-180 min) were studied by RSM. Kinetic parameters of mathematical models were obtained in order to predict concentration of xylose, glucose, furfural and acetic acid in the hydrolysate and to optimize the process. The optimum condition was the 2% (v/v) H_2SO_4 with solid/liquid ratio (SLR) of 1:10 (w/v) at 120 ºC for 30 min, giving the highest xylose and glucose yields of 28.7 and

3.5 g/l, respectively. The concentrations of the inhibitors acetic acid and furfural were 10.56 g/l and 0.55 g/l, respectively. In addition, this optimal condition was also suitable for production of xylose from PPF giving the highest xylose and glucose yields of 27.23 and 2.3 g/l with lower acetic acid (5.99 g/l) and furfural (0.42 g/l) production.

The ethanol production in synthetic medium by *Candida shehatae* TISTR5843 was studied. Cultivation in synthetic medium with glucose as a sole carbon source, the optimum glucose concentration was found to be 24 g/l, whereas cultivation in the synthetic medium containing xylose as a sole carbon source, the optimum xylose concentration was 20 g/l. The optimum glucose to xylose ratio in the medium was 2:8 w/w with initial pH of 5.0 at room temperature (30°C) and the shaking speed of 180 rpm. The highest ethanol yields, ethanol productivities and ethanol concentration under the optimum condition were 0.42-0.45 g ethanol/ g sugar, 0.103-0.343 g/l/h and 0.37-1.30%, respectively.

Ethanol production from cellulosic hydrolysate using two yeast strains (*Candida shehatae* TISTR5843 and *Saccharomyces cerevisiae* TISTR5017) and bacteria (*Zymomonas mobilis* TISTR405) were studied. *S. cerevisiae* TISTR5017 was found to be the best strain for ethanol production giving the highest ethanol concentration and ethanol yield of 2.82 g/l and 0.34 g ethanol/g sugar, respectively. Three parameters, initial pH (4-6), shaking speed (120-240 rpm), and initial cell concentration (0.4-1.0 g/l), were studied by using RSM. The optimal condition was initial pH 5.40 with shaking speed of 137 rpm and initial cells concentration of 0.56 g/l. After 24 h fermentation, ethanol concentration and ethanol yield were 3.98 ± 0.42 g/l and 0.48 g ethanol/g sugar (R^2=0.97).

The effects of nitrogen source and concentration, C/N ratio as well as inhibitory compounds on ethanol production by *C. shehatae* TISTR5843 were studied in the synthetic xylose medium. The highest ethanol production (4.75 g/l) was obtained at 3 g/l peptone, which corresponded to a C/N ratio of 9.3. The inhibitory compounds presented in PPF hydrolysate such as acetate, furfural, and vanillin, should be less than 2.5, 0.5, and 0.5 g/l, respectively in order to get the productive ethanol. Moreover, dilution of PPF hydrolysate played a major role in ethanol

production by *C. shehatae* TISTR5843. The 1/2 dilution was a suitable dilution for production of ethanol, giving the maximum yield of 0.32 g ethanol/g sugar and ethanol productivity of 0.125 g/l/h.

The PPF hydrolysate containing xylose and glucose of 27.23 and 2.3 g/l, respectively, was supplemented with nutrients and adjusted pH to 5.0 was used for ethanol production by *C. shehatae* TISTR5843. Optimization on the environmental factors (initial pH, shaking speed and inoculum concentration) affecting on ethanol production were conducted using response surface methodology (RSM). The optimal condition was the initial pH 5.25 with shaking speed of 135 rpm and inoculum concentration of 1.08 g/l. The maximum ethanol concentration and ethanol yield were 5.25 ± 0.72 g/l and 0.40 g ethanol/g xylose (R^2=0.98). The final acetic acid and furfural in the hydrolysate medium were 0.08 ± 0.02 g/l and 0.30 ± 0.01 g/l, respectively.

Fed batch and semi-continuous processes were studied to enhance the ethanol production from PPF hydrolysate by *C. shehatae* TISTR5843. The experiments were carried out in 3 cycles of 1/2 dilution of fresh medium in 3 liters fermenter. The maximum ethanol productions of fed batch and semi-continuous process were 3.92 and 4.02 g/l, respectively, which were similar to ethanol production from batch process (4.07 g/l). However, the ethanol productions of fed batch and semi-continuous process were slightly decreased in the following cycles because of the accumulation of toxic compounds.

Potential use of palm pressed fiber (PPF) and delignified palm pressed fiber (DPPF) as immobilization supports of *C. shehatae* TISTR5843 for ethanol production in synthetic glucose medium was studied. A small size less than 5 mm of DPPF (sDPPF) gave the maximum ethanol production of 11.5 g/l (0.47 g ethanol/g glucose) at 36 h cultivation. In batch system, the ethanol concentration and ethanol yield by immobilized cells were higher than the free cell for 6.20% and 6.82%, respectively. There was no significant difference between immobilized yeast on sPPF and sDPPF (P>0.05). The sPPF was used as a support in four repeated batches fermentation. The ethanol concentrations, ethanol yields and ethanol productivities of the immobilized cells in the range of 10.78-30.12%, 9.52-22.22% and 11.90-32.35%,

respectively, were higher than those of the free cells. The immobilized cells on sPPF could be reused 4 times with retaining activity of 93%.

ACKNOWLEDGEMENT

This thesis could not be successfully completed without the kindness of my major advisor, Assoc. Prof. Dr. Poonsuk Prasertsan, for pushing me to reach my potential. I appreciate her confidence in me as her graduate student to make my own mistakes and learn from them. She opened me to biotechnological world and provided lots of good ideas and advices. Special thanks to Prof. Dr. Kenji Iiyama, for his comments and suggestions at the initial stage of my experimental work.

Many thanks to my fellow lab mates in Environmental Biotechnology Laboratory: Dr. Maneewan (Joy) Suwansaard for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had during my Ph. D study. I would like to thanks Environmental Biotechnology Lab members for their kind assistance. Also I thanks P' Sa, P' Pan, P' Pu and P' Da, the officers in Scientific Equipment Center (SEC), Prince of Songkla University for all technical suggestions.

I would like to express my sincere appreciation to The Joint Graduate School of Energy and Environment (JGSEE), Palm Oil Products and Technology Research Center (POPTEC) at Faculty of Agro-Industry, and Graduate School of Prince of Songkla University, Thailand for the research grant.

Lastly, and most importantly, I am grateful to my wonderful family, parents Soraphum -Suwanna Riansa-ngawong, brothers and sisters. They provided the unconditional love, supports, and encouragement to help me learn that I can truly accomplish anything. To them I dedicate this thesis.

Wiboon Riansa-ngawong

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LIST OF ABBREVIATIONS AND SYMBOLS

ADH	Alcohol dehydrogenase
AFEX	Ammonia fiber explosion
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
CCD	Central composite design
CH_3COOK	Potassium acetate
CMC	Carboxymethylcellulose
COD	Chemical oxygen demand
CO_2	Carbon dioxide
C/N	Carbon to nitrogen ratio
CV	Coefficient of variation
°C	Degree celcious
Da	Dalton
DCW	Dry cell weight
dPPF	Delignified palm pressed fiber
EDTA	Ethylenediaminetetraacetic acid
EFB	Empty fruit bunch
FFB	Fresh fruit bunch
FPA	Filter paper activity
FPU	Filter paper unit
GC	Gas chromatography
g	Gram
g/l	Gram per liter
g/l/h	Gram per liter per hour
g/g	Gram per gram
x g	Gravitational force
h	Hour
HMF	Hydroxymethylfurfural

LIST OF ABBREVIATIONS AND SYMBOLS (Cont.)

HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
KH_2PO_4	Potassium phosphate
KOH	Potassium hydroxide
LSR	Liquid to solid ratio
ml	Milliliter
mM	Millimolar
ml/min	Milliliter per minute
$MgCl_2$	Magnesium chloride
MSW	Municipal solid wastes
MTHF	Methyltetrahydrofuran
$NaClO_2$	Sodium chlorite
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium nitrate
$(NH_4)_3PO_4$	Ammonium phosphate
$(NH_4)_2SO_4$	Ammonium sulfate
NRE	The national renewable energy
P_{max}	Maximum product production
PKS	Palm kernel shell
PPF	Palm pressed fiber
POME	Palm oil mill effluent
Qp	Ethanol productivity
Qs	Substrate uptake rate
R_f	Retention factor
rpm	Round per minute

LIST OF ABBREVIATIONS AND SYMBOLS (Cont.)

R_{max}	Maximum product production rate
RSM	Response Surface Methodology
SEC	Scientific Equipment Center
SEM	Scanning electron microscopy
SLR	Solid to liquid ratio
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TISTR	Thailand Institute of Scientific and Technological Research
TLC	Thin layer chromatography
v/v	Volume by volume
w/v	Weight by volume
λ	Lag time
μ	Specific growth rate
X_{max}	Maximum cells concentration
Yp/s	Ethanol yield

CHAPTER 1

INTRODUCTION

Palm oil is one of the major agro-industries in Southern Thailand. The palm oil industry has developed very fast in the last few years due to the use of palm oil as raw material for biodiesel production. The Thai government energy policy stated clearly to increase the national renewable energy (NRE) from 0.5% in 2002 to 8.0% in 2011 (Prasertsan and Sajjakulnukit, 2006). The continued increase of oil price in 2007-8 stimulated the increase production of ethanol from cassava and biodiesel from palm oil. To have adequate supply of raw material, the oil palm plantation has been planned to expand to 6.4 x 10^5 hectare (4 x 10^6 Rai) in 2011. This increased not only palm oil production but also its wastes. Conversion of these wastes to valuable products would be beneficial to both the industry and the country's economics.

At present there are more than 65 factories employing the standard oil extracting process (or the wet process) (Kasikorn Research Center, 2009). The standard palm oil milling process has a lot of wastes consisting of 20-28% empty fruit bunch (EFB), 11% palm pressed fiber (PPF) and 0.87 m^3/ton FFB of wastewater or palm oil mill effluent (POME) (Prasertsan and Prasertsan, 1996). Most of palm oil mill wastes are lignocellulosic materials that are biomass consisting of cellulose (34%), hemicellulose (26%) and lignin (28%) (Kaddami *et al*., 2006). Hemicellulose and cellulose can be converted by chemical reagent to pentose and hexose, respectively, then further converted to furfural and hydroxymethylfurfural, respectively, which finally converted to levulinic acid. Furfural is important as it is used as a selective solvent for separating saturated (ethane, propane, and butane) from unsaturated (ethelene, propylene, naptha, and aromatic compounds such as benzene, toluene, and p-xylene) in petroleum refining, gas, oil, and diesel fuel (Mansilla *et al*., 1998). On the other hand, furfural is an inhibitor of ethanol production from hemicellulose hydrolysis, furfural is therefore reduced and controlled (Gutierrez *et al*., 2006). A minimum content of pentosans in lignocellulosic material is around 18-20%

but only one third of this amount can be converted to furfural (Jaeggle, 1975; Montane *et al*., 1998).

In addition, hemicellulose can also be converted by chemical reagent or enzyme to monomeric sugars mostly as xylose, and then further converted by fermentation to bio-ethanol. PPF has been reported to its component, that consisting of cellulose, pentosan, and lignin (Aziz, *et al*., 2002); hence, its composition indicated the potential to use PPF as one of the suitable raw materials for bio-ethanol production. Bio-ethanol can be mixed with gasoline in the suitable ratio such as E10 (E10 means 10 % (v/v) ethanol mixed with 90 % (v/v) gasoline). The use of biotechnological science overcomes the environmental potentials such as greenhouse effect relating to the combustion of fossil fuels in chemical process.

The biological process of ethanol fuel production utilizing lignocellulose as substrate requires: (1) delignification to liberate cellulose and hemicellulose from their complex with lignin, (2) depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and (3) fermentation of mixed hexose and pentose sugars to produce ethanol (Lee, 1997). Bio-ethanol production by microorganisms from renewable biomass can reduce fossil fuel use and wastes from agro-industry can be appropriately utilized.

Review of literature

1. Standard palm oil milling process

Palm oil milling process can be classified into two types, dry process and wet process (or standard process), which is regardless the use of decanter (Prasertsan *et al*., 1990). Both processes have many differences such as process details, yield percentage, oil properties (color, viscosity, and smell) and amount of waste including advantages and disadvantages of each process. Palm oil milling process is known to generate large quantity of liquid and solid wastes which are lignocellulosic materials.

The wet process can be sub-classified into two types, decanter using and no decanter usage, and was usually used in large factories (Prasertsan *et al*.,

1990). The wet process using decanter is shown in Fig. 1. Initial step, fresh bunches were heated by steam at 120-130➡C pressure 40 pound/in^2 for 45 minutes. Heated bunches were fed to separator machine in order to separate seeds from bunches and then pericarps fibre were separated from nuts by digester machine. The pericarp fibre were fed in the screw press in order to extract palm oil and palm oil then was fed to decanter for separating palm oil from water and particles fibre including soil and dust. After that, palm oil was stored in the tank.

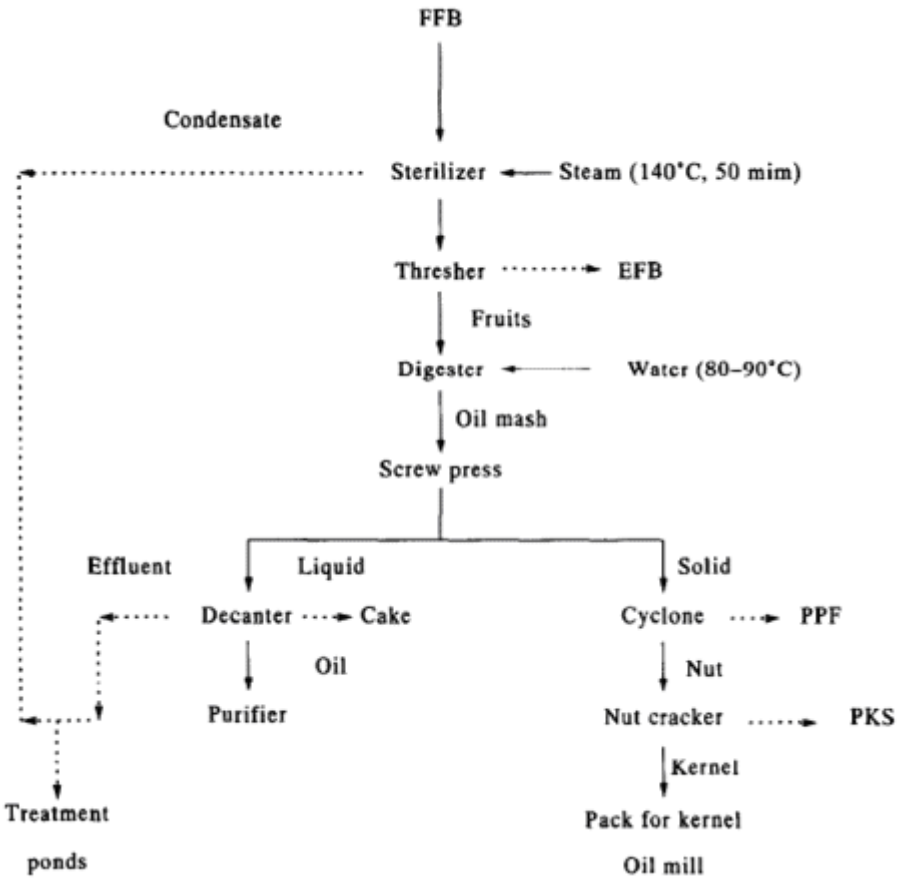


Figure 1. Schematic diagram of wet processing palm oil production using decanter (———) process; (·····) waste.

Source: Prasertsan and Prasertsan (1996)

2. Palm oil industry wastes and their utilization

Oil palm fruit consists of 68.3% pericarp and 31.7% nuts. The pericarp itself contains 23.3% pericarp fibre which has 6.4% (dry weight) holocellulose consisting of cellulose and hemicellulose (Kirdaldy and Sutanto, 1976). Due to the many components of oil palm fruit, the standard palm oil milling process have a lot of generating wastes consisting of 20-28% empty fruit bunch (EFB), 11-13% palm pressed fiber (PPF) and 0.87-1.50 m^3/ton FFB of wastewater or palm oil mill effluent (POME) (Prasertsan and Prasertsan, 1996; Sumathi *et al*., 2008).

PPF has been utilized as fuel for boiler, fiber board production (Prasertsan and Prasertsan, 1996; Abdul Khalil *et al*., 2007; Yin *et al*., 2008) and mushroom cultivation (Ayodele and Okhuoya, 2007). In addition, huge quantities of biomass by-products are developed to produce value added products such as methane gas, bio-plastic, organic acids, bio-compost, plywood, activated carbon, and animal feedstock (Sumathi *et al*., 2008). Even waste effluent, palm oil mill effluent (POME) has been converted to produce energy (Sumathi *et al*., 2008). Nevertheless, it is still surplus and left unused in some palm oil mills.

An alternative approach of utilizing PPF for ethanol production was proposed in this study because bioethanol production is growing rapidly worldwide due to a substitute for fossil fuels in the transportation sector. The USA is the largest ethanol producer in the world, closely followed by Brazil. Both of them produce approximately 70% to the world's total production (Linde *et al*., 2008). However, there is a material's difference for ethanol production in both countries; Brazil uses cane sugar while USA mainly utilizes starch from corn (Linde *et al*., 2008).

2.1 Empty fruit bunch (EFB)

EFB is the major component solid waste of oil palm industry. It is rich in sugars, especially glucose (42% dry basis) and xylose (24% dry basis). EFB was used as a raw material for xylose production (Rahman *et al*., 2006; Rahman *et al*., 2007). Operational conditions were controlled at 120➡C using various concentration of diluted sulfuric acid (2-6%) and reaction time (0-90 min). The optimal condition at reaction temperature of 120➡C was 6% sulfuric acid for 15 min giving the maximum xylose of 29.4 g/l (Rahman *et al*., 2006). Maximum concentration of xylose, 30.81 g/l

was achieved when reaction was carried out at 115➡C for 60 min with 4%sulfuric acid concentration (Rahman *et al*., 2007).

2.2 Palm pressed fiber (PPF)

Hemicellulose, a second major composition of PPF (Aziz *et al*., 2002; Kelly-Yong *et al*., 2007), could be converted to pentose sugars by using chemical (Abad *et al*., 1997; Karimi *et al*., 2006) or enzymes (Saha *et al*., 2005; Karimi *et al*., 2006). The pentose sugar, on the other hand, can be fermented to produce xylitol from xylose (Téllez-Luis *et al*., 2002) and ethanol from xylose/arabinose (Limtong *et al*., 2000; Sun and Cheng, 2005; Cheng *et al*., 2007). Through chemical process, the pentose sugars can be converted directly to furfural and levulinic acid (Mansilla *et al*., 1998). Furfural normally has been used in petroleum refining, gas, oil and diesel fuel as a selective solvent (Mansilla *et al*., 1998) while levulinic acid could be used as plasticizer, textile, animal feed, fuel extender (methyltetrahydrofuran, MTHF), coating material and antifreeze (Bozell *et al*., 2000). Furfural can be produced by either one-stage or two-stage process (Mansilla *et al*., 1998; Punsuvon *et al*., 2008). In one-stage process, pentosan is hydrolyzed into xylose and then dehydrated to furfural within the same reactor. This process gave low yield of furfural (0.7-3.3 wt %) (Mansilla *et al*., 1998) and the residue solid can be used as a fuel. For two-stage process, hydrolysis and dehydration reaction process took places in two different reactors. The advantages of the two-stage process are higher furfural yield (3-15 wt %) than the one-stage process (Mansilla *et al*., 1998). The residue solid can be utilized for production of cellulose, glucose and ethanol via fermentation (Punsuvon *et al*., 2008).

2.3 Palm kernel shell (PKS)

PKS is the most hardly waste to decompose and usually used in the factory as firewood in boiler or disposed of by the land-fill method. Moreover, PKS has been successfully used to produce the activated carbon because of high carbon content (20.3%) and physically similar to the coconut shell. The demand of activated carbon in the future will be increased due to the stringent environmental control measurement (Prasertsan *et al*., 1996).

2.4 Palm oil mill effluent (POME)

POME is the mixed effluent generated from two major wastewaters; sterilizer and decantor during the palm oil extraction in the wet process (Prasertsan *et al*., 1990). The compositions of POME are given in Table 1 (Suwansaard, 2010). However, the milling process produces a huge volume of POME. Disposal of these wastes is already an economic burden on communities and industries, so creating a marketable product from this waste would reduce the treatment cost. Recovery of energy from waste might reduce the cost of wastewater treatment, and contribute to reducing our dependence on fossil fuel. Hydrogen and energy production could mitigate these problems. Hydrogen production by microorganisms can be divided into two main categories: one involves the use of photosynthetic bacteria (Suwansaard *et al*., 2009) and algae under light conditions and the other, anaerobic fermentative bacteria under dark conditions (O-Thong *et al*., 2008).

Table 1. Characteristics of palm oil mill effluent (POME)

Compositions	Unit	POME from JK Import Export Co., Ltd.	POME from Trang Palm Oil Co., Ltd.
pH		4.50	4.2-4.5
Color		Brown	Brown
Biochemical oxygen demand (BOD)	mg l^{-1}	38,740	22,000-54,300
Chemical oxygen demand (COD)	mg l^{-1}	50,057	75,200-96,300
Total nitrogen	mg l^{-1}	460	830-920
Acetic acid	mM	76.43	nr
Propionic acid	mM	27.98	nr
Butyric acid	mM	24.71	nr

nr: no reported

Source: O-Thong et al. (2008); Suwansaard, (2010)

3. Characteristics of lignocellulosic materials

In general, lignocellulosic wastes contain 70-80% carbohydrates consisting of 40–55% cellulose, 15–35% lignin and 25–40% hemicellulose (dry basis) (Pushpamalar *et al*., 2006) whereas oil palm biomass consists of 32-42% cellulose, 21-38% hemicellulose and 11-27% lignin (Fig. 2) (Hamelinck *et al*., 2003; Aziz *et al*., 2002; Kelly-Yong *et al*., 2007; Gutiérrez *et al*., 2009).

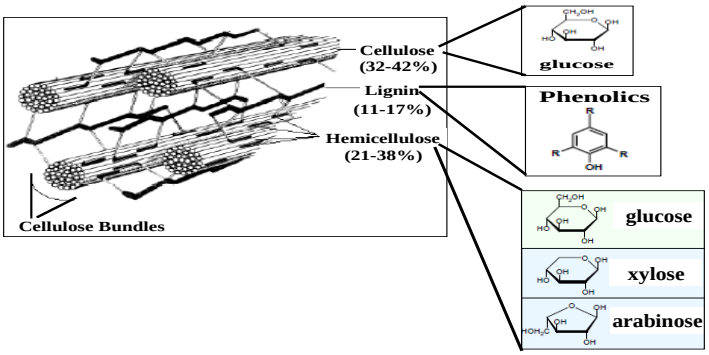


Figure 2. The composition of palm pressed fiber (PPF) and the final products.
Source: Modified from Hamelinck *et al*. (2003)

3.1 Cellulose

Cellulose with a molecular weight of about 100,000 is essentially a polymer with linear chains of glucopyranose units linked to each other by its ⇆-1, 4 in the a configuration (Goyal *et al*., 2006). However, the basic building block of cellulose is actually cellobiose, a dimer of two-glucose unit linked by β-(1-4) glycosidic bonds between C(4) of one sugar unit and the anomeric C(1) of the other (Fig. 3) (Ramos, 2003; Pushpamalar *et al*., 2006). As glucose units are linked together into polymer chains, a molecule of water is lost, which makes the chemical formula $C_6H_{10}O_5$ for each monomer unit of "glucan".

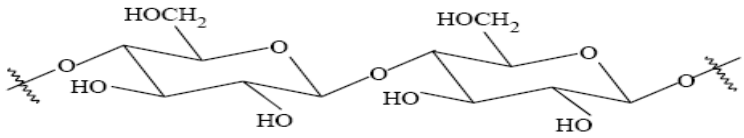


Figure 3. Fragment of a cellulose chain.

Source: Pushpamalar *et al*. (2006)

3.2 Hemicellulose

Hemicellulose, component of cell wall, is a complex mixture of several polysaccharides such as pentoses (xylose, rhamnose and arabinose), hexoses (glucose, mannose and galactose) and uronic acids (4-*O*-methyl-glucuronic and galacturonic acids) (Fig. 4). Its average molecular weight is of about 30,000, (Goyal *et al*., 2006). In plant, hemicelluloses are normally connected to lignin (Ramos, 2003) which is a polymer of *p*-hydroxyphenylpropane units.

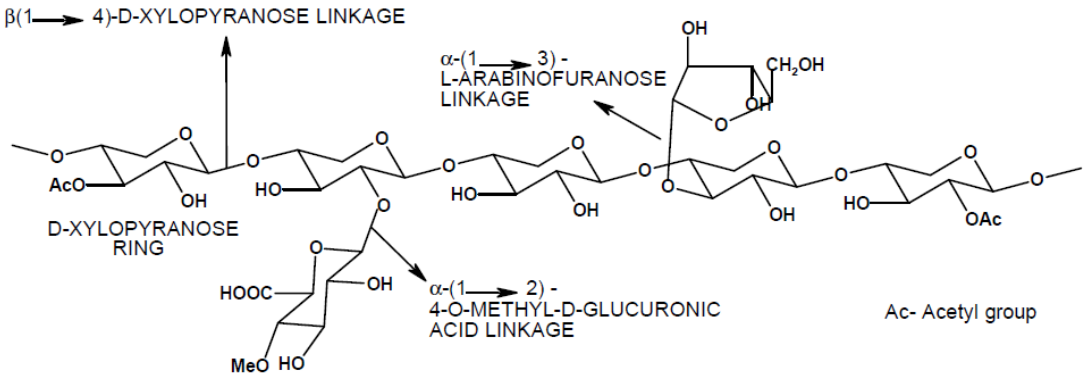


Figure 4. Fragment of a hemicellulose chain.

Source: Lachke (2002)

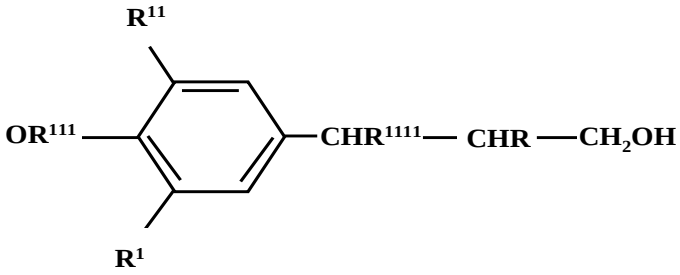
3.3 Lignin

Lignin, component of cell wall, is a mononuclear aromatic polymer of phenylpropane units linked in a three dimensional structure (Fig. 5) (Lee, 1997) also found in the cell wall. Due to the near position of hemicellulose and lignin in the cell wall, adjacent to each other, both these compounds can form a complex termed as lignocellulose (Goyal *et al*., 2006). The principal structure elements of lignin have been clarified. Biosynthetically, lignin releases from lignocellulosic materials via formation of three precursor alcohols (Lee, 1997):

(1) *p*-hydroxycinnamyl (coumaryl) alcohol, which gives rise to *p*-hydroxyphenyl units in the polymer

(2) 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units

(3) 4-hydroxy-3,5-dimethoxy-cinnamyl (sinapyl) alcohol, the syringyl units



R = another phenyl propane unit
R^{111} = H or R
R^{1111} = OH or R

Guaiacyl: $R^1 = OCH_3$, R^{11} = H
Syringyl: $R^1 = R^{11} = OCH_3$
p-hydroxyphenyl: $R^1 = R^{11}$ = H

Figure 5. The elementary phenylpropane building blocks of various lignin.
Source: Lee (1997)

The chemical bonds linked between lignin and hemicellulose are ester, ether and glycosidic bonds. The ether linkages are more common and stronger than ester bonds between lignin and carbohydrates. These units and bonds, therefore, it makes lignins extremely resistant to chemical and enzymatic degradation. However, chemical degradation can be achieved by alkali and some catalysts hydrolysis. Biological degradation of lignin can be achieved by white rot fungi (Ohkuma *et al*., 2001).

4. Lignocellulosic pretreatment

It is evident that the importance of lignocellulosic biomass as a feedstock for ethanol production. Lignocellulosic complex is the most abundant biopolymer in the Earth. It is considered that lignocellulosic biomass comprises about 50% of world biomass. Its annual production was estimated in 10–50 billion ton (Claassen *et al*., 1999). In general, prospective lignocellulosic materials for fuel ethanol production can be divided into six main groups: crop residues (cane bagasse,

corn stover, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose wastes (newsprint, waste office paper, recycled paper sludge), herbaceous biomass (alfalfa hay, switchgrass, reed canary grass, coastal bermudagrass, thimothy grass), and municipal solid wastes (MSW). The composition of most of these materials can be found elsewhere (Sun and Cheng, 2002; Sánchez *et al*., 2008).

Lignocellulosic materials are difficultly converted to sugars by directly biological methods because a lignin network covers the layer of cell walls. Therefore, various physical, chemical and biological pretreatments have been developed in order to degrade or remove the lignin in the biomass (Asada *et al*., 2005).

4.1 Physical methods

4.1.1 Mechanical comminution

The objective of the mechanical pretreatment is a reduction of particle size and cristallinity of lignocellulosic in order to increase the specific surface and reduce the degree of polymerization. This can be produced by a combination of chipping, grinding or milling depending on the final particle size of the material (10–30 mm after chipping and 0.2–2 mm after milling or grinding) (Sun and Cheng, 2002). Different milling processes (ball milling, two-roll milling, hammer milling, colloid milling and vibro energy milling) can be used to improve the enzymatic hydrolysis of lignocelullosic materials (Taherzadeh and Karimi, 2008). The energy requirements of mechanical comminution of lignocellulosic materials depend on the final particle size and biomass characteristics. Although mechanical pretreatment methods increase cellulose reactivity towards enzymatic hydrolysis, they are unattractive due to their high energy and capital costs (Ghosh and Ghose, 2003; Sánchez *et al*., 2008).

4.1.2 Pyrolysis

Pyrolysis has also been tested as a physical method for pretreatment of lignocellulosic biomass since cellulose rapidly decomposes when is treated at high temperatures (>300➡C) (Sánchez *et al*., 2008).

4.1.3 Extrusion

Extrusion process is a novel and promising physical pretreatment method for biomass conversion to ethanol production. In extrusion, the materials are subjected to heating, mixing and shearing, resulting in physical and chemical modifications during the passage through the extruder. Screw speed and barrel temperature are believed to disrupt the lignocellulose structure causing defibrillation, fibrillation and shortening of the fibers, and, in the end, increasing accessibility of carbohydrates to enzymatic attack. The different bioreactor parameters must be taken into account to achieve the highest efficiency in the process. In recent studies application of enzymes during extrusion process is being considered as a promising technology for ethanol production (Alvira *et al*., 2010).

4.2 Physical-chemical methods

4.2.1 Steam explosion

Steam explosion is a process for separation of cellulose, hemicellulose and lignin using high temperature (more than 200➡C) and high pressure (1.5 Mpa) for a short time (1-10 min.). The hydrolysis of glycosidic linkages in hemicelluloses and the β-*O*-4 ether bonds in lignin are cleaved by acetic acid formed at high temperature from acetyl groups present in hemicelluloses (autohydrolysis) (Sun *et al*., 2005; Asada *et al*., 2005; Chen *et al*., 2005). Therefore, during steam explosion, significant amounts of hemicelluloses are partially hydrolysed and some lignin is depolymerised. Sugars and phenolic compounds are soluble in water whereas the exploded solid residue is cellulose. Some of the possible end products of steam-exploded wood are ethanol, xylitol, lactic acid and furfural or furfural derivatives (Sun *et al*., 2005). After treating with steam explosion, hemicelluloses are recovered from the exploded fiber by three times of washing with water at 90➡C for 15 min at a fiber to water ratio of 1/20 w/w. The washed solution is concentrated under vacuum at 60➡C in an evaporator (Montane *et al*., 1998). Lignin removal from wheat straw has been studied by using a two-stage process based on steam explosion pre-treatment and followed by hydrogen peroxide in alkaline condition post-treatment gave the maximum lignin removal yield (92.3-99.4%) when the steam explosion pre-treatment was performed at 220➡C, 22 atm for 3 min with a solid to liquid ratio of 2:1 (Sun *et*

al., 2005). Steam explosion pretreatment of bagasse has also been studied. The effective removal of lignin under 206➡C for 4 min was 50.7% (Punsuvon *et al*., 2008).

4.2.2 Liquid hot water (LHW)

One of the most promising methods is the pretreatment in the neutralization process with liquid hot water (LHW) or thermohydrolysis that does not employ any catalysts (Sánchez *et al*., 2008; Pérez *et al*., 2008). LHW pretreatment, in which pressure is utilized to maintain water in the liquid state at elevated temperatures, has been reported to have the potential to remove most hemicellulose while minimizing cellulose hydrolysis and sugar degradation reactions. For example, it has been shown to remove up to 80% of the hemicellulose and to enhance the enzymatic digestibility of pretreated material in herbaceous feedstocks, such as corn fiber and sugarcane bagasse (Párez *et al*., 2008). Two fractions are obtained after filtration of the slurry generated in LHW pretreatment: one cellulose-enriched (water-insoluble solids fraction) and another one rich in hemicellulose-derived sugars (HDS, liquid fraction or prehydrolyzate).

Laser et al. (2002) mention that under optimal conditions, this method is comparable to dilute acid pretreatment but without addition of acids or production of neutralization wastes. In addition, this technology presents elevated recovery rates of pentoses and does not generate inhibitors (Sánchez *et al*., 2008). Nevertheless, solid load is much less than for steam explosion, which is usually greater than 50%. Wheat straw was also studied by using LHW. The optimal conditions were 188➡C and 40 min, leading to HDS recovery yield of 43.6% of HDS content in raw material and enzymatic hydrolysis (EH) yield of 79.8% of theoretical was obtained (Párez *et al*., 2008).

4.2.3 Microwave hydrolysis

Microwave-based pretreatment can be considered a physicochemical process since both thermal and non-thermal effects are often involved (Alvira *et al*., 2010). Pretreatments were carried out by immersing the biomass in dilute chemical reagents and exposing the slurry to microwave radiation for residence times ranging from 5 to 20 min (Keshwani, 2009). Preliminary experiments identified alkalis as

suitable chemical reagents for microwave-based pretreatment. An evaluation of different alkalis identified sodium hydroxide as the most effective alkali reagent (Alvira *et al*., 2010). Wheat bran pretreatment was achieved by microwave giving high yields of glucose and xylose when performed under 170°C with no sulfuric acid for various reaction time (20, 30 and 40 min). In additionally, pretreatment temperatures below 170°C have been shown to yield low concentration of furfural and HMF (Palmarola-Adrados *et al*., 2005). The advantages of this pretreatment are not only to decrease amount of sample required and the vessel also provides a close system with a constant amount of material throughout the process (Palmarola-Adrados *et al*., 2005).

4.2.4 Ammonia fiber explosion (AFEX)

In the AFEX process, biomass is treated with liquid anhydrous ammonia at temperatures between 60 and 100➡C and high pressure for a variable period of time (Alvira *et al*., 2010). The pressure is then released, resulting in a rapid expansion of the ammonia gas that causes swelling and physical disruption of biomass fibers and partial decrystallization of cellulose. While some other pretreatments such as steam explosion produce slurry that can be separated in a solid and a liquid fractions, AFEX produces only a pretreated solid material. AFEX has been reported to decrease cellulose crystallinity and disrupt lignin–carbohydrates linkages (Laureano-Pérez *et al*., 2005). During the pretreatment only a small amount of the solid material is solubilized; little hemicellulose and lignin is removed (Wyman *et al*., 2005a). Deacetylation of hemicellulose is also observed. AFEX removes the least acetyl groups from certain lignocellulosic materials (Kumar *et al*., 2009b). Digestibility of biomass is increased after AFEX pretreatment (Galbe and Zacchi, 2007) and therefore the enzymatic hydrolysis results in greater yields. Both cellulases and hemicellulases will be required in hydrolysis process due to the considerable remaining hemicellulose in the pretreated material (Alvira *et al*., 2010).

Ammonia recovery and recycle is feasible despite of its high volatility (Teymouri *et al*., 2005) but the associated complexity and costs of ammonia recovery may be significant regarding commercial potential of the AFEX pretreatment (Mosier *et al*., 2005b). No formation of inhibitors for the downstream biological processes is

one of the main advantages of the ammonia pretreatment, even though some phenolic fragments of lignin and other cell wall extractives may remain on the cellulosic surface (Alvira *et al*., 2010). The AFEX pretreatment is more effective on agricultural residues and herbaceous crops, with limited effectiveness demonstrated on woody biomass and other high lignin feedstocks (Wyman *et al*., 2005a). There have been reported recent strategies to optimize the conditions in the AFEX pretreatment in studies using different materials (Teymouri *et al*., 2005). At optimal conditions AFEX can achieve more than 90% conversion of cellulose and hemicellulose to fermentable sugars for a broad variety of lignocellulosic materials. In fact, despite of little removal of lignin or hemicellulose in the AFEX process, enzymatic digestion at low enzyme loadings results very high comparing other pretreatment alternatives (Wyman *et al*., 2005b). This may suggest that ammonia affects lignin and possibly hemicellulose differently than other chemicals, reducing the ability of lignin to adsorb enzyme and/or to make its access to cellulose more difficult (Alvira *et al*., 2010). Barley hull was pretreated by using aqueous ammonia, to be converted into sugars. The best pretreatment conditions were 75°C for 48 h with 15 wt% aqueous ammonia and 1:12 of solid to liquid ratio resulting in saccharification yield of 83 % glucan and 63 % xylan with decrease of 50-66% of the original lignin (Kim *et al*., 2008).

4.2.5 CO_2 explosion

Carbon dioxide explosion is also used for lignocellulosic biomass pretreatment. The method is based on the utilization of CO_2 as a supercritical fluid, which refers to a fluid that is in a gaseous form but is compressed at temperatures above its critical point to a liquid like density. Supercritical pretreatment conditions can effectively remove lignin increasing substrate digestibility (Alvira *et al*., 2010). Addition of co-solvents such ethanol can improve delignification. Supercritical carbon dioxide (SC-CO_2) has been mostly used as an extraction solvent but it is being considered for non-extractive purposes due to its many advantages (Schacht *et al*., 2008). In aqueous solution CO_2 forms carbonic acid, which favors the polymers hydrolysis. CO_2 molecules are comparable in size to water and ammonia and they can penetrate in the same way the small pores of lignocellulose. This mechanism is facilitated by high pressure. After the explosive release of CO_2 pressure, disruption of

cellulose and hemicellulose structure is observed and consequently accessible surface area of the substrate to enzymatic attack increases. Operation at low temperatures compared to other methods prevents monosaccharides degradation, but in comparison to steam and ammonia explosion sugar yields obtained are lower. Nevertheless a comparison of different pretreatment methods on several substrates showed that CO_2 explosion was more cost-effective than ammonia explosion and formation of inhibitors was lower compared to steam explosion (Zheng *et al*., 1998).

4.2.6 Irradiation pretreatment

Ionizing irradiation can modify and disrupt the structure of lignocellulose and can be an effective method of pretreatment of lignocellulosic biomass for sugar production (Chunping *et al*., 2008). The irradiation degradation of various lignocellulosic materials for increasing sugar yield has been reported, such as bagasse (Han *et al*., 1981), rice straw, chaff, sawdust (Kumakura and Kaetsu, 1979, 1984a), corn stalk, peanut husk (Chosdu *et al*., 1993), oil palm empty fruit bunch (Matsuhashi *et al*., 1995). The radiationinduced reactions in the macromolecules of the cellulose materials are known to be initiated through rapid localization of the absorbed energy within the molecules to produce long- and short-lived radicals which caused the secondary degradation of materials through chemical reactions such as chain scission, cross-linking, and so forth (Khan *et al*., 2006). The efficiency of these two types of reactions depends mainly on the polymer structure and radiation dose (Charlesby, 1981).

4.2.7 Ultrasound pretreatment

The effect of ultrasound on lignocellulosic biomass have been employed for extracting hemicelluloses, cellulose and lignin but less research has been addressed to study the susceptibility of lignocellulosic materials to hydrolysis (Alvira *et al*., 2010). In spite of the minor research on ultrasound pretreatment from lignocellulose, some researchers have also shown that saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment (Yachmenev *et al*., 2009). Higher enzymatic hydrolysis yields after ultrasound pretreatment could be explained because cavitation effects caused by introduction of ultrasound field into the enzyme processing solution greatly enhance the transport of enzyme macromolecules toward

the substrate surface (Alvira *et al*., 2010). Furthermore, mechanical impacts, produced by the collapse of cavitation bubbles, provide an important benefit of opening up the surface of solid substrates to the action of enzymes, in addition, the maximum effects of cavitation occur at 50➡C, which is the optimum temperature for many enzymes (Yachmenev *et al*., 2009).

4.3 Chemical methods

Chemical processes are an efficient method for breaking the large complex structure of biomass to the small molecules including separation of their components (cellulose, hemicellulose and lignin). These methods have to control various parameters such as temperature, reaction time and chemical concentration, etc because of the vigor reaction.

4.3.1 Alkali pretreatment

The effect that some bases have on lignocellulosic biomass is the basis of alkaline pretreatments, which are effective depending on the lignin content of the biomass. Alkali pretreatments increase cellulose digestibility and they are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydrothermal processes (Carvalheiro *et al*., 2008). Alkali pretreatment can be performed at room temperature and times ranging from seconds to days. It is described to cause less sugar degradation than acid pretreatment and it was shown to be more effective on agricultural residues than on wood materials (Kumar *et al*., 2009). Nevertheless, possible loss of ethanol production due to the presence of toxic compounds during alkaline hydrolysis must be taken into consideration to optimize the pretreatment conditions (Alvira *et al*., 2010).

Sodium, potassium, calcium and ammonium hydroxides are suitable alkaline pretreatments. NaOH causes swelling, increasing the internal surface of cellulose and decreasing the degree of polymerization and cristallinity, which provokes lignin structure disruption (Taherzadeh and Karimi, 2008). For example, wheat straw was pretreated with a 0.5 M NaOH solution for 6 h at 80➡C in a thermostated 2 liters batch stirred reactor (300 rpm) using a solid–liquid ratio of 5% (w/v) and after that, it was cool down immediately (Ramos, 2003). NaOH has been

reported to increase hardwood digestibility from 14% to 55% by reducing lignin content from 24–55% to 20% (Kumar *et al*., 2009).

$Ca(OH)_2$, also known as lime, has been widely studied. Lime pretreatment removes amorphous substances such as lignin, which increases the crystallinity index (Alvira *et al*., 2010). Lignin removal increases enzyme effectiveness by reducing non-productive adsorption sites for enzymes and by increasing cellulose accessibility (Kim and Holtzapple, 2006). Lime also removes acetyl groups from hemicellulose that is an obstacle of enzymes and enhancing cellulose digestibility (Mosier *et al*., 2005a). Lime has been proven successfully at temperatures from 85–150➡C and for 3–13 h with corn stover (Kim and Holtzapple, 2006) or poplar wood (Chang *et al*., 2001). Pretreatment with lime has lower cost and less safety requirements compared to NaOH or KOH pretreatments and can be easily recovered from hydrolysate by reaction with CO_2 (Mosier *et al*., 2005a).

Addition of an oxidant agent (oxygen/H_2O_2) to alkaline pretreatment (NaOH/$Ca(OH)_2$) can improve the performance by favoring lignin removal (Carvalheiro *et al*., 2008). Ethanol yields of 0.33 g/g have been obtained in simultaneous saccharification and fermentation (SSF) processes with *Escherichia coli* FBR5 from wheat straw pretreated with alkali peroxide (Saha and Cotta, 2006). Furthemore, no furfural or HMF were detected in hydrolysates obtained with alkaline peroxide pretreatment which favours the fermentation step in an ethanol production process (Taherzadeh and Karimi, 2008).

4.3.2 Ozonolysis pretreatment

Ozone pretreatment is a process for reducing the lignin content of lignocellulosic materials. Ozone was used to degrade lignin and hemicellulose in many biomasses i.e. cotton straw, wheat straw, bagasse, pine, peanut, green hay and poplar sawdust. The degradation is mainly limited to lignin. Hemicellulose is slightly affected, but cellulose is not degraded (Kumar *et al*., 2009). The pretreatment is usually performed at room temperature and normal pressure and does not lead to the formation of inhibitory compounds that can affect the subsequent hydrolysis and fermentation (Alvira *et al*., 2010). After wheat straw pretreatment by ozone, the rate of enzymatic hydrolysis increased following 60% removal of the lignin. Enzymatic

hydrolysis yield of poplar sawdust increased from 0% to 57% as the lignin decreased from 29% to 8% (Kumar *et al*., 2009). Despite of some interesting results further research has to be performed regarding ethanol production from lignocellulosic materials pretreated with ozone. An important drawback to consider is the large amounts of ozone needed, which can make the process economically unviable (Sun and Cheng, 2002).

4.3.3 Acid hydrolysis pretreatment

The main objective of the acid pretreatments is to solubilize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes (Alvira *et al*., 2010). Diluted acid pretreatment appears as more favorable method for industrial applications and have been studied for pretreating wide range of lignocellulosic biomass. Different types of reactors such as percolation, plug flow, shrinking-bed, batch and countercurrent reactors have been applied for pretreatment of lignocellulosic materials (Taherzadeh and Karimi, 2008). It can be performed at high temperature (e.g. 180➡C) during a short period of time; or at lower temperature (e.g. 120➡C) for longer retention time (30–90 min). It presents the advantage of solubilizing hemicellulose, mainly xylan, but also converting solubilized hemicellulose to fermentable sugars (Saha, 1999). Nevertheless, depending on the process temperature, some sugar degradation compounds such as furfural and hydroxyl methyl furfural (HMF) and aromatic lignin degradation compounds are detected (Rahman *et al*., 2006; Rahman *et al*., 2007), and affect the microorganism metabolism in the fermentation step (Saha *et al*., 2005). Anyhow, this pretreatment generates lower degradation products than concentrated acid pretreatments. Concentrated acid are toxic, corrosive, hazardous and required the specific reactor that are resistant to corrosion, which results the pretreatment process very expensive. Moreover, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sun and Cheng, 2002).

High hydrolysis yields have been reported when pretreating lignocellulosic materials with diluted H_2SO_4 which is the most studied acid. Hydrochloric acid, phosphoric acid and nitric acid have also been tested (Mosier *et al*., 2005a). Saccharification yield as high as 74% was shown when wheat straw was

subjected to 0.75% v/v of H_2SO_4 at 121➡C for 1 h (Saha *et al*., 2005). Olive tree biomass was pretreated with 1.4% H_2SO_4 at 210➡C resulting in 76.5% of hydrolysis yields (Cara *et al*., 2008). Recently, ethanol yield as high as 0.47 g/g glucose was achieved in fermentation tests with cashew apple bagasse pretreated with diluted H_2SO_4 at 121➡C for 15 min (Rocha *et al*., 2009).

Organic acids such as fumaric or maleic acids are appearing as alternatives to enhance cellulose hydrolysis for ethanol production. In this context, both acids were compared with sulfuric acid in terms of hydrolysis yields from wheat straw and formation of sugar degradation compounds during pretreatment. Results showed that organic acids can pretreat wheat straw with high efficiency although fumaric acid was less effective than maleic acid. Furthermore, less amount of furfural was formed in the maleic and fumaric acid pretreatments than with sulfuric acid (Kootstra *et al*., 2009).

4.3.4 Oxidative delignification pretreatment

Degradation of lignin could be catalyzed by the peroxidase enzyme with the presence of hydrogen peroxide (H_2O_2). The pretreatment of bagasse with H_2O_2 greatly enhanced its susceptibility to enzymatic hydrolysis. 50% of the lignin content and most of the hemicellulose were solubilized by 2% H_2O_2 at 30➡C within 8 hours, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45➡C for 24 hours (Azzam, 1986).

4.3.5 Organosolv pretreatment

In the organosolvation process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (sulfuric acid, hydrochloric acid, oxalic acid, acetylsalicylic acid and salicylic acid) is used to break the internal lignin and hemicellulose bonds (Chum *et al*., 1988). A high yield of xylose can usually be obtained with the addition of acid. However, this acid addition can be avoided for a satisfactory delignification by increasing process temperature (above 185➡C) (Alvira *et al*., 2010). The solvents commonly used in the process are methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Chum *et al*., 1988). Pulps and its lignin content between 6.4% and 27.4% (w/w) have been prepared from mixed softwoods using a biorefining technology or lignol process,

which is based on an aqueous ethanol organosolvation extraction (Pan *et al*., 2005). This process uses a blend of ethanol and water in the ratio of 50:50 (w/w) at 200➡C with a pressure of 400 psi to extract most of the lignin content from wood chips or other lignocellulosic materials. All pulps were readily hydrolyzed and lignin was removed to less than 18.4%). More than 90% of the cellulose in low lignin pulps was hydrolyzed to glucose in 48 hours.

Comparing to other chemical pretreatments the main advantage of organosolv process is the recovery of relatively pure lignin as a by-product (Zhao *et al*., 2009a). Removal of solvents from the system is necessary using appropriate extraction and separation techniques, e.g., evaporation and condensation, and they should be recycled to reduce operational costs. Solvents need to be separated because they might be inhibitory to enzymatic hydrolysis and fermentative microorganisms (Sun and Cheng, 2002). The high commercial price of solvents is another important factor to consider for industrial applications. For economic reasons, among all possible solvents, the low-molecular weight alcohols with lower boiling points such as ethanol and methanol are favored (Alvira *et al*., 2010).

4.3.6 Wet oxidation (WO)

In wet oxidation, the lignocellulosic biomass is treated with water and high pressure oxygen or air at elevated temperatures (more than 120➡C) (Talebnia *et al*., 2010). Typical oxygen pressure range is 120–480 psi (Schmidt and Thomsen, 1998). WO is an effective pretreatment method for the fractionation of wheat straw into a solubilized hemicellulose fraction and a cellulose-rich solid fraction with high susceptibility to enzymatic hydrolysis. Combination of alkali and WO not only improves the rate of lignin oxidation (and in turn enzymatic hydrolysis) but also prevents formation of furfural and HMF (Bjerre *et al*., 1996). Acids formed during initial reaction in WO due to solubilization of hemicellulose components catalyze the subsequent hydrolytic reactions through which hemicelluloses are broken down into lower molecular weight fragments that are soluble in water (Talebnia *et al*., 2010). Lignin degradation is also significant especially at the higher temperatures because phenol-like compounds and carbon–carbon linkage are very reactive under wet

oxidation conditions. Lignin is decomposed to CO_2, H_2O and carboxylic acids (Klinke *et al*., 2002).

4.3.7 Ionic liquids (ILs) pretreatment

The use of ILs as solvents for pretreatment of cellulosic biomass has recently received much attention. ILs are salts, typically composed of large organic cations and small inorganic anions, which exist as liquids at relatively low temperatures; often at room temperature. Their solvent properties can be varied by adjusting the anion and the alkyl constituents of the cation. These interesting properties include chemical and thermal stability, non-flammability, low vapor pressures and a tendency to remain liquid in a wide range of temperatures (Hayes, 2009). Since no toxic or explosive gases are formed, ILs is called ‘‘green” solvents. Carbohydrates and lignin can be simultaneously dissolved in ILs with anion activity (e.g. the 1-butyl-3 methylimidazolium cation $[C4mim]^+$) because ILs form hydrogen bonds between the non-hydrated chloride ions of the IL and the sugar hydroxyl protons in a 1:1 stoichiometry. As a result, the intricate network of non-covalent interactions among biomass polymers of cellulose, hemicellulose, and lignin is effectively disrupted while minimizing formation of degradation products. However, most data showing the effectiveness of ILs has been developed using pure crystalline cellulose, and its applicability to a more complex combination of constituents in lignocellulosic biomass requires further studies (Alvira *et al*., 2010). Nevertheless, the use of ILs has also been already demonstrated on some lignocellulosic feedstocks such as straw (Li *et al*., 2009) or wood (Lee *et al*., 2009). Toxicity to enzymes and fermentative microorganisms must be studied before ILs can be considered a real option for biomass pretreatment (Yang and Wyman, 2008; Zhao *et al*., 2009b). Depending on the amount of ILs residues remaining, significative negative effect on cellulase activity may be observed. Thus, ILs residues removal would be required to prevent decrease of final sugars concentrations (Alvira *et al*., 2010).

In a pretreatment study using 1-ethyl-3-methyl imidazolium diethyl phosphate, the yield of reducing sugars from wheat straw pretreated with this ionic liquid at 130➡C for 30 min was 54.8% after being enzymatically hydrolyzed for 12 h

(Li *et al*., 2009). The fermentability of the hydrolysates obtained after enzymatic saccharification of the regenerated wheat straw was also evaluated. Results obtained using *Saccharomyces cerevisiae* indicated that wheat straw pretreated by this IL did not bring any negative effect on the growth of *S. cerevisiae* (Li *et al*., 2009).

4.4 Biological methods

Fungal pretreatment has been previously explored to upgrade lignocellulosic materials for feed and paper applications. Recently, this environmentally friendly approach has received renewed attention as a pretreatment method for enhancing enzymatic saccharification of lignocellulosic biomass in ethanol production processes (Alvira *et al*., 2010). The potential method for removing lignin and releasing fermentable sugars is pretreatment followed by enzymatic and acidic hydrolysis. Lignin could be degraded by several fungal enzymes such as lignin peroxidase, Mn-dependent peroxidase, and laccase (mono-phenol oxidase) and its degradability depend on fungal strain, accessibility of lignin to enzyme, culture condition, and reactor design (Ohkuma *et al*., 2001). Biological pretreatments employ microorganisms mainly brown, white and soft-rot fungi which degrade lignin and hemicellulose and very little of cellulose, more resistant than the other components (Sánchez, 2009). Lignin degradation by white-rot fungi is the most effective for biological pretreatment of lignocellulosic materials (Alvira *et al*., 2010).

Several white-rot fungi such as *Phanerochaete chrysosporium*, *Ceriporia lacerata*, *Cyathus stercolerus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnarbarinus* and *Pleurotus ostreaus* have been examined on different lignocellulosic biomass showing high delignification efficiency (Kumar *et al*., 2009; Shi *et al*., 2008). Biological pretreatment by white-rot fungi has been combined with organosolv pretreatment in an ethanol production process by simultaneous saccharification and fermentation (SSF) from beech wood chips (Itoh *et al*., 2003). Results from other recent studies have shown that fungal pretreatment of wheat straw for 10 days with a high lignin-degrading and low cellulose-degrading fungus (fungal isolate RCK-1) resulted in a reduction in acid loading for hydrolysis, an increase in the release of fermentable sugars and a reduction in the concentration of fermentation

inhibitors. Ethanol yield and volumetric productivity with *Pichia stipitis* were 0.48 g/g and 0.54 g/l/h, respectively (Kuhar *et al*., 2008).

The major sugars in enzyme hydrolysates are glucose and xylose released from cellulose and hemicellulose, respectively. The advantages of biological delignification may include higher product yields, fewer side reactions, less energy demand and less reactor resistance because of mild reaction condition. However, the main drawback to develop biological methods is the low hydrolysis rate obtained in most biological materials compared to other technologies (Sun and Cheng, 2002).

To move forward a cost-competitive biological pretreatment of lignocellulose, and improve the hydrolysis to eventually improve ethanol yields, there is a need to keep on studying and testing more basidiomycetes fungi for their ability to delignify the plant material quickly and efficiently (Alvira *et al*., 2010).

5. Key factors for an effective pretreatment of lignocellulosic biomass

There are several key properties to take into consideration for low-cost and advanced pretreatment process (Yang and Wyman, 2008; Alvira *et al*., 2010):

5.1 High yields for multiple crops, sites ages, harvesting times

Various pretreatments have been shown to be better suited for specific feedstocks. For example, alkaline-based pretreatment methods such as lime, ammonia fiber explosion (AFEX), and ammonia recycling percolation (ARP), can effectively reduce the lignin content of agricultural residues but are less satisfactory for processing recalcitrant substrate as softwoods. Acid based pretreatment processes have been shown to be effective on a wide range of lignocellulose substrate, but are relatively expensive.

5.2 Highly digestible pretreated solid

Cellulose from pretreatment should be highly digestible with yields higher than 90% in less than five and preferably less than 3 days with enzyme loading lower than 10 FPU/g cellulose (Yang and Wyman, 2008).

5.3 No significant sugars degradation

High yields close to 100% of fermentable cellulosic and hemicellulosic sugars should be achieved through pretreatment step (Alvira *et al*., 2010).

5.4 Minimum amount of toxic compounds

The liquid hydrolyzate from pretreatment must be fermentable following a low-cost, high yield conditioning step. Harsh conditions during pretreatment lead to a partial hemicellulose degradation and generation of toxic compounds derived from sugar decomposition that could affect the proceeding hydrolysis and fermentation steps (Oliva *et al*., 2003). Toxic compounds generated and their amounts depend on raw material and harshness of pretreatment. Degradation products from pretreatment of lignocellulose materials can be divided into the following classes: carboxylic acids, furan derivatives, and phenolic compounds. Main furan derivates are furfural and 5-hydroxymethylfurfural (HMF) derived from pentoses and hexoses degradation, respectively (Palmqvist and Hahn-Hägerdal, 2000b).Weak acids are mostly acetic and formic and levulinic acids Phenolic compounds include alcohols, aldehydes, ketones and acids (Klinke *et al*., 2002).

5.5 Biomass size reduction not required

Milling or grinding the raw material to small particle sizes before pretreatment is energy-intensive and costly technologies.

5.6 Operation in reasonable size and moderate cost reactors

Pretreatment reactors should be low in cost through minimizing their volume, employing appropriate materials of construction for highly corrosive chemical environments, and keeping operating pressures reasonable.

5.7 Non-production of solid-waste residues

The chemicals formed during hydrolyzate conditioning in preparation for subsequent steps should not present processing or disposal challenges.

5.8 Effectiveness at low moisture content

The use of raw materials at high dry matter content would reduce energy consumption during pretreatment.

5.9 Obtaining high sugar concentration

The concentration of sugars from the coupled operation of pretreatment and enzymatic hydrolysis should be above 10% to ensure an adequate ethanol concentration and to keep recovery and other downstream cost manageable.

5.10 Fermentation compatibility

The distribution of sugar recovery between pretreatment and subsequent enzymatic hydrolysis should be compatible with the choice of an organism able to ferment pentoses (arabinose and xylose) in hemicellulose.

5.11 Lignin recovery

Lignin and other constituents should be recovered to simplify downstream processing and for conversion into valuable co-products (Yang and Wyman, 2008).

5.12 Minimum heat and power requirements

Heat and power demands for pretreatment should be low and/or compatible with the thermally integrated process.

6. Detoxification of lignocellulosic hydrolyzates

During pretreatment and hydrolysis of lignocellulosic biomass, a great amount of compounds that can seriously inhibit the subsequent fermentation are formed in addition to fermentable sugars (Sánchez and Cardona, 2008). Inhibitory substances are generated as a result of the hydrolysis of the extractive components, organic and sugar acids esterified to hemicellulose (acetic, formic, glucuronic, galacturonic), and solubilized phenolic derivatives. In the same way, inhibitors are produced from the degradation products of soluble sugars (furfural, HMF) and lignin (cinnamaldehyde, *p*-hydroxybenzaldehyde, syringaldehyde), and as a consequence of corrosion (metal ions) (Lynd, 1996; Palmqvist and Hahn-Hägerdal, 2000b). For this reason and depending on the type of employed pretreatment and hydrolysis, detoxification of the streams that will undergo fermentation is required (Sánchez and Cardona, 2008).

Detoxification methods can be physical, chemical or biological. As pointed out by Palmqvist and Hahn-Hägerdal (2000a), these methods cannot be

directly compared because they vary in the neutralization degree of the inhibitors. In addition, the fermenting microorganisms have different tolerances to the inhibitors. The main features of the detoxification methods employed for ethanol production from biomass and some examples are summarized in Table 2. Alkali treatment is considered one of the best detoxification methods. By this method, furaldehydes and phenolic compounds are mainly removed leading to great improvement in fermentability, especially in the case of dilute-acid hydrolyzates (Persson *et al*., 2002). Treatment with calcium hydroxide (overliming) or ammonia has shown better results than treatment with sodium or potassium hydroxide, but this difference has not been understood (Sánchez and Cardona, 2008). Martinez et al. (2001) performed the experimental optimization of the amount of added lime, which depends on the content of acids in each hydrolyzate. These authors developed a method for predicting the optimal addition dosage based on the titration of hydrolyzate with 2 N NaOH. Persson et al. (2002) indicate that the positive effects of alkali treatment cannot be completely explained by the removal of inhibitors and that this method could have possible stimulatory effects on fermenting microorganisms. Other very diverse detoxification methods have been proposed as: neutralization with lime followed by the addition of activated carbon and filtration for acetic acid removal; partial removal of acetic acid, furfural and soluble lignin by molecular sieves; vapor stripping for removal of volatile inhibitors (Olsson and Hahn-Hägerdal, 1996); and adsorption using activated carbon, diatomite, bentonite and zeolite after neutralization or overliming (Yu and Zhang, 2003). An alternative biological method for detoxification of dilute solutions resulting from biomass pretreated by pyrolysis has been proposed (Khiyami *et al*., 2005). It is based on a bio-film reactor that uses a mixed culture of aerobic bacteria cells naturally immobilized on a plastic support. In this way, the bio-film associated cells are more resistant to the toxic substances released during the biomass pretreatment.

The presence of inhibitors directly influences on the course of ethanolic fermentation. In continuous or fed-batch fermentations, feed of the bioreactors is carried out with not very high flow rates allowing a low concentration of inhibitors in the broth. In continuous systems, inhibitors reduce the growth rate and, therefore, the process productivity that is directly linked to the dilution rate. In

systems with cell retention (e.g. by cell recirculation using filtration, sedimentation or centrifugation), the increase of accumulables, including the inhibitors, makes the productivity to fall down imposing the need of implementing purge streams (Sánchez and Cardona, 2008). Taherzadeh (1999) developed a simple strategy for on-line feedback control of fed-batch cultivation for in situ detoxification of spruce and birch hydrolyzates. Through this strategy, the same yeast cells converted the inhibitors and maintained their concentration at low levels without the need of any detoxification treatment. Thus, the maximal specific productivity of ethanol increased in more than 10 times (Nilsson *et al*., 2001). Purwadi et al. (2007) employed a continuous cultivation system using a flocculating strain of *S. cerevisiae* to ferment a non-detoxified spruce hydrolyzate. Results obtained demonstrated that high-cell system with recycling of cells allow the in situ detoxification of the pretreated biomass at high dilution rates without the need of any detoxification method. It has shown the possibility of converting the hydrolyzates into ethanol in two hours (at dilution rates of 0.5 h^{-1}), which represents an important outcome in the cultivation of toxic pretreated lignocellulosic biomass. Most of the studies on the effect of toxic compounds on growth and ethanol production have been performed for *S. cerevisiae* and xylose-fermenting yeast (Sánchez and Cardona, 2008). Palmqvist et al. (1999) carried out extensive experiments for assessing the effect of acetic acid, furfural and *p*-hydroxybenzoic acid on growth and ethanol productivity of *S. cerevisiae* and *C. shehatae* through full factorial design.

One new approach to tackle the presence of inhibitors in biomass hydrolyzates is the development of inhibitor-tolerant strains of microorganisms by means of genetic modification and metabolic engineering. However, Belkacemi et al. (2002) point out that due to the synergistic interactions among inhibitors and lack of information about the mechanisms of these interactions, it is not clear against what inhibitor resistance is desired. In this way, intense efforts are being carried out for the identification of inhibitory substances, as well as the determination of their inhibition mechanisms. Palmqvist and Hahn-Hägerdal (2000b) have reviewed the main works carried out in this field applied to wood hydrolyzates. These authors emphasize that these studies will allow the minimization of inhibitors formation during pretreatment

and hydrolysis, the prediction of hydrolyzates fermentability and the development of more efficient detoxification methods (Sánchez and Cardona, 2008).

7. Conversion of lignocellulosic materials to value added products

7.1 Hemicellulose (xylose) production

7.1.1 Diluted acid hydrolysis

Due to the fossil fuel crisis, bioconversion of lignocellulosic materials to chemicals and fuel are significantly interesting in recent decade as a low cost, renewable and widespread in nature (Rhaman *et al*., 2006). These abundant lignocellulosic materials contain approximately 34% of cellulose, 26% of hemicellulose, and 28% of lignin (Kaddami *et al*., 2006). Therefore, the utilizations of these invaluable material wastes to be valuable products i.e. furfural, ethanol, xylitol, and high grade paper by biochemical and chemical processes have been studied worldwide (Rhaman *et al*., 2006). Autohydrolysis (Garrote *et al*., 2001) and acid hydrolysis of various raw materials have been focused (Pessor *et al*., 1997; Neureiter *et al*., 2002; Rhaman *et al*., 2006; Cheng *et al*., 2007). Under autohydrolysis and controlled acid hydrolysis conditions, xylose is a mainly product from both processes because the lignin protective layer around the hemicellulose is weak under high temperature and pressure which allows the acid to hydrolyze the layer and amorphous xylan to form xylose (Rhaman *et al*., 2006). On the other hand glucose could not been produced so much in the diluted acid hydrolysis because of the crystalline structure of cellulose (Rhaman *et al*., 2006). Due to this problem, two-stage acid hydrolysis process can be constructed to produce xylose and glucose, respectively. Dilute acid at moderate temperatures, the first stage of acid hydrolysis, has established to be an efficiency of xylose production. In the second stage, more severe reaction conditions are engaged and glucose can be produced from cellulose hydrolysis (Pessor *et al*., 1997).

Acid hydrolysis's advantages

1. To increase the enzymatic digestibility of the material (Palmarola-Adrados *et al*., 2005; Linde *et al*., 2008).

2. Acid recovery might not be required and there will be no significant losses of acid (Iranmahboob *et al*., 2002).

3. The reaction time is faster than enzymatic hydrolysis (Garrote *et al*., 2001).

4. This method can be used in many lignocellulosic waste materials.

Acid hydrolysis's disadvantages

By-products can be produced by dilute acid hydrolysis, i.e. acetic acid and furfural (Pessoa *et al*., 1997; Rhaman *et al*., 2006). Moreover, by-products (acetic acid and furfural) generated from dilute acid hydrolysis do not only reduce the yield on monomeric sugar but also act as the inhibitors in the fermentation (Pessoa *et al*., 1997; Neureiter *et al*., 2002). These inhibitors have affected on cell morphological change or ultimate death of the organism (Rhaman *et al*., 2006).

Acetic acid has been produced from degradation of acetyl group, which contains in the hemicellulose structure (Garrote *et al*., 2001) whereas xylose degradation is a cause of furfural production (Rhaman *et al*., 2006). It was demonstrated that acid concentration is an important parameter for sugars production whereas temperature is mainly responsible for decomposition of sugars to various by-products (Neureiter *et al*., 2002; Rhaman *et al*., 2006). Moreover, temperature and reaction time are also affected on xylose decomposition (Delgenes *et al*., 1996). Therefore, to keep the concentration of by-products in the hydrolysate at low level it is necessary to operate the hydrolysis reaction at less severe conditions (Rhaman *et al*., 2006). Furaldehyde or furfural, acetate and hydroxymethylfuraldehyde (HMF) are main inhibitory compounds from acid hydrolysis of lignocellulosic materials. These toxic compounds have a negative affected on cell growth and ethanol production by yeast and bacteria (Delgenes *et al*., 1996).

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of lignocellulosic biomass for bioethanol production.

Methods	Procedure/agents	Samples	Stains	Results
Physical methods;				
Evaporation	Evaporation, separation of volatile and nonvolatile fractions and dilution of non-volatile fraction	Willow	*S. cerevisiae*	Reduction of acetic acid and phenolic compounds in non-volatile fraction.
		Aspen	*P. stipitis*	Roto-evaporation 93% yield of ref. fermn.; removal: 54% acetic acid, 100% furfural, 29% vanillin
Extraction	Organic solvents, 3:1 org. phase: aqueous phase volumetric ratio	Spruce	*S. cerevisiae*	Solv: diethyl ether (solv.); yield comparable to ref. fermn.; removal of acetic, formic and levulinic acids, furfural, HMF.
		Aspen	*P. stipitis*	Solv.: ethyl acetate; 93% yield of ref. fermn.; removal: 56% acetic acid, 100% furfural, 100% vanillin, 100% hydroxybenzoic acid.
		Pine	*S. cerevisiae*	Solv.: ethyl acetate; removal of low molecular phenolic compounds.
		Steam-exploded Poplar	*S. cerevisiae*	Solv.: ethyl acetate; EtOH yield (SSF): detoxified 0.51 g/g, undetox. 0 g/g; high degree of phenolic removal.
	Supercritical solvent in counter-current with the hydrolyzate, 20 MPa, 40°C; then, depressuri-zation	Dilute-acid spruce	*S. cerevisiae*	Solv.: supercritical CO_2; 98% yield of ref. fermn.; removal 93% furfural, 10% HMF.

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of lignocellulosic biomass for bioethanol production (**cont.**).

Methods	Procedure/agents	Samples	Stains	Results
Physical methods; Adsorption	Activated carbon, 0.05–0.20 g/g glucose	Steam-exploded concentrated oak	*S. cerevisiae*	Detoxified with 140–170 g/l initial glucose was utilized; undetox. with 100 g/l initial glucose could not be completely utilized.
	Amberlite hydrophobic polymeric adsorbent XAD-4, 8% (w/v), 1.5 h, 25°C; regeneration with EtOH; then, neutraliz. with lime.	LHW-pretreated corn fiber Poplar	Recombinant *E. coli*	Reduction of furfural conc. from 1–5 to <0.01 g/l; 90% yield of theoretical; sugars are not adsorbed.
Chemical methods; Neutralization	$Ca(OH)_2$ or CaO, pH = 6, then membrane filtration or adsorption.	Acid hydrolysis of cotton waste pyrolysate	*S. cerevisiae*, *Pichia* sp.	Precipitation or removal of toxic compounds; 10% lower yield for *Pichia* sp.
		Steam-exploded Poplar	*S. cerevisiae*	EtOH yield (SSF): detoxified 0.86 g/g, undetox. 0 g/g.
Ionic exchange	Weak base resins Amberlyst A20, regenerated with NH_3.	Dilute-acid poplar	Recombinant *Z. mobilis*	Removal: 88% acetic acid, 100% H_2SO_4; 100% sugars recovery.
	Poly(4-vinyl pyridine)	Corn-stover	Recombinant *S. cerevisiae*	Sugars eluted earlier than all tested inhibitors; ferment. results were similar to that using pure sugars.

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of lignocellulosic biomass for bioethanol production (**cont.**).

Methods	Procedure/agents	Samples	Stains	Results
Chemical methods; Alkaline detoxification (overliming)	$Ca(OH)_2$, pH = 9–10.5, then pH adjustment to 5.5–6.5 with H_2SO_4 or HCl	Dilute-acid of spruce	*S. cerevisiae*	Yield comparable to ref. fermn.; 20% removal of furfural and HMF.
		Steam-exploded bagasse	Recombinant *S. cerevisiae*	Removal of acid acetic, furfural and part of phenolic compounds.
		Rice hulls	Recombinant *E. coli*	39% reduction in fermentation time.
		Wheat straw	Recombinant *E. coli*	Reduction in fermn. time: SSF =18%, SHF = 67%.
		Dilute-acid bagasse hydrolysate	Recombinant *E. coli*	Removal: 51% furfural, 51% HMF, 41% phenolic compounds, 0% acetic acid; overliming at 60°C or 25°C, at high temperature.
Combined alkaline detoxification	KOH, pH = 10, then pH adjustment to 6,5 with HCl and addition of 1% sodium sulfite.	Bagasse hydrolysate	*P. stipitis*	Reduction of ketones and aldehydes, removal of volatile compounds when hydrolyzate is heated at 90°C.

Table 2. Detoxification methods of streams resulting of pretreatment and hydrolysis of lignocellulosic biomass for bioethanol production (**cont.**).

Methods	Procedure/agents	Samples	Stains	Results
Biological methods; Enzymatic detoxification	Laccase (phenol oxidase) and lignin peroxidase from Trametes versicolor: 30°C, 12h	Willow	*S. cerevisiae*	2–3-fold increase of EtOH productivity compared to undetox.; laccase selectively removes phenolic low molecular weight compounds and phenolic acids.
		Steam-exploded bagasse	Recombinant *S. cerevisiae*	80% removal of phenolic compounds.
Microbial detoxification	*Trichoderma reesei*	Steam-exploded Willow	*S. cerevisiae*	3-fold increase of EtOH productivity compared to undetox.; 4-fold increase of yield; removal of acetic acid, furfural and benzoic acid derivatives.
	Immobilized to PCS mixed culture of *Pseudomonas putida* and *Streptomyces setonii* cells (biofilm reactor: PCS tubes attached to CSTR agitator shaft)	Diluted pyrolysate of corn stover		Detoxification of 10 and 25 vol.% of pyrolysate medium, and partially detoxification of 50 vol.% of pyrolysate medium.

Observations: Reference fermentation (ref. fermn.) refers to fermentation carried out in a glucose-based medium without inhibitors; undetox.– undetoxified; PCS – plastic composite support.
Source: Sánchez and Cardona, (2008)

7.1.2 Steam explosion

Steam explosion is a thermomechnochemical process. The breakdown of structural components is aided by heat in the form of steam (thermo), shear forces due to the expansion of moisture (mechano) and hydrolysis of glycosidic bonds (chemical) (Jeoh, 1998). By this pretreatment, the biomass is usually treated with high pressure at high temperature between 120➡C and 240➡C corresponding to the pressure between 5 and 34 bars and gave reaction time of for several seconds to a few minutes. During pretreatment, hemicellulose is solubilized in the liquid phase as oligomeric and monomeric sugars. The cellulose is in the solid phase then becomes more easily to the enzymatic hydrolysis (Galbe *et al*., 2007).

Chen and Liu. (2006) studied on steam explosion for separating hemicellulose from chipped wheat straw which composed of 35.1% cellulose, 27.1% hemicellulose, 5.3% Klason lignin and 6.04% ash. After hydrolysis by steam explosion at 160-180➡C, 1.5 MPa for 4.5 min and determination using HPLC, the ratio of monosaccharides to oligosaccharides was found to be 1:9 and the main component was xylose (85.9%) in content. The total recovery rate of hemicellulose was 80%.

7.1.3 Alkali method

Sun *et al*. (1996) studied NaOH concentration to hydrolyze wheat straw and produce hemicellulose. Solid residues were hydrolyzed 6 times to be hemicellulose. It was found that the condition of hydrolysis (alkali concentration, temperature and retention time) will be increased every steps in order to break down the complex structure of wheat straw. After 6 times of NaOH hydrolysis, 33.9% hemicellulose was obtained.

7.1.4 Combination of chemical or physical techniques for hemicellulose production

Hemicellulose production from wheat straw, in the first step ligonin content was obtained 11.2–12.3% of the total lignin due to the hydrolysis of substantial amounts of hemicelluloses during the steam pretreatment, even though small amounts of lignin present in the middle lamella were also degraded during the steam explosion. After that, 80.6–88.2% of the total lignin was obtained by alkaline

peroxide hydrolysis. Therefore, the two-stage treatment degraded 92.3–99.4% of the original lignin from wheat straw (Sun *et al*., 2005). Moreover, hemicellulose production from olive stone by steam explosion combined with and without 0.1% sulfuric acid pretreatment were also studied (Fernandez-Bolanos, 2001). The maximum yield of the pentosan recovered in the water solution from steam explosion was 63% pentose via treated at 200➡C for 2 min with 0.1% sulfuric acid or 215➡C for 2 min without acid. This indicated that treated with acid can decrease level of temperature because acid is a promoter of hydrolysis.

Extraction of hemicellulose from EFB and sterilizer condensate was conducted using alkali treatment and solvent method. The optimal ratios of EFB to 12% KOH were found to be 1:50 (w/v) while extraction at 80➡C for 20 min gave significantly higher hemicellulose concentration than other treatments. Addition of ethanol to precipitate the hemicellulose from the extracted solution in the ratio of 1:1 (v/v) gave the highest hemicellulose yield of 8.67 g/100 g EFB. For extraction of hemicellulose from sterilizer condensate, the optimal ratio of ethanol to the condensate was 2:1 (v/v) which gave a hemicellulose yield of 6.42 g/100 ml (Prasertsan and Oi, 2001).

Pretreatment of wood components by steam explosion at 180-230➡C for 2-20 min gave the xylose yield of 10-20% and about 50% of the wood was obtained as solid residues in which the lignin and residual hemicelluloses might be removed by a subsequent alkali extraction (Shimizu, 1998). In addition, hemicellulose could also be produced from un-utilized bamboo, which provided 48 g holocellulose/100 g bamboo (Asada *et al*., 2005). Hemicellulose could be also hydrolyzed from residual corrugated cardboard using acid hydrolysis; 1-3% sulfuric acid and heated at 130➡C for 30-180 min. The suitable condition consisted of 1% sulfuric acid, heated at 130➡C for 30 min and gave 14.1% w/w hemicellulose (Yanez *et al*., 2004). Softwood chips (*pine* and *spruce*) (30x30x2 mm^3, moisture content of 20-30%) were treated at 175➡C in combination with 4.5%SO_2 for 7.5 min. SO_2–catalyzed steam explosion was found to be an effective method to enhance percentages of hemicellulose (80-90%) into solution because of the partial conversion of SO_2 into sulfuric acid during the process (Shevchenko *et al*., 2000). The

components of Eucalyptus wood were studied by various conditions of steam explosion. The optimal condition of C-6 sugar (hexose) and C-5 sugar (xylose) production was high temperature at 180➡C, pressure at 19 bars for 6 min and then treated with cellulase (20 FPU/g substrate) at 50➡C for 24 h, giving total sugar of 56.40 g/l (Nunes and Pourquie, 1996). Mulberry or *Morus alba Linn* hydrolyzed by steam explosion was achieved after heating at 190➡C for 5 min. The retention time (min) of xylose was 15.019 min and its quantity was 82.242 mg/l (Punsuwan *et al*., 2004).

The characteristic of exploded fibre of hemp or *Cannabis sativa*, which was an annual plant used in the pulp and paper industry, was studied by scanning electron microscopy (SEM). The original hemp's fibres were associated in the bundles containing 15-30 fibres while the exploded fibres were separated into packets containing 2-5 fibres. In addition, most of pectic substances at the surface of the bundles were removed after treated with steam explosion and water washing. These fibres were well separated (1-3 fibres) after using steam explosion in combination with a 2% sodium hydroxide extraction. The best hemp bundles (1-2 fibers) were achieved after steam explosion, water extraction, NaOH extraction and bleaching, respectively (Garcia-Jaldon *et al*., 1998).

7.1.5 Xylanase hydrolysis

Xylanase (E.C. 3.2.1.8) is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, which is one of the major components of plant cell walls (Wulandari, 2009). Xylanase represents one of the largest groups of industrial enzymes with increasing market demands due to its applications in prebleaching of kraft pulps, bioconversion of agricultural residues, extraction of coffee and plant oils, improvement of the nutritional properties of agricultural silage and degumming of plant fibers, such as flax, sun hemp and ramie (Subramaniyan and Prema, 2002). A variety of microorganisms including bacteria, yeast, actinomycetes and filamentous fungi have been reported to produce xylanolytic enzymes. Each organism or strain has its own special conditions for maximum enzyme production and activity (Kapoor *et al*., 2008). Complete depolymerization of xylan is accomplished by the synergistic

action of endo-xylanases and xylosidases along with arabinofuranosidases, ferulic acid esterases, uronidases, and other enzymes which, respectively, act on the xylan backbone, side chains and decorating units, producing fermentable xylooligomers and monomers (Collins *et al*., 2005; Pastor *et al*., 2007; Squina *et al*., 2009). Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss and side product generation (Chapla *et al*., 2010).

Xylanases have commercial uses in various forms: (a) biobleaching agents in pulp and paper industry; (b) enhanced utilization of biomass in the biofuel industry; (c) production of xylitol (a low calorie sweetener); (d) foodstuff additive in bread, juice and wine manufacturing and (e) additive in animal feedstuff preparation (Kapoor *et al*., 2008).

7.2 Glucose production

7.2.1 Concentrated acid hydrolysis

Concentrated acid have been used for decrystallization of cellulose followed by dilute acid hydrolysis to sugars. In order to save the chemical cost, separation of acid from sugars, acid recovery, and acid reconcentration are critical unit points. The concentrated acid disrupts the hydrogen bonding between cellulose chains, converting it to a completely amorphous state. Once the cellulose has been decrystallized, it forms a homogeneous gelatin with the acid. The cellulose is extremely susceptible to hydrolysis at this point. Therefore, dilution with water at modest temperatures provides complete and rapid hydrolysis to glucose, with little degradation (U.S. Department of Energy, 2008).

Concentrated acid have been reported by U.S. Department of Energy (2008). The first stage, materials were mixed with 70-77% (v/v) H_2SO_4 and then followed by adding water into the system to dilute the acid concentration to 20-30% H_2SO_4 for an hour at less than 50°C.

In addition, concentrated sulfuric acid had been used directly to produce glucose from ⇨-cellulose (Xiang *et al*., 2003). The ⇨-cellulose form treated by concentrated sulfuric acid of 65% at high temperature (more than 200°C) can be

changed from fibrous form to gelatinous form. The results indicated that 65% H_2SO_4 pretreatment for 4 hours was successfully hydrolyzed ⇨-cellulose around 95% after carrying out at 120°C, 4% H_2SO_4 for 90 min (Xiang *et al*., 2003).

7.2.2 Cellulase hydrolysis

Cellulase is a class of enzymes produced chiefly by fungi, bacteria and protozoans that catalyze the cellulolysis. There are fives commonly types of cellulase based on the type of reaction catalyzed, consist of:

7.2.2.1 Endo-cellulase

It breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains (Zhou *et al*., 2009).

7.2.2.2 Exo-cellulase

It cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide in term of cellubiose. Two main types of exo-cellulase were identified based on the cleaved positions of cellulose; one type digests at the reducing end of cellulose while another type digests at the non-reducing end of cellulose (Zhou *et al*., 2009).

7.2.2.3 Cellubiase or ⇦-glucosidase

This cellulolytic enzyme digests disaccharides into individual monosaccharides (Zhou *et al*., 2009).

7.2.2.4 Oxidative cellulase

It depolymerizes cellulose by radical reactions, as for instance cellulobiose dehydrogenase.

7.2.2.5 Cellulose phosphorylase

It also depolymerize cellulose using phosphates instead of water.

7.2.3 Cellulase production by microorganisms

The extracellular cellulolytic system of *Trichoderma reesei* is composed of 60–80% cellobiohydrolases or exogluconases, 20–36% of endogluconases and 1% of ⇦-glucosidases, which all act synergistically in the conversion of cellulose into glucose (Ahamed and Vermette, 2008) whereas, the well studied fungus *T. reesei* can produce cellulases, including at least two cellobiohydrolases (EC 3.2.1.74), five endoglucanases (EC 3.2.1.4) and two ⇦-

glucosidases (EC 3.2.1.21), which act synergistically during the conversion of cellulose to glucose (Zhang and Cai, 2008; Zhou *et al*., 2009). *T. reesei* cellulase system is deficient in cellobiase, causing the accumulation of the disaccharide cellobiose, which produces repression and end product inhibition of the enzyme, both of which limit enzyme synthesis and activity (Ahamed and Vermette, 2008). The cellulase is an inducible enzyme system in which several carbon sources have been tested to find the best inducer such as cellulose and lactose whereas, glucose is an inhibitor in cellulase biosynthesis (Ahamed and Vermette, 2008).

Cellulase could be produced from several stains of bacteria and fungi. There were many research works reporting production of cellulase. For example, the cellulase production by *Bacillus* spp. isolated from compost giving the highest activity of 1.33 mg glucose released ml^{-1} min^{-1} at 70°C was studied (Mayende *et al*., 2006). Ahamed and Vermette (2008) have reported that a mixture of lactose and lactobionic acid was added into the bioreactor as cellulase inducers. The use of a cellulose–yeast extract culture medium yielded the highest enzyme and cell production with a volumetric enzyme activity of 69.8 U/l/h, a filter paper activity of 5.02 U/ml, a CMCase activity of 4.2 U/ml, and a fungal biomass of 14.7 g/l. Zhang and Cai (2008) have reported that the production of reducing sugars and filter paper activity (FPA) could achieve 2.231 g/l and 12.92 U/ml, respectively, under enzymatic hydrolysis at 35°C, pH 4.5 for 96 h by broth of *Trichoderma reesei* ZM4-F3 in 2% NaOH pretreated rice straw as a substrate cultured for 36 h.

⇦-glucosidase could be produced by *Aspergillus phoenicis*. The ⇦-glucosidase activity and FPA were 0.64 IU/ml and 1.54 FPU/ml, respectively, cultured in manure solid at 27 °C and pH 5.5, which is close to the optimal values for both fungi (Wen *et al*., 2005). *Fusarium oxysporum VTT*-80134 has been found to produce insufficient amounts of enzymes to convert cellulose directly into ethanol. *F. oxyspoerum* produces sufficient activities of ⇦-glucosidase to prevent severe product inhibition by cellobiose during the hydrolysis process (Panagiotou *et al*., 2005). Fermentation performance by the fungus *F. oxysporum* under aerobic and anaerobic cultivation on cellulose was investigated. It was found that *F. oxysporum* grow with a maximum specific growth rate of 0.023 h^{-1} on cellulose under aerobic conditions

giving final endoglucanase, ⇦- glucosidase and cellobiohydrolase activities of 55, 1.25 and 0.43 U/ml, respectively. Under anaerobic conditions it can produce ethanol with a yield of 0.35 g/g cellulose and significant amounts of acetic acid, with a yield of 0.2 g/g cellulose, as a by-product (Panagiotou *et al*., 2005).

7.2.4 Factors affecting on enzymatic hydrolysis

The pretreatment is a necessary step to alter some structural characteristics of lignocellulose, increasing glucan and xylan accessibility to the enzymatic attack. These structural modifications of the lignocellulose are highly dependent on the type of pretreatment employed and have a great effect on the enzymatic hydrolysis (Kumar *et al*., 2009) and subsequent steps. The choice of pretreatment technology for a particular raw material depends on several factors, some of them directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate, type of pretreatment, and dosage and efficiency of the enzymes used for the hydrolysis have a great influence on biomass digestibility; although the individual impacts of these factors on the enzymatic hydrolysis are still unclear (Alvira *et al*., 2010).

Main factors that influence the enzymatic hydrolysis of cellulose in lignocellulosic feedstocks can be divided in two groups: enzyme-related and substrate-related factors, though many of them are interrelated during the hydrolysis process. Composition of the liquid fraction and solid process streams resulting from different pretreatment approaches can be widely different. These differences will have a great influence on the requirements for effective enzymatic saccharification in subsequent processing steps (Alvira *et al*., 2010).

The reduction of pretreatment severity is sometimes required to reduce economic cost. Low severity factor results in less sugar-release and consequently higher amount and different types of enzymes will be required to achieve high sugar yields from both cellulose and hemicellulose fraction. In this context, development of hemicellulases and other accessory enzymes needed for complete degradation of lignocellulose components has become an important issue. Recent studies show the importance of new balanced enzymatic complexes containing optimal combinations

to effectively modify the complex structure of lignocellulosic materials (Garĉa-Aparicio *et al*., 2007; Merino and Cherry, 2007; Alvira *et al*., 2010).

Substrate-related factors limiting enzymatic hydrolysis are directly connected to the pretreatment employed. These factors are described separately below although their effect is normally interrelated (Alvira *et al*., 2010).

7.3 Furfural production

Furfural is a liquid aldehide in hetero cyclic group, an organic solvent, and almond odor, less color or yellow when reacted with oxygen (auto-oxidation). It is dissolved in organic solvents but in inorganic solvents. Furfural is usually used as a solvent in many industries such as brewery, perfume, herbicide, insecticide and especially in petroleum fuel. The composition of diesel is almost large hydrocarbon, i.e. paraffin, olefin, naphthen and aromatic hydrocarbon. These compounds are the cause of uncompleted incineration, soot and smoke. Therefore, furfural is usually used as a solvent for dissolving these compounds in order to enhance the efficiency of incineration, to reduce soot and smoke. There are three solvent used in diesels; furfural, phenol and 1-methyl-2-pyrrolidone (MP). However, furfural is a favorite solvent due to cheaper, lower toxicity and simplifies.

Furfural and hydroxymethylfurfural (HMF) were produced from agricultural wastes such as corncobs, sugarcane bagasse, cottonseed hull and rice hull by acidic degradation process (Gutierrez *et al*., 2006; Rahman *et al*., 2006). The precursors of furfural were pentoses (mainly xylose), whereas hexoses (fructose, sucrose, and inulin) were the precursors of HMF production (Karimi *et al*., 2006). The metabolism reaction of furfural production is given in Fig. 6. There were many parameters affecting on the efficiency of production such as acid concentration, temperature, and retention time. The optimal conditions were 6% sulfuric acid, 120➡C and 15 min providing the highest furfural and xylose about 0.87 and 29.4 g/l, respectively (Rahman *et al*., 2006).

Rice straws were hydrolyzed by dilute sulfuric acid (0-1%) at high temperature (180-230➡C) and pressure (1.5-2.0 MPa) in one and two stages, which could produce cellulose (82.3 g/kg of rice straw), hemicellulose (56.4 g/kg of rice

straw), furfural (8.5 g/kg of rice straw), and hydroxymethylfurfural (HMF) (12.7 g/kg of rice straw) (Karimi *et al*., 2006). HMF could be converted to levulinic acid by hydrolysis at 195-215➡C for 15-30 min. (Bozell *et al*., 2000). Production of furfural by acid hydrolysis of olive stones was also reported (Montané *et al*., 2002). The hydrolysis was conducted in dilute sulfuric acid (0.05-0.250 mol/l) at high temperature (220–240➡C) and short reaction time (a few minutes at the most) and performed in a tubing-bomb reactor system. The maximum yield of 65% was achieved at the acid concentration of 0.250 mol/l at 240➡C for 150 seconds. Pentosan content of 18.5% (dry basis) in olive stones produced furfural equivalent to 135 kg furfural/ton of dry olive stones.

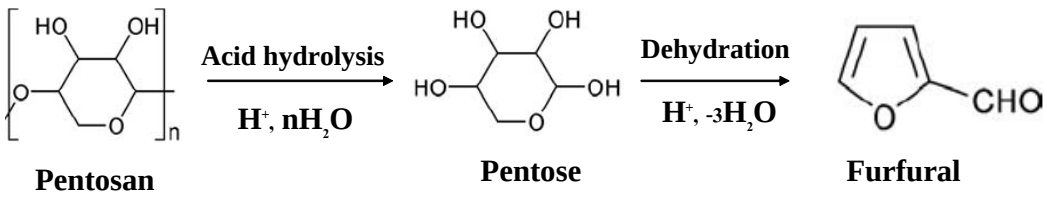


Figure 6. Reaction mechanism of acid hydrolysis of pentosan to furfural.
Source: Dias *et al*. (2005); Riansa-ngawong and Prasertsan (2010)

The market price of furfural was around 1700 US dollars/ton (Montané *et al*., 2002). Furfural was mainly produced from lignocellulosic biomass by pentose dehydration (Gutierrez *et al*., 2006; Karimi *et al*., 2006). The capacities of furfural production were different depending on sources (dry biomass) such as 220 kg furfural/ton corncobs, 170 kg furfural/ton bagasse, 165 kg furfural/ton corn stalks, 160 kg furfural/ton sunflower hulls, 120 kg furfural/ton rice hulls, and 150-170 kg furfural/ton hardwoods (Montané *et al*., 2002).

7.4 Ethanol production from biomass

The fuel ethanol can be obtained from energy crops and lignocellulosic biomass (Lee, 1997; Sánchez *et al*., 1997). The complexity of the production process depends on the feedstock. In this way, the spectrum of designed and implemented technologies goes from the simple conversion of sugars by fermentation, to the multi-

stage conversion of lignocellulosic biomass into ethanol. The big diversity of technological alternatives requires the analysis of the global process along with the design and development of each one of the involved operations. Among the new research trends in this field, process integration has the key for reducing costs in ethanol industry and increasing bioethanol competitiveness related to gasoline (Sánchez and Cardona, 2008).

Table 3. Fuel ethanol programs in some countries.

Country	Feedstocks	Percentage of ethanol in gasoline blends, % (v/v)
Brazil	Sugar cane	24
USA	Corn	10
Cananda	Corn, wheat, barley	7.5-10
Colombia	Sugar cane	10
Spain	Wheat, barley	-
France	Sugar beet, wheat, corn	-
Sweden	Wheat	5
China	Corn, wheat	-
India	Sugar cane	5
Thailand	Cassava, sugar cane, rice	10

Source: Sánchez and Cardona, (2008)

7.4.1 Bio-ethanol as fuel

Ethanol (ethyl alcohol, bioethanol) is the most employed liquid biofuel either as a fuel or as a gasoline enhancer. Ethanol has some advantages when it is used as an oxygenate. Firstly, it has a higher oxygen content that implies a less amount of required additive. The increased percentage of oxygen allows a better oxidation of the gasoline hydrocarbons with the consequent reduction in the emission of CO and aromatic compounds. Related to methyl tertiary butyl ether (MTBE) which is a gasoline additive used as an oxygenate and to raise the octane number, ethanol

has greater octane booster properties, it is not toxic, and does not contaminate water sources. Nevertheless, ethanol production costs are higher than those of MTBE, gasoline mixed with alcohol conduces the electricity, and vapor pressure is higher that entails a greater volatilization, which can contribute to ozone and smog formation (Thomas and Kwong, 2001; Sánchez and Cardona, 2008). Many countries have implemented or are implementing programs for addition of ethanol to gasoline (Table 3). Fuel ethanol production has increased remarkably because many countries look for reducing oil imports, boosting rural economies and improving air quality.

The world ethyl alcohol production has reached about 51,000 mill liters, being the USA and Brazil the first producers (Table 4). In average, 73% of produced ethanol worldwide corresponds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol (Sánchez and Cardona, 2008).

Table 4. World production of ethyl alcohol (mill liters).

Country	2006
1. USA	18,376
2. Brazil	16,998
3. China	3,849
4. India	1,900
5. France	950
6. Germany	765
7. Russia	647
8. Canada	579
9. Spain	462
10. South Africa	386
11. Thailand	352
12. United Kingdom	280
13. Ukraine	269
14. Colombia	269
15. Poland	250

Total	51,056

7.4.2 Ethanol and its properties

Ethanol is the most common alcohol, which is produced from several sources i.e. starch (from corn and cassava), cellulose and hemicellulose (from lignocellulosic materials) through fermentation of these carbohydrates.

7.4.2.1 Physical properties

Ethanol is a volatile, colorless liquid that has a strong characteristic odor. It burns with a smokeless blue flame that is not always visible in normal light. The physical properties of ethanol stem primarily from the presence of its hydroxyl group and the shortness of its carbon chain. Ethanol's hydroxyl group is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight. Ethanol is a versatile solvent, miscible with water and with many organic solvents, including acetic acid, acetone, benzene, carbon tetrachloride, chloroform, diethyl ether, ethylene glycol, glycerol, nitromethane, pyridine, and toluene. It is also miscible with light aliphatic hydrocarbons, such as pentane and hexane, and with aliphatic chlorides such as trichloroethane and tetrachloroethylene.

7.4.2.2 Chemical properties

The chemical structure of ethanol is CH_3CH_2OH and has a density of 0.789 g/ml at 20°C with a molecular weight of 46.07 g/mol, a melting point of 144➡C and a boiling point of 78➡C. Ethanol is normally used to form blended gasoline fuels in concentration between 5-85% (Minteer, 2006).

7.4.3 Bio-ethanol production process from biomass

Biological process of bio-ethanol production utilizing lignocellulose as substrate requires: delignification, depolymerization, and fermentation (Lee, 1997).

(1). Deliginification

The potential method for removing lignin and releasing fermentable sugars is pretreatment followed by enzymatic and acidic hydrolysis. Lignin can be degraded by several fungal enzymes such as lignin peroxidase, Mn-dependent peroxidase, and laccase (mono-phenol oxidase) and its degradability depend on fungal

strain, accessibility of lignin to enzyme, culture condition, and reactor design (Lee, 1997). The major sugars in enzyme hydrolysates are glucose and xylose released from cellulose and hemicellulose, respectively (Ahamed and Vermette, 2008; Zhang and Cai, 2008; Zhou *et al*., 2008). The advantages of biological delignification may include higher product yields, fewer side reactions, less energy demand and less reactor resistance because of mild reaction condition (Lee, 1997).

Diluted acetic acid in combination with catalyst such as sodium chlorite is used to degradate lignin in biomass (Iiyama and Wallis, 1990). The residue is holocellulose containing cellulose and hemicellulose. Furthermore, steam explosion is also used to remove the lignin (Punsuvon *et al*., 2008).

(2). Depolymerization

Hemicellulose was hydrolyzed to monosaccharides by many methods such as alkali hydrolysis, acid hydrolysis, enzyme hydrolysis as hemicellulase, and steam explosion. Hemicellulose could be hydrolyzed by diluted sulfuric acid and heated at 120➡C for 30-60 min (Nigam, 2002). Acid and heat are the important parameters affecting on degradation of ⇦ 1,4 glycosidic linkages and ⇦ 1,6 glycosidic linkages of hemicellulose (Rhaman *et al*., 2006). In the enzymatic system, hemicellulase can specifically break bond at glycosidic linkages. Finally, monomeric sugars are obtained.

Recently, some engineered bacteria that produce some enzymes required for the depolymerization of cellulose and efficiently fermentation all of the released sugars to ethanol have been developed (Nigam, 2002). For example, genetic engineering technique was used to improve *Zymomonas mobilis* 8b that both could use both glucose and xylose for ethanol production giving the ethanol yield of 0.42 g ethanol/g sugar (Mohagheghi *et al*., 2004). Therefore, these recombinant microorganisms could be promising candidates to be applied to the direct ethanol fermentation.

(3). Fermentation

The ethanol fermentation process by fungi and bacteria has been well developed with glucose as a carbon and energy source. Xylose known as a hardly fermentable sugar by microorganisms is also compost mostly 50% in biomass

(Nigam, 2002). The production of xylose can be done by enzymatic hydrolysis (Lachke, 2002) and acid hydrolysis (Rhaman *et al*., 2006). It was found that there are some of yeasts that are able to ferment xylose to ethanol such as *Candida shehatae* (Delgenes *et al*., 1996), *Pachysolen tannophilus* (Bravo *et al*., 1995) and *Pichia stipitis* (Abbi *et al*., 1996; Lee, 1997; Nigam, 2002). To enhance the overall efficiency of biomass utilization, the fermentation process from both C-6 sugar and C-5 sugarneed to develop. The fermentation reactions of those sugars are represented by these following equations (Lachke, 2002).

Glucose; $C_6H_{12}O_6 \longrightarrow 2\ CH_3CH_2OH + 2\ CO_2$

Xylose; $3C_5H_{10}O_5 \longrightarrow 5\ CH_3CH_2OH + 5\ CO_2$

Arabinose; $3C_5H_{10}O_5 \longrightarrow 5\ CH_3CH_2OH + 5\ CO_2$

Ethanol yield is criteria to evaluate ethanol production. It is well known that 0.51 g ethanol is produced from 1 g glucose. However, the carbon flow in cells is also used for biomass production. Therefore, the theoretical ethanol yield is approximately 0.46-0.48 g ethanol/g glucose (Kopsahelis *et al*., 2007).

7.4.3.1 Simultaneous saccharification and fermentation (SSF)

The avoidance of end products inhibition and thereby increasing the saccharification rate and the ethanol yield are one of the significant reasons for using SSF; however there are several additional potential advantages as the presence of ethanol in the culture medium causes the mixture to be less vulnerable to invasion by undesired microorganisms (Menon *et al*., 2010). Moreover, the SSF process shows more attractive indexes than the separate hydrolysis and fermentation (SHF) as higher ethanol yields, less energetic consumption, decrease the number of vessels needed and thereby reduces the investment costs (Alkasrawi *et al*., 2003; Menon *et al*., 2010). In this case, the cellulases and microorganisms are added to the same process unit allowing that the glucose formed during the enzymatic hydrolysis of cellulose be immediately consumed by the microbial cells converting it into ethanol. Thus, the inhibition effect caused by the sugars over the cellulases is neutralized. However, the

need of employing more dilute media to reach suitable rheological properties makes that final product concentration be low. In addition, this process operates at non-optimal conditions for hydrolysis and requires higher enzyme dosage, which positively influences on substrate conversion, but negatively on process costs. Considering that enzymes account for an important part of production costs, it is necessary to find methods reducing the cellulases doses to be utilized. With this aim, addition of surfactants has been proposed (Alkasrawi *et al*., 2003). The addition of the non-ionic surfactant Tween-20 to the steam exploded wood during a batch SSF using *S. cerevisiae* has some effects: 8% increase in ethanol yield, 50% reduction in cellulases dosage (from 44 FPU/g cellulose to 22 FPU/g cellulose), increase of enzyme activity at the end of the process, and decrease in the time required for reaching the highest ethanol concentration. It is postulated that the surfactant avoids or diminishes the non-useful adsorption of cellulases to the lignin (Sánchez and Cardona, 2008).

7.4.3.2 Separate hydrolysis and fermentation (SHF)

When sequential process is utilized, solid fraction of pretreated lignocellulosic material undergoes hydrolysis (saccharification). This fraction contains the cellulose in an accessible to acids or enzymes form. Once hydrolysis is completed, the resulting cellulose hydrolyzate is fermented and converted into ethanol. One of the main features of the SHF process is that each step can be performed at its optimal operating conditions. The most important factors to be taken into account for saccharification step are reaction time, temperature, pH, enzyme dosage and substrate load (Sánchez and Cardona, 2008).

By testing lignocellulosic material from sugar cane leaves, Hari Krishna et al. (1998) have found the best values, 65–70% cellulose conversion was achieved at 50➡C and pH of 4.5. Although enzyme doses of 100 FPU/g cellulose caused almost a 100% hydrolysis, this amount of cellulases is not economically justifiable. Hence, 40 FPU/g cellulose dosage was proposed obtaining only 13% reduction in conversion. Regarding the substrate concentration, solids loads of 10% was defined as the most adequate considering arising mixing difficulties and accumulation of inhibitors in the reactioning medium (Sánchez and Cardona, 2008).

Hydrolysis tests for steam-pretreated spruce also indicate the need of high enzyme loadings of both cellulases and β-glucosidase to achieve cellulose conversions greater than 70% due to the less degradability of the softwood (Tengborg *et al*., 2001).

Saha and Cotta (2006) obtained 96.7% yield of monomeric sugars using an enzymatic cocktail of cellulase, β-glucosidase and xylanase for saccharification of wheat straw pretreated by alkaline peroxide method. An ethanol concentration of 18.9 g/l and a yield of 0.46 g/g of available sugars were achieved in the subsequent fermentation using a recombinant *E. coli* strain capable of assimilating both hexoses and pentoses.

Nguyen et al. (1999) employed a mixed solids waste (construction lumber waste, almond tree prunings, wheat straw, office waste paper, and newsprint) for producing ethanol by SHF using yeasts. In this process, a recycling of enzymes was implemented through microfiltration and ultrafiltration achieving 90% cellulose hydrolysis at a net enzyme loading of 10 FPU/g cellulose.

7.4.4 Microbial key players in bio-ethanol production from lignocellulosic materials

Olsson and Hahn–Hägerdal (1996) presented a list of bacteria, yeasts, and filamentous fungi, producing ethanol from xylose. Among naturally occurring organisms, certain species of the yeasts *Candida*, *Pichia*, *Schizosaccharomyces*, *Kluyveromyces*, and *Pachysolen*, the filamentous fungi *Fusarium*, *Mucor*, *Monilia*, and *Paecilomyces*, and the bacteria *Clostridium*, *Bacillus*, *Bacteroides*, *Thermoanaerobacter*, and *Erwinia* produce ethanol. Among these microorganisms, *Candida shehatae*, *Pichia stipitis*, and *Fusarium oxysporum* resulted in high yields (>0.45 g ethanol/g xylose) and reasonable productivities (>0.17 g/l h). The characteristics required for an industrially suitable microorganism have been reported and are summarized in Table 5 (Dien *et al*., 2003).

Table 5. Important characteristics for industrially ethanol production.

Characteristics	Requirement
Ethanol yield	>90% of theoretical
Ethanol tolerance	>40 g/l
Ethanol productivity	>1 g/l/h
Able to grow in undiluted hydrolysate	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperature

Source: Dien *et al*. (2003)

(1) Bacteria

1.1 *Clostridium* sp.

The bioconversion of abundant and renewable cellulosic biomass into ethanol as an alternative to petroleum is gaining importance due to the realization of diminishing natural oil and gas resources. The single-step conversion of cellulosic biomass to ethanol by *Clostridium thermocellum* has advantages over the multiple-step process in which fungal cellulases and yeasts are used. However, the low ethanol tolerance (up to 1.5% v/v) and low ethanol yields (0.08-0.29 g/g) of the organism are the major limiting factors for its industrial exploitation. Furthermore, most of the studies have been carried out at low substrate concentrations using pure cellulosics. Therefore, it is necessary to conduct experiments with natural cellulosic materials using high ethanol yielding and ethanol tolerant *C. thermocellum* strains (Sudha Rani *et al*., 1998).

Clostridium thermocellum produces an exocellular, multienzyme complex, termed cellulosome, which comprises numerous cellulases and hemicellulases. Searches for *C. thermocellum* genes involved in cellulose degradation were performed by several groups, resulting in the cloning of genes encoding 21 endoglucanases, 3 exoglucanases, 2 β-glucosidases and 4 xylanases (Guglielmi and Béguin, 1998). Thermophilic bacteria have a distinct advantage over conventional

yeasts for ethanol production in their ability to use a variety of inexpensive biomass feedstocks and their ability to withstand temperature extremes. Because these bacteria are inhibited by relatively low levels of ethanol, extractive fermentation using compressed solvents could prevent this toxicity and greatly enhance the economic viability of ethanol production by thermophilic organisms (Knutson *et al*., 1999).

Thermophilic and anaerobic *C. thermocellum* strains, SS21 and SS22, which produced 0.37 and 0.33g ethanol/g cellulose consumed, respectively, were recently obtained. The strains are tolerant to 4.0 and 5.0% (v/v) ethanol and on addition of ethanol at different culture ages, there was increase in ethanol tolerance up to 7.0 and 8.0% (v/v), respectively (Sudha Rani *et al*., 1998).

1.2 *Zymomonas mobilis*

Zymomonas mobilis is an obligatorily fermentative Gram-negative bacterium that utilizes sucrose, glucose, and fructose by the Entner–Doudoroff (ED) pathway leading to the production of ethanol and CO_2 (Fig. 7) (Sprenger, 1996; Kang and Kang, 1998; Lee and Huang, 2000; Tao *et al*., 2005). Morphology of *Z. mobilis* is shown in Fig. 8 (Davis *et al*., 2006). Most *Z. mobilis* strains are capable of growth in the presence of up to 10% ethanol and of fermentation in media with up to 25% glucose (Kang and Kang, 1998).

Fermentation technologies utilizing strains of *Zymomonas mobilis*, in place of the traditional yeast, have been proposed by a number of authors for starch-based ethanol production, as they have been shown to ferment under fully anaerobic conditions with faster specific rates of glucose uptake and ethanol production as well as ethanol yields close to theoretical (Davis *et al*., 2006). Ethanol productions from synthetic medium (Mohagheghi *et al*., 2006) to liquid wastes such as agro-industry wastes (Ruanglek *et al*., 2006) and hydrolysates such as wheat waste steam (Davis *et al*., 2006), corn stover hydrolysate (Mohagheghi *et al*., 2004) by *Z. mobilis* have been studied. Moreover, genetic engineering techniques were used as tools to mutant wild type strain of *Z. mobilis* for consuming of other sugars such as xylose (Mohagheghi *et al*., 2004) and arabinose (Deanda *et al*., 1996).

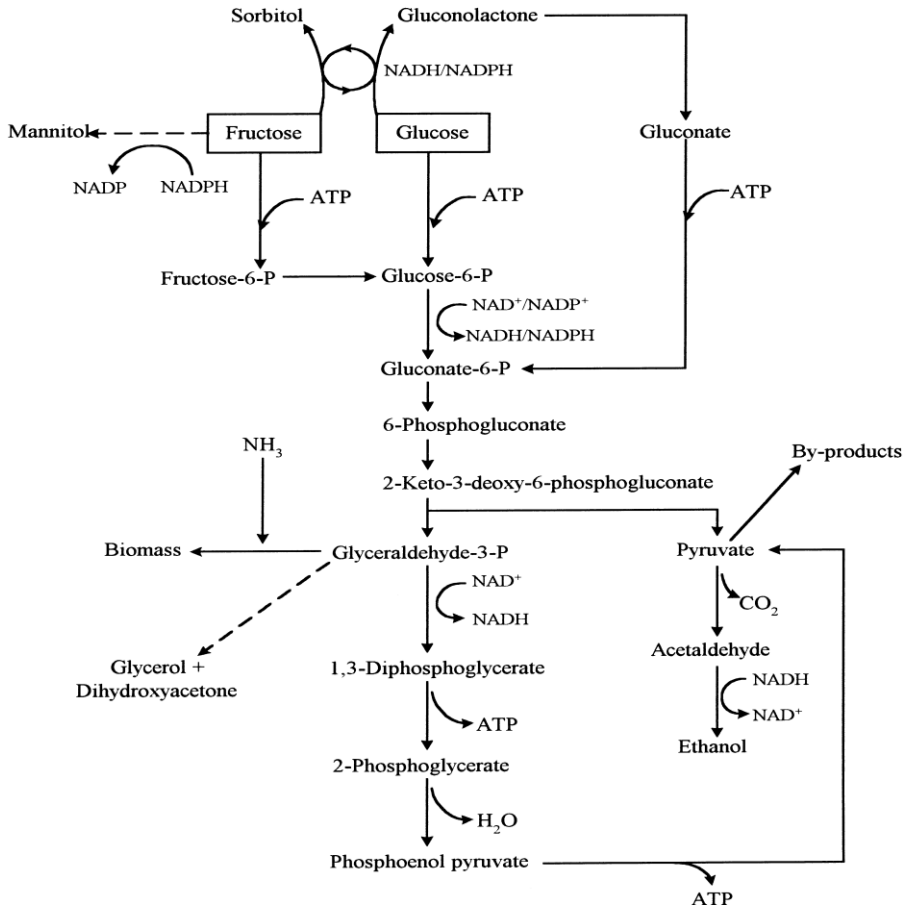


Figure 7. Schematic representation of glucose and fructose metabolism in *Z. mobilis*. ⟶common pathway, - - -▸ exclusive fructose pathway.

Source: Lee and Huang (2000)

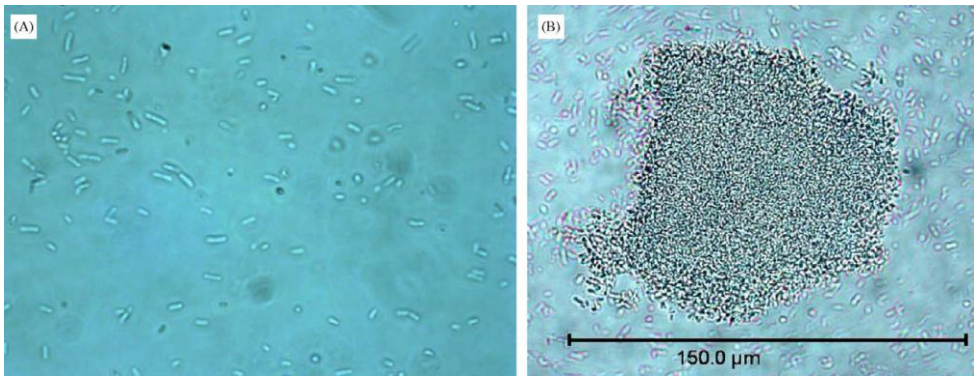


Figure 8. Photograph showing single cells of (A) *Z. mobilis* ZM4 and (B) its flocculent.

Source: Davis *et al*. (2006)

1.3 Enterobacter

Enterobacter aerogenes HU-101, isolated as a high-rate H_2 producer from methanogenic sludge, can convert various carbohydrates, such as sugars and sugar alcohols, to H_2, ethanol, 2,3-butanediol, lactate and acetate. *E. aerogenes* HU-101 mainly produces H_2 and ethanol with a minimal production of other by-products when glycerol was used as the substrate. Thus, the microorganism can be utilized for the high-yield production of H_2 and ethanol from biodiesel wastes containing glycerol (Ito *et al*., 2005).

Ethanol production from glycerol-containing wastes discharged after a manufacturing process for biodiesel fuel (biodiesel wastes) using *Enterobacter aerogenes* HU-101 was studied (Ito *et al*., 2005). The yield of ethanol decreased with an increase in the concentrations of biodiesel wastes and commercially available glycerol (pure glycerol). Furthermore, the rate of ethanol production from biodiesel wastes was much lower than those at the same concentration of pure glycerol, partially due to a high salt content in the wastes. In continuous culture with a packed-bed reactor using self-immobilized cells, the maximum rate of ethanol production from pure glycerol was 0.8 mol/mol-glycerol. However, using porous ceramics as a support material to fix cells in the reactor, the maximum ethanol production rate from biodiesel wastes reached 0.85 mol/mol-glycerol (Ito *et al*., 2005).

(2) Yeast

Various yeasts are capable of fermenting D-xylose along with D-glucose. These are *Pachysolen tannophilus, Pichia stipitis,* and *Candida shehatae.* This has led to a growing interest in the use of lignocellulose residues for the industrial production of ethanol since the conversion of both the hemicellulose and cellulose fractions substantially increases the yield of ethanol (Sánchez *et al*., 1997).

2.1 *Saccharomyces cerevisiae*

Baker's yeast (*Saccharomyces cerevisiae*) is the most commonly used microorganism for ethanol production due to its excellent characteristics of growing at high sugar concentrations and producing high yields of ethanol. However, it brings

about two major problems for ethanol production from wood hydrolyzates. The first one is due to the presence of toxic compounds in some of the hydrolyzates, which make the cells unable to grow (Taherzadeh *et al*., 1997). This problem is usually tackled by detoxification. The second problem is related to its lack of capability to ferment xylose (Kötter and Ciriacy, 1993).

2.2 *Candida shehatae*

C. shehatae is one of a few yeasts, which can ferment both glucose and xylose to ethanol (Delgenes *et al*., 1996). Moreover, mannose and galactose were also fermented by this yeast (Sreenath *et al*., 2000). Bio-ethanol production from D-xylose of *C. shehatae* is via xylose pathway (Seiboth *et al*., 2003), before passing through pentose phosphate pathway and glycolysis in order to produce ethanol (Fig. 9). Studies on the possible effects of the availability of oxygen on the metabolism of D-xylose by *C. shehatae* found that, in principle, an extra supply of oxygen was unnecessary, although ethanol production was indeed enhanced by added oxygen when using either D-xylose or D-glucose as the carbon source (Sánchez *et al*., 1997). When the oxygen supply was restricted, some growth occurred, but no ethanol was produced. This indicates that for the efficient conversion of D-xylose into ethanol, the aeration rate should be higher than 0.02 v/v/min (Delgenes *et al*., 1986). These publications suggested that an optimum aeration rate must be achieved in order to obtain maximum productivity and ethanol yield (Sánchez *et al*., 1997). However, these explanations are contrast with Alexander et al. (1988). They demonstrated that *C. shehatae* exhibits three different types of metabolic behavior; (1) under fully aerobic conditions, in which oxygen is available in excess, respirative growth occurs without fermentation, (2) fermentation and respirative growth occur simultaneously under semi-aerobic conditions wherein growth is limited by the oxygen supply, (3) Under anaerobic conditions fermentation occurs without growth.

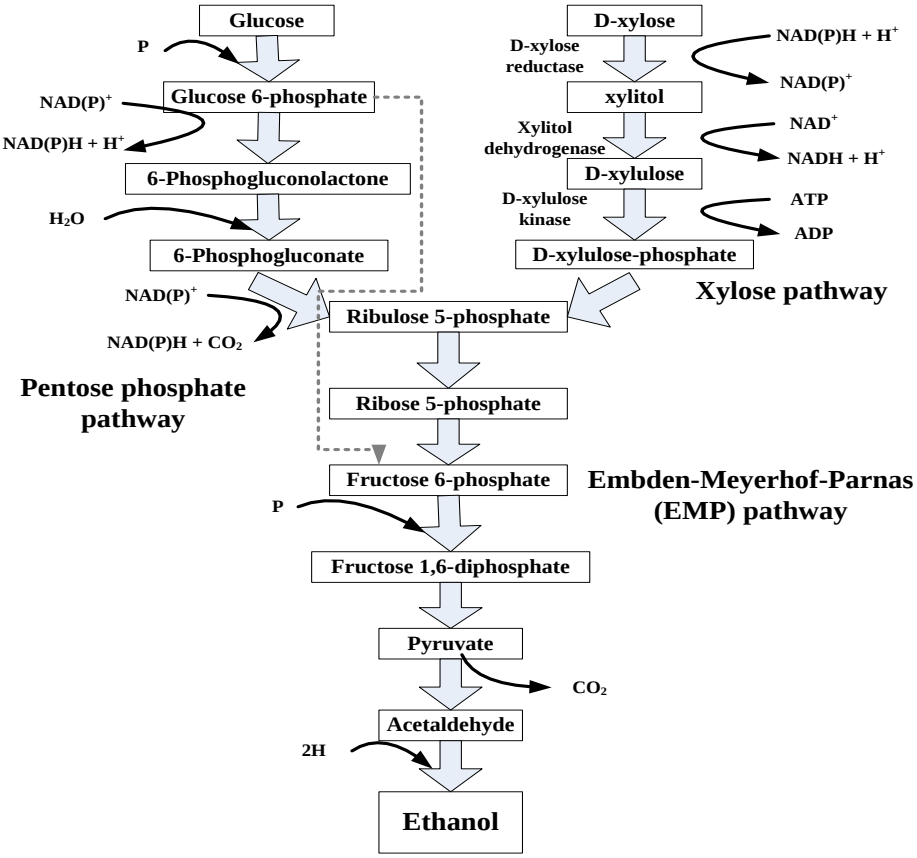


Figure 9. Diagram of ethanol production via xylose pathway, pentose phosphate pathway, and Embden-Meyerhof-Parnas (EMP) pathway.

Source: Modified from Seiboth *et al*. (2003)

Aerobic and anaerobic xylose metabolism may be limited by different factors. Aerobic xylose uptake in continuous culture appears to be transport-limited. Alternatively, aerobic xylose consumption could be affected by the levels of xylose reductase (XOR) or glucose6-phosphate dehydrogenase (GPD) (this enzyme provides the reductant necessary for NADPH-linked xylose reduction). Anaerobic metabolism proceeds at only a third the aerobic rate and may be limited by low levels of key enzymes such as NADH-linked XOR activity, xylitol dehydrogenase (XID) or alcohol dehydrogenase (ADH). NADH-linked XOR can relieve reductant imbalance that arises in cells under anoxic conditions (Bruinenberg *et al*., 1984). Alcohol dehydrogenase is responsible for ethanol production and may be implicated in the lack of anaerobic fermentation by fully-aerobic cells (Alexander *et al*., 1988).

With some xylose-metabolizing yeasts, their inability to produce ethanol anaerobically has been accounted for by an imbalance between NADH production and NADH consumption. The imbalance arises because xylose is first reduced to xylitol by an NADPH-linked aldehyde reductase (= xylose reductase or XOR, EC 1.1.1.21), and the resulting xylitol is converted to D-xylulose by an NAD^+ -linked xylitol dehydrogenase (Fig. 10). After phosphorylation, xylulose-5-phosphate is rearranged by non-oxidative reactions to yield hexose phosphate and triose phosphate. A portion of the hexose phosphate can be oxidized via glucose-6-phosphate dehydrogenase to yield NADPH for assimilation. Otherwise, metabolism continues through the glycolytic pathway to yield ethanol in a balanced fermentation (Fig. 10). Yeasts known to convert xylose to ethanol under anoxic conditions (i.e., ferment) also possess an XOR that is active with NADH as well as NADPH (Alexander *et al*., 1988).

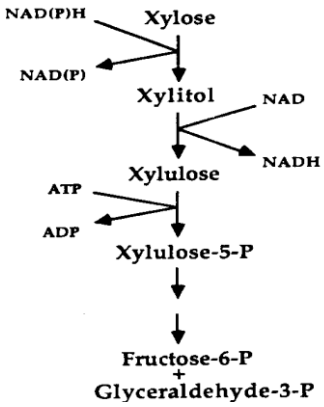


Figure 10. Initial steps in the metabolism of D-Xylose by yeasts.
Source: Alexander *et al*. (1988)

The most adequate pH for the growth of *C. shehatae* was between 3.5 and 4.5 (Du Preez *et al*., 1984). Whereas Sreenath and Jeffries (2000) reported that the ethanol production rate from wood hydrolysate by *C. shehatae* Y-049 was optimum in the pH range of 5.5-6.0 giving ethanol yield of 0.41-0.46 g/g.

2.3 *Pachysolen tannophilus*

Pachysolen tannophilus can ferment both hexose and pentose sugars to ethanol. The optimal temperature for growth of this strain was at 30-32➡C (Rorbuck

et al., 1995) while the optimal temperature for producing ethanol under anaerobic condition was at 37➡C (Converti *et al*., 2001). The highest values for ethanol yield (0.39 g ethanol/g substrate) and the specific ethanol production rate (0.06 kg/kg.h) were obtained from fermentation at 30➡C, pH 4.5 for 50 h (Sánchez *et al*., 2004).

2.4 *Pichia stipitis*

Among the wild-type xylose-fermenting yeast strains for ethanol production, *Pichia stipitis* reportedly provides one of the best overall performances in terms of complete sugar utilization, minimal by-product formation, low sensitivity to temperature, and substrate concentration (Taherzadeh *et al*., 2003). Furthermore, *P. stipitis* has no absolute vitamin requirement for xylose fermentation and is able to ferment a wide variety of sugars to ethanol (Taherzadeh *et al*., 2003; Synowiecki and AL-Khateeb, 1997; Karimi *et al*., 2006a). Ethanol yield between 0.24 and 0.47 g/g has been obtained by *P. stipitis* on different hydrolyzates (Taherzadeh *et al*., 2003; Synowiecki and AL-Khateeb, 1997). However, *P. stipitis* has some limitations, among which the requirement of oxygen to maintain cell viability, xylose transport and ethanol productivity can be mentioned. While the yeast rapidly loses viability without sufficient oxygen, excess oxygen completely stops ethanol production and the cells respire the substrate to form biomass (Taherzadeh *et al*., 2003). *P. stipitis* had a better performance in ethanol production at identical conditions with ethanol yield 0.38 g/g of the sugars within the hydrolyzate.

(3) Filamentous fungi

Although filamentous fungi such as *Rhizopus* sp. and *Mucor (please check) indicus* have been industrially used for a long time for several purposes, a number of process engineering problems are associated with these organisms due to their filamentous growth. Problems can appear in mixing, mass transfer, and heat transfer. Furthermore, attachment and growth on bioreactor walls, agitators, probes, and baffles cause heterogeneity within the bioreactor and problems in measurement of controlling parameters and cleaning of the bioreactor. Such potential problems might hinder industrial application for ethanol production from lignocellulose hydrolyzate (Karimi *et al*., 2008). However, filamentous fungi have been used for ethanol

production from several lignocellulosic hydrolysates because of their high tolerant to toxic compounds in hydrolysates (Karimi *et al*., 2008).

7.4.5 Factors affecting on bio-ethanol production from lignocellulosic materials

7.4.5.1 Type of microorganisms

Most of microorganisms used in bio-ethanol production by fermentation are yeast and bacteria, which can convert rapidly mono-sugars to ethanol. For example, *Pachysolen tannophilus* (yeast) is able to convert glucose and xylose to ethanol (Bravo *et al*., 1995), *Zymomonas mobilis* (bacteria) can use glucose, fructose or sucrose and convert to ethanol (Ahring *et al*., 1996), *Pichia stipitis* is a specie of yeast that can convert xylose to ethanol, and *Candida shehatae* NCL-3501 is a good specie of yeast because it can convert both glucose and xylose rapidly to ethanol in the fermentation (Abbi *et al*., 1996; Lee, 1997; Nigam, 2002). Recently, genetic engineering technique was used to improve some strains of yeast and bacteria *Zymomonas mobilis* 8b, that can use both glucose and xylose as substrates for ethanol production (Mohagheghi *et al*., 2006). Ethanol productions of above microorganisms are performed in Table 6.

Table 6. Ethanol production from glucose and/or xylose by microorganisms.

Microorganism	Carbon source	Ethanol yield (g/g)	Ethanol productivity (g/l/h)	References
S. cerevisiae	Glu. and Xyl.	0.45	-	Laplace *et al*., 1991
C. shehatae	Xyl.	0.48	0.19	Jeffries and Jin, 2000
	Glu. and Xyl.	0.37-0.47	-	Abbi, *et al*., 1996
	Glu. and Xyl.	0.39	-	Laplace *et al*., 1991
P. stipitis	Xyl.	0.45	0.34	Jeffries and Jin, 2000
	Xyl.	0.35-0.41	-	Nigam, 2002
	Glu. And Xyl.	0.44	-	Laplace *et al*., 1991
P. tannophilus	Xyl.	0.24	0.13	Jeffries and Jin, 2000
	Glu. and Xyl.	0.34-0.39	-	Bravo *et al*., 1995; Sanchez *et al*., 2004

Z. mobilis	Glu. and Xyl.	0.42	-	Mohagheghi, *et al*., 2006
	Glu. and Xyl.	0.43	-	Laplace *et al*., 1991
	Sucr.	0.40	3.82	Lee and Huang, 1995

Remark: Xyl. = Xylose, Glu. = Glucose, Sucr. = Sucrose

7.4.5.2 Inhibitors

With acid hydrolysate and autohydrolysate, the maximum ethanol yields based on sugar consumption were 0.37 and 0.47 g/g with free cells, respectively (Abbi *et al*., 1996). The lower ethanol production in acid hydrolysate compared to autohydrolysate may be due to the presence of inhibitory compounds such as furfural and phenolics, which are almost absent in autohydrolysates (Abbi *et al*., 1996).

Acetic acid is one of the most prevalent. At the pH optimum for fermentation (5.5-6.0), acetic acid is largely undissociated. This permits diffusion into the cell cytoplasm, where it dissociates and decreases the intracellular pH. As a result, the proton gradient across the membrane cannot be maintained and the transport of various nutrients is impaired (Sreenath and Jeffries, 2000). Hence, in the presence of acetate, yeast fermentation of wood hydrolyzates is poor.

Several detoxification methods like neutralisation, overliming with calcium hydroxide, activated charcoal, ion exchange resins (Carvalheiro *et al*., 2005) and enzymatic detoxification using laccase (Jönsson *et al*., 1998) are known for removing various inhibitory compounds from lignocellulosic hydrolysates as described in Table 2.

Sugarcane bagasse hydrolysis with 2.5% (v/v) HCl yielded 30.29 g/l total reducing sugars along with various fermentation inhibitors such as furans, phenolics and acetic acid. The acid hydrolysate when treated with anion exchange resin brought about maximum reduction in furans (63.4%) and phenolics compounds (75.8%). Treatment of hydrolysate with activated charcoal caused 38.7% and 57.5% reduction in furans and total phenolics, respectively. Laccase reduced the most phenolics compounds (77.5%). Fermentation of these hydrolysates with *Candida shehatae* NCIM 3501 showed maximum ethanol yield (0.48 g/g) from ion exchange treated hydrolysate, followed by activated charcoal (0.42 g/g), laccase (0.37 g/g), overliming (0.30 g/g) and neutralized hydrolysate (0.22 g/g) (Chandel *et al*., 2007).

7.4.5.3 Effect of pH

As far as the pH of the culture medium is concerned, it should be borne in mind that this variable affects cell growth and its influence may vary considerably among yeast strains. The cell membranes are not completely permeable to hydrogen ions and so the intracellular pH and that of the culture medium may not be the same (Sánchez *et al*., 1997). Apart from affecting cell membrane permeability, pH may also determine the solubility of some components of the medium: thus, a modification in the pH might also cause some micronutrient to precipitate and so become impossible to be assimilated. The ethanolic producing yeast and bacteria have the difference of pH for growth and fermentation. For example, the optimal pH for growth by *P. tannophilus* was 3.7 (Roebuck *et al*., 1995), while the optimal pH from another researcher was 5.2 (Xu and Taylor, 1993). The optimal pH of *Zymomonas mobilis* was 4.93 (Bandaru *et al*., 2006). The ethanol production from wood hydrolysate by *C. shehatae* Y-049 was optimum in the pH range of 5.5-6.0 giving ethanol yield of 0.41-0.46 g/g (Sreenath and Jeffries, 2000). Du Preez *et a1*. (1986) report that the most adequate pH for the growth of *C. shehatae* was 3.5-4.5. Moreover, Sánchez *et al*. (1997) found that the best initial pH for ethanol production from xylose by *C. shehatae* in batch fermentation was 4.5. Under these conditions, the maximum specific growth rate (μ_{max}) was 0.329 h^{-1} and the specific ethanol production rate (qE) was 0.72 g/g/h and ethanol yield was 0.41 g/g.

7.4.5.4 Effect of oxygen

The dissolved oxygen tension (DOT) is particularly critical in attaining maximal ethanol production with xylose-fermenting yeasts. *P. stipitis* and *C. shehatae* require aeration for maximal ethanol production. Under anoxic conditions, the specific ethanol productivity of *P. stipitis* and *C. shehatae* decreased (Table 7), and especially in the case of *C. shehatae*, xylitol production increased (Jeffries and Jin, 2000).

Sánchez *et al*., 1997 studied the effect of air supply on the production of ethanol from xylose using the yeast *C. shehatae* in a batch reactor. The aeration via the stirring vortex of the bioreactor was sufficient. Under these conditions, the maximum specific growth rate was 0.329 h^{-1}; overall biomass yield was 0.036 g/g; the

specific uptake rate of xylose was 2.0 g/g/h; and the specific ethanol production rate was 0.72 g/g/h. The overall ethanol yield was 0.41 g/g.

In addition, rotary of the shaker should be effective enough to provide gentle mixing and surface aeration during the first period of the growth phase (Phisalaphong *et al*., 2006). The oxygen requirement for ethanol production was considered, but it is apparent that oxygen plays various roles in the metabolism of xylose by eukaryotes. It is very important for a xylose-fermenting yeasts to possess an aldose reductase that is active with both NADH and NADPH in order to maintain redox balances during xylose assimilation (Verduyn *et al*., 1985).

In the absence of aeration, ethanol accumulation is still continues, but at a much lower rate, and xylitol production increased (Jeffries and Jin, 2000). *C. shehatae* requires oxygen to maintain viability. Oxygen starvation induces cell death in *C. shehatae* when it is grown on xylose, but not when it is grown on glucose. Growth of *C. shehatae* was limited to one division or less when cells cultivated aerobically on either glucose or xylose are shifted from aerobic to anaerobic conditions. The cultivation of *P. stipitis* on glucose increases the activity of plasma membrane ATPase 3-folds in comparison to the activity obtained when cells are grown on xylose. These results indicated that plasma membrane ATPase activity, which is critical for transport, correlates with ethanol tolerance and the inhibitory effect of ethanol on growth. Plasma membrane ATPase is essential for maintaining the proton gradient that is responsible for uptake of nutrients. These yeasts require active electron transport for the synthesis of uracil, and hence can not make mRNA under anaerobic conditions (Jeffries and Jin, 2000).

Table 7. Performance of xylose-fermenting yeasts on aeration.

Strains	Substrate (g/l)	Condition	Ethanol yield (g/g)	Productivity (g/l/h)	Biomass yield (g/g)
P. stipitis CBS7126	Xylose (40)	Aerobic	0.18	0.17	0.39
		Facultative	0.47	0.20	0.05
		Anaerobic	0.40	0.02	0.03
	Glucose (40)	Aerobic	0.26	0.17	0.23
		Facultative	0.38	0.28	0.14
		Anaerobic	0.33	0.13	0.10
C. shehatae CBS2779	Xylose (40)	Aerobic	0.22	0.21	0.33
		Facultative	0.37	0.32	0.01
		Anaerobic	0.41	0.15	0.01
	Glucose (40)	Aerobic	0.33	0.35	0.21
		Facultative	0.42	0.51	0.03
		Anaerobic	0.44	0.29	0.02
P. tannophilus NRRL Y-2460	Xylose (40)	Aerobic	0.10	0.04	0.25
		Facultative	0.28	0.10	0.01
		Anaerobic	0.26	0.07	0.01
	Glucose (40)	Aerobic	0.31	0.38	0.14
		Facultative	0.43	0.49	0.06
		Anaerobic	0.42	0.18	0.04

Source: Jeffries and Jin (2000)

7.4.5.5 Effect of immobilized cell

Cell immobilization support for ethanol production has been classified into two types based on source: (i) synthetic supports such as gelatin, carrageen (Yu *et al*., 2007), Ca-alginate (Behera *et al*. 2010), agar-agar (Behera *et al*. 2010), polyurethane (Fujii *et al.*, 1999) and ceramic beads or porous glass (Kourkoutas *et al*., 2006), and (ii) natural supports such as chitosan (Fujii *et al*., 1999), sawdust, wood chip, rice husk, rice straw, spent grain, delignified spent grain (Kopsahelis *et al.*, 2007), apple piece (Kourkoutas *et al.*, 2006), sorghum bagasse (Yu *et al*., 2007) and watermelon pieces (Reddy *et al.*, 2008). The benefits of natural supports are wide spread in the nature, low cost, and ease to operate in bioprocess fermentation i.e.

better operational stability, less contamination, protect cell from shear force, ease to separate cell in downstream process, less effect by inhibitory compounds and remain cell viability for several cycles of operations (Chandel *et al*., 2007; Reddy *et al*., 2008; Behera *et al*., 2010).

The immobilized yeast cells, *Debaromyces hansenii*, in Ca-alginate matrix produced ethanol with a yield of 0.46 g/g from hemicellulosic hydrolysates and were reused six times with 100% fermentation efficiency (Menon *et al*., 2010).

7.4.5.6 Effect of temperature

Temperatures that provide for optimum biomass and ethanol productivities do not necessarily enable maximum ethanol accumulation. This implies that ethanol toxicity affects production. In *P. stipitis*, xylitol increases with high temperature. Maximum ethanol selectivity was achieved at 25-26➡C (Jeffries and Jin, 2000). Numerous studies have shown that temperatures above 37➡C are detrimental for ethanol production (Cazetta *et al*., 2007). The deleterious effects of high temperature were considered to be due to the denaturation of ribosomes and enzymes and problems associated with the fluidity of membranes (Phisalaphong *et al*., 2006). *P. tannophilus* can ferment both hexose and pentose sugars to ethanol. The optimal temperature for growth of this strain was at 30-32➡C (Rorbuck *et al*., 1995) while the optimal temperature for producing ethanol under anaerobic condition was at 37➡C (Converti *et al*., 2001). The highest values for ethanol yield (0.39 g ethanol/g substrate) and the specific ethanol production rate (0.06 g/g/h) were obtained from fermentation at 30➡C, pH 4.5 for 50 h (Sánchez *et al*., 2004).

7.4.5.7 Effect of carbon source and concentration

The maximum ethanol production attained by *P. stipitis* and *C. shehatae* doesn't affect by xylose utilization (Jeffries and Jin, 2000). The cell metabolism was strongly affected by the substrate and product concentrations, which could be classified into two types: limitation and inhibition (Phisalaphong *et al*., 2006). However, the ethanol concentration resulting in growth inhibition depended on the sugar consumption. In the case of xylose, growth inhibition occurred at 30 g ethanol/l, but with glucose, cells continued to grow up to 34 g ethanol/l by *P. tannophilus* (Jeffries and Jin, 2000). The higher ethanol tolerance observed with

glucose as a carbon source correlated with higher plasma membrane H^+-ATPase activity. The ethanol tolerance of *P. tannophilus* changes with the carbon source used for growth. When cultivated on xylose as a sole carbon source, this yeast produces only 20 g/l of ethanol. However, *P. tannophilus* produces up to 55 g/l when cultivated on glucose. *P. tannophilus* also produces ethanol much more rapidly on glucose than on xylose (Jeffries and Jin, 2000).

Substrate concentration also affected on the ethanol production. The optimal D-xylose concentration for ethanol production by *P. tannophilus* was 25 g/l, which gave a maximum specific growth rate of 0.26 h^{-1}, biomass productivity of 0.023 g/l h, specific ethanol production rate of 0.065 g/g h and ethanol yield of 0.34 g/g (Bravo *et al*., 1995).

7.4.5.8 Effect of glucose to xylose ratio

In the fermentation on mixture of glucose and xylose, glucose is utilized first. After glucose is used up (12 h), xylose is fermented (Zhao *et al*., 2008). Ethanol concentration and biomass increased quickly at the beginning of fermentation according to the fast glucose consumption then increased slowly after glucose was exhausted. The maximum ethanol production by *P. tannophilus* were 5.80, 4.80 and 3.85 g/l appeared at the 36 h in fermentation on mixed glucose and xylose at 3:1, 1:1 and 1:3, respectively (Zhao *et al*., 2008).

7.4.5.9 Type of fermentation

Fed-batch culture is a batch culture. It is fed continuously or sequentially with substrate without the removal of fermentation broth. It is widely used for the production of microbial biomass, ethanol, organic acids, antibiotics, vitamins, enzymes and other compounds. Fed-batch culture compared to the conventional batch culture has several advantages including very low concentration of residual sugars, higher dissolved oxygen in the medium, decreased fermentation time, higher productivity and reduced toxic effects of the medium components which are present at high concentrations (Roukas, 1996) as well as eliminating substrate inhibition (Ozmichi and Kargi, 2007).

When a portion of the fermentation broth is withdrawn at intervals and the residual part of the culture is used as an inoculum for the next fed-batch culture,

the system is operated as a repeated fed-batch culture or semi-continuous culture. In addition to increased productivity, semi-continuous culture has the advantages which are (i) it does not require new inocula for each consecutive fed-batch and (ii) the contamination of the medium is also lower than in the continuous culture. Thus semi-continuous culture is considered one of the most useful systems for economical ethanol production (Roukas, 1996).

OBJECTIVES

1. To extract holocellulose from palm pressed fiber (PPF) by alkaline extraction.
2. To produce hemicellulose from the extracted holocellulose by chemical process.
3. To optimize furfural production from hemicellulose using two-stage process.
4. To produce monomeric sugars from holocellulose and/or PPF and optimize the condition by chemical process.
5. To produce bio-ethanol from monomeric sugars by various microorganisms
6. To optimize bio-ethanol production from the selected source and microorganism.
7. To scale-up the ethanol production process by the selected strain.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Palm pressed fiber

Palm pressed fiber (PPF) was kindly provided by Thai-Taro & Oil, Co., Ltd., Suratthanee Province, Thailand.

1.2 Microorganisms and fermentation condition

1.2.1 *Zymomonas mobilis* TISTR405

Z. mobilis TISTR405 was purchased from Thailand Institute of Scientific and Technological Research (TISTR), Thailand. For short-term storage, stock cultures were stored on Zymomonas medium agar at 4➡C and subcultured every 3-4 weeks. For long-term storage, the cultures were suspended in sterile 40% (v/v) glycerol and stored in 1 ml volumes at -70➡C. When required, individual aliquots were thawed rapidly and regenerated at 30➡C in liquid Zymomonas medium (Davis *et al*., 2006).

Seed culture of *Z. mobilis* TISTR405 was prepared by liquid Zymomonas medium (pH 5.5) and incubated at 30➡C. Fermentation was started with 10% (v/v) inoculum at an absorbance of 0.5 at 660 nm which indicated cells in the exponential growth phase (Davis *et al*., 2006).

1.2.2 *Candida shehatae* TISTR5843

C. shehatae TISTR5843 purchased from TISTR, Thailand, was maintained on yeast-malt (YM) agar slant. Stock cultures were stored at 4➡C and subcultured every 3-4 months (Chandel *et al*., 2007; Lebeau *et al*., 2007).

The inoculum of *C. shehatae* TISTR5843 was prepared by harvesting the cells grown for 24 h at 30➡C in the culture medium containing 15.0 g/l glucose and a cell suspension was prepared in sterile water containing 0.85% NaCl. The cell number was maintained by adjusting the OD at 600 nm in the range of 0.6–0.8 (corresponding to dry weight of cell mass 0.8–0.95 g/l) (Appendix E). Each 250 ml

Erlenmeyer flask containing 100 ml working volume was inoculated with 10% (v/v) of starter and incubated at 30➡C on a rotary shaker (150 rpm) (Chandel *et al*., 2007).

1.2.3 *Saccharomyces cerevisiae* TISTR5017

S. cerevisiae TISTR5017 obtained from Microbiological Laboratory, Faculty of Agro-Industry, Prince of Songkla University, Thailand, was stocked on YM agar (Lebeau *et al*., 2007), kept at 4ºC and subcultured every 2 weeks.

The inoculum of *S. cerevisiae* TISTR5017 was prepared as described above for *C. shehatae* TISTR5843 (Chandel *et al*., 2007; Lebeau *et al*., 2007).

1.3 Media

Yeast malt (YM) agar contained (g/l): malt extract 5.0, glucose 10.0, yeast extract 3.0, peptone 5.0, agar 20, and pH 6.4 (Chandel *et al*., 2007; Lebeau *et al*., 2007).

Zymomonas medium contained (g/l): glucose 20, yeast extract 10 bacteriological peptone 10 and agar 15 (Davis *et al*., 2006).

1.4 Enzyme

The liquid cellulase, Accellerase 1000, from *Trichoderma reesei* was kindly provided by Danisco US Inc. (Genencor Division). Carboxymethyl cellulose (CMC) (Sigma-Aldrich) was used as a substrate for determination of cellulase (CMCase) activity. Cellulose-PPF, obtained from delignification of PPF, was a substrate for glucose production.

1.5 Chemicals and reagents

All chemicals and reagents employed were of analytical or reagent grade and commercial grade. Their lists and sources were shown in Appendix A

1.6 Instruments

Most of the instruments used in this work were in Faculty of Agro-Industry, and some equipments were supported by Scientific Equipment Center (SEC), Prince of Songkla University, Thailand. All lists and sources of instrument are given in Table 8.

Table 8. List of some instruments.

Instrument name	Model	Source
Autoclave	SX-700	Tomy, USA
Balance	BP 221S	Sartorius, Germany
Bio-safety cabinet	25 MANOMETER	DWYER Instruments Inc., USA
Centrifuge	UNIVERSAL 32R	Hettich Zentrifugen
Fermentor	MDL 300	B.E. Marubishi, Japan
Gas chromatography (GC)	GC Model 6850	Hewlett-Packard, USA
High performance liquid chromatography (HPLC)	HPLC Model 1100	Hewlett-Packard, USA
Hot air oven	ULM 500	Memmert, USA
Micro centrifuge	Centrifuge A 14	Jouan, France
pH meter	Delta 320	TOLEDO, China
Rotary Evaporator	SB 651	Rikakai Co.LTD., Tokyo
Scanning electron microscope	Quanta400	
Shaker	SK3-PO SSeriker II	Welsource CO.,LTD
Spectrophotometer	Anthos Zenyth 200rt	Anthos labtec instruments, Austria
Water bath	MP	Judabo, Perkin-elmer (Thailand) Co., LTD

2. Analytical methods

2.1 Sugars analysis

2.1.1 Qualitative method

The sugar compositions of palm pressed fiber (PPF)'s hemicellulose were determined by Thin Layer Chromatography (TLC). TLC Silica gel 60 F_{254} aluminum sheet (Merck) employing isopropyl alcohol, ethyl acetate and water (3:3:1) were used. N-(1-napthyl)-ethylenediamine (0.3 g in 5 ml of conc. H_2SO_4 and 100 ml of methanol) was a spray dye. Standard sugars were arabinose, rhamnose, xylose,

fructose, galactose, glucose and mannose (50 mg/ml of each sugar) (Bandaiphet, 2007). The hemicellulose extracted from PPF were digested by 5N TFA at 120➡C for 90 min (Marzialetti *et al*., 2008). Hydrolysates and standard sugars were spotted on TLC, sprayed with N-(1-napthyl)-ethylenediamine, and then dried at 103➡C for 20 min. Colors and R_f values of standard sugars were considered to estimate the sugar type and constituents of these hydrolysates.

2.1.2 Quantitative method

2.1.2.1 High performance liquid chromatography (HPLC)

The sugars were determined by HPLC (Hewlett-Packard, Agilent 1100, USA) with Zorbax NH_2 column (4.6×250 mm, 5-Micron, Agilent, USA) combined with RI detector. Mobile phase was acetonitrile and water in the ratio of 75:25 v/v, operating temperature was controlled at 25➡C and flow rate was set at 0.7 ml/min. Standard sugars were run in the same condition (Rahman *et al*., 2006).

2.1.2.2 Somogyi-Nelson method

Residual reducing sugar was determined by Somogyi-Nelson method as described in Appendix B (Somogyi, 1952; Nelson, 1944).

2.2 Enzyme assay

Cellulase enzyme solution and CMC (1%) were prepared in sodium acetate buffer (0.05 M, pH 4.8). 0.5 ml of 1% CMC was incubated with 0.5 ml of enzyme solution at 50°C for 30 min. The reaction was terminated by boiling for 10 min (Sharma *et al*., 2001). The reducing sugar produced as a result of CMC hydrolysis was determined by the Somogyi-Nelson method (Somogyi, 1952; Nelson, 1944). One enzyme unit (U) liberates 1 ↘mol of reducing sugar per minute at 50°C, pH 4.8 and for 30 min. The saccharification values were calculated by using the formula (Eq. 1) given by Sharma et al. (2004) and Chen et al. (2008):

$$Saccharification\ value\ (\%) = \frac{\text{Reducing sugar } (g) \times 0.9}{\text{Polysaccharides in substrate } (g)} \times 100 \quad \ldots\ldots\ldots (1)$$

2.3 Ethanol, furfural and acetate determination

Ethanol, furfural (or furaldehyde) and acetate were analyzed by GC-FID (HP 6850, Hewlett Packard). The Stabilwax→-DA column and operating condition were modified from Suwansaard *et al*., 2009. Briefly, flow rate of helium was 1.2 ml/min. The temperatures of injection port and detection port were 230ºC and 250ºC, respectively. Injection volume was 1 ↘l. The initial temperature of oven was 70ºC for 1 min followed with a ramp of 20ºC/min to final temperature of 180ºC and then hold for 2 min.

2.4 Protein estimation

The protein was determined by Folin's method using bovine serum albumin as a standard protein (Appendix C) (Chapla *et al*., 2010).

2.5 Electron microscopic scanning

The samples were soaked in 3.5% glutaraldehyde for 6 h, and dried by treatment with 50, 70, 90, 95, and 100% ethanol, followed by overnight retention of samples in a desiccator for the removal of moisture. The samples were then detected by a scanning electron microscope (SEM) (Quanta400, FEI) (Yu *et al*., 2007).

3. Methods

This research aimed to produce value added products from palm press fiber (PPF) such as hemicelluloses, furfural, xylose, cellulose, glucose, and ethanol. All experiments were carried out as outline in Fig. 11.

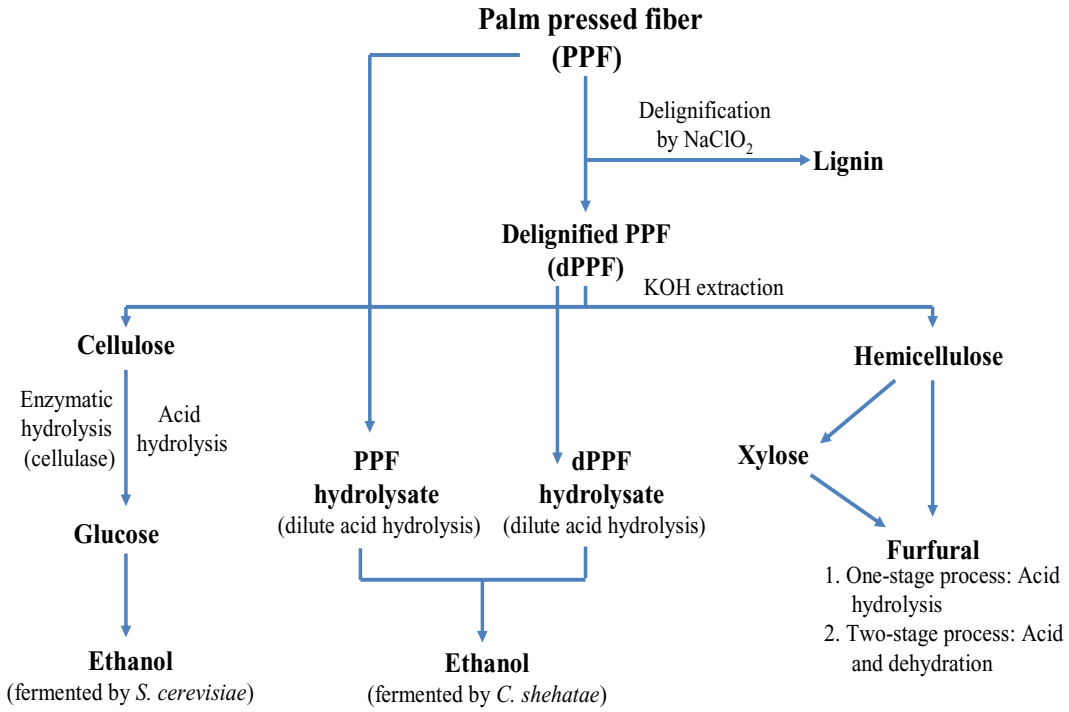


Figure 11. Flow chart of this research work.

3.1 PPF preparation and its composition

The PPF was sun-dried and ground to a particle size of <0.5 mm by sieve cut off (35 mesh analytical sieve, Fritsch, Germany). After oven-dried at 103➡C overnight, the PPF powder was kept in a plastic bag and stored at room temperature (30ºC). Prior to pretreatment, composition of PPF was analyzed, according to the standard methods (A.O.A.C., 1990).

3.2 Delignification process

PPF to sodium chlorite ($NaClO_2$) in the ratio of 10:1 (w/w) was dissolved in 0.01% acetic acid solution, at 70➡C for 1 h and repeated 3-4 times (Collings *et al.*, 1978). The delignified PPF (dPPF) was then separated by centrifugation (5,720 x *g* for 20 min) and twice washed with warm water (< 60➡C in order to prevent autohydrolysis of C5 sugars), then centrifuged again, and finally added acetone to remove water. After incubation at 45➡C overnight, the amount of

the dPPF was calculated using the equation (2). The composition of PPF and dPPF were analyzed according to the standard methods (A.O.A.C., 1990).

$$Delignifie\ d\ PPF\ (\%) = \frac{Weight\ of\ Delignifie\ d\ PPF\ (g)}{Weight\ of\ PPF\ (g)} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

3.3 Extraction and optimization of hemicellulose by alkaline hydrolysis

3.3.1 Procedure of hemicellulose extraction by alkaline hydrolysis

The dPPF was mixed with potassium hydroxide (KOH) solution and kept in ice waterbath with shaking to obtain the highest efficiency of extraction (Aquino *et al*., 2002). After centrifugation (7,244 x *g* for 30 min), the supernatant was neutralized with 5% (v/v) acetic acid and then hemicellulose was precipitated by adding 95% ethanol in the ratio of 1:1, v/v (Prasertsan and Oi, 2001). The hemicellulose after centrifugation (7,244 x *g* for 10 min) was then freeze dried. The hemicellulose yield was calculated using Eq. (3) (A.O.A.C., 1990) and the experimental protocol is illustrated in Fig. 12.

$$Hemicellul\ ose\ (\%) = \frac{Weight\ of\ hemicellul\ ose\ (g)}{Weight\ of\ PPF\ (g)} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

3.3.2 Hemicellulose component

The sugar composition of hemicellulose was determined after hydrolysis with 5% (v/v) sulfuric acid at 120➡C for 30 min (Rahman *et al*., 2006), the supernatant was neutralized (2 N NaOH) and filtered through 0.2 µm syringe filter, then analyzed by HPLC as described in Section 2.1.2.1.

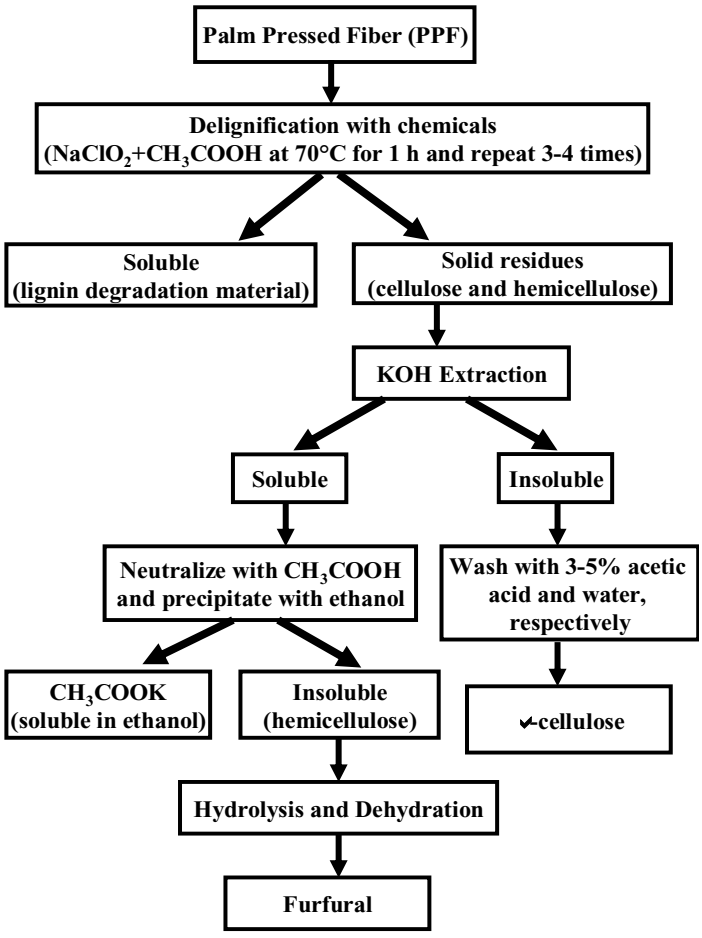


Figure 12. Experimental protocol used to determine the yields of hemicellulose and furfural.

3.3.3 Optimization of hemicellulose extraction by using RSM

Three parameters affecting KOH hydrolysis; KOH concentration (10-50% w/v), ratio of delignified PPF to KOH (1:20-1:50 (w/v)) and reaction time (20-60 min) were studied. In order to describe these parameters (as the independent variables) on hemicellulose production (as the dependent variable), 20 batch experiments were conducted by central composite designs (CCD). The concentration ranges were 10-50% w/v KOH (central value = 30% w/v), the PPF to KOH ratio of 1:20-1:50 (w/v) (central value = 1:35), and reaction time of 20-60 min (central value = 40 min). The data analysis was developed by fitting the experimental data in a smooth curve, which is plotted by calculation of specific predicted response (Khanna and Srivastava, 2005). The variables are coded according to Eq. (4) whereas a

quadratic model (Eq. (5) (Box *et al*., 1978; Sangkharak and Prasertsan, 2007; O-Thong *et al*., 2008) was used to evaluate the optimization of environmental parameters and stepwise regression analysis (Rahman *et al*., 2007).

$$\overline{Xi} = \frac{Xi - Xi^{*}}{\Delta Xi}, \quad i = 1, 2, 3, \ldots, k \quad \ldots\ldots\ldots \quad (4),$$

where $\overline{Xi}$ is the code value of an independent variable, Xi is a real value of an independent variable, Xi^{*} is the real value of an independent variable at the center point of the experiment, and ΔXi is the step change value.

$$Y_1 = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^{2} + \beta_{22} X_2^{2} + \beta_{33} X_3^{3} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad \ldots\ldots\ldots\ldots (5),$$

where Y_1 is the expected response value predicted from RSM of hemicellulos ; β_i, β_j and β_{ij} are the parameters estimated from regression results. The response variable (Y_1) was fitted using a predictive polynomial quadratic equation (5) in order to correlate the response variable to the independent variables. Regression analyze of hemicellulose was illustrated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA) (trial version). The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface plots. The quality of the fit of quadratic model was expressed by the coefficient of determination R^2 and its statistical significance was checked by the F-test. The confirmation of this experiment was conducted by the Design Expert v. 7.

3.4 Production and optimization of furfural from extracted hemicellulose

3.4.1 Production of furfural by one-stage process

The extracted hemicellulose was then used for production of furfural using one-stage process. This experiment was carried out in COD test tube and heated by COD heating box. Amount of substrate (hemicellulose extracted from dPPF) used in this research was 0.5 g. The reaction temperature was tested at 120 and 150 ➡C, the sulfuric acid concentration at 5 and 10% (v/v), the 3% (v/v) sulfuric acid to hemicellulose ratio (liquid to solid ratio, LSR) was 1:8 and 1:10 (w/v), and the reaction time of 30-120 min was studied. Furfural was analyzed by gas chromatography (GC-FID) as described in Section 2.3.

3.4.2 Production of furfural by two-stage process

3.4.2.1 Optimization of hydrolysis process by RSM

PPF was delignified by using sodium chlorite and acetic acid (Collings *et al*., 1978) and hemicellulose was extracted (as described above). Hemicellulose was then used for production of furfural using two-stage process. Each stage of furfural production was optimized independently. This experiment was carried out in flask and heated by hot air oven. Amount of substrate (hemicellulose extracted from dPPF) used in this research was 10 g. In the first stage process or xylose production process, 30 batch experiments were also conducted by the central composite design (CCD). The range of reaction temperature (X_1) was 100-150➡C (central value = 125➡C), the sulfuric acid concentration (X_2) was ranged from 1-10% v/v (central value = 5.50% v/v), sulfuric acid to hemicellulose ratio (liquid to solid ratio, L/S ratio) (X_3) was ranged from 8- 10 (v/w) (central value = 1:9 v/w), and the reaction time (X_4) of 30-120 min (central value = 75 min) was studied. The variables are coded according to Eq. (4) whereas a quadratic model (Eq. (5)) was used to evaluate the optimization of environmental parameters and stepwise regression analysis (Rahman *et al*., 2007). Data analysis was calculated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA) (trial version) as described in Section 3.3.3.

3.4.2.2 Optimization of dehydration process by RSM

For the second stage or furfural production process, sulfuric acid concentration was fixed at the same acid concentration obtained from hydrolysis process. In this study, 13 batch experiments were conducted by CCD. Reaction temperature was ranged from 120-160➡C (central value = 140➡C) and reaction time at 30-150 min (central value = 90 min) was tested. Furfural was analyzed in the liquid phase by gas chromatography (GC-FID) (modified from Suwansaard *et al*., 2009). The variables are also coded according to Eq. (4) similarly with a quadratic model (Eq. (5)) was also used to evaluate the optimization of environmental parameters and stepwise regression analysis. Regression analyze of furfural was illustrated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA).

3.5 Hemicellulosic hydrolysate production

3.5.1 Hemicellulosic hydrolysate production

Dilute sulfuric acid was varied from 0%, 5% and 10% (v/v) and carried out in 100 ml Duran bottoms. The delignified PPF (dPPF) was mixed with dilute acid in the ratio of 1:10 (w/w) (Herrera *et al*., 2003; Téllez-Luis *et al*., 2002; Yáñez *et al*., 2004; Rahman *et al*., 2006). Operating temperatures of hydrolysis were varied between 75 and 148ºC. 5 ml of samples were taken at various time intervals in the range of 30-180 min. After reaction, solids were separated from aqueous solution by filtration and used as a substrate for glucose production by enzymatic hydrolysis. The filtrate was analyzed for xylose, glucose, furfural and acetic acid.

3.5.2 Kinetics models

The acid hydrolysis models for cellulose hydrolysis involves polymer glucan of cellulose is degraded to glucose and then subsequently converted to decomposition products (Eq. 6) (Rahman *et al*., 2006).

$$glucan \longrightarrow glucose \longrightarrow decomposition\ products \quad \ldots\ldots\ldots\ldots\ldots (6)$$

Hemicellulose can be hydrolyzed by acid and proposed by different studies, which can be divided into two different kinetic mechanisms (Rahman *et al*., 2006). The first mechanism, xylan is first converted to xylooligosaccharide, which is future converted to xylose by acid hydrolysis and then xylose is subsequently decomposed to furfural. Another mechanism, xylan is converted to xylose without include the intermediate formation of xylooligosaccharide and finally xylose is decomposed to furfural (Eq. 7). The final results of both mechanisms are same.

$$xylan \rightarrow xylose \rightarrow decomposition\ products \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Therefore, it can be generalized as Eq. 8:

$$polymer \rightarrow monomer \rightarrow decomposition\ products \quad \ldots\ldots\ldots\ldots\ldots (8)$$

where k_1 is the rate of monomer production (min^{-1}) and k_2 is the rate of monomer decomposed (min^{-1}). Based on this reaction, model and solving differential equations, monomer concentration *(M)* as a function of time *(t)* can be represented by equation 9:

$$M = \frac{k_1 P_0}{(k_2 - k_1)}\left(e^{-k_1 t} - e^{-k_2 t}\right) + M_0 e^{-k_2 t} \qquad (9)$$

where P and M represents concentration of polymer and monomer, respectively. The subscript 0 represents at time $t = 0$. P_0 was fixed to 35.77 g/l (see in Chapter 3 section 3.7 and Eq. 27). Assuming M_0 to be nearly equal to 0, Eq. 9 can be modified to Eq. 10:

$$M = \frac{k_1 P_0}{(k_2 - k_1)}\left(e^{-k_1 t} - e^{-k_2 t}\right) \qquad (10)$$

The kinetic model for cellulose hydrolysis involves pseudo-homogeneous irreversible first order reactions represented by equation (11):

$$glucan \xrightarrow{k_3} glucose \xrightarrow{k_4} decomposition\ products \qquad (11)$$

where k_3 is the rate of glucose production (min^{-1}) and k_4 is the rate of glucose decomposed (min^{-1}). Actually, the operating conditions in this investigation did not want decomposition products. Thus the Eq. (11) can be modified as Eq. (12):

$$glucan \xrightarrow{k_3} glucose \qquad (12)$$

According to solving differential equations, concentration of glucose *(G)* as a function of time can be represented as Eq. (13):

$$G = G_0(1 - e^{-k_3 t}) \qquad (13)$$

where G_0 is the potential glucose concentration calculated by regression analysis. In addition, the kinetic model of furfural generation is from xylose degradation as Eq. (14). Thus the model of furfural concentration *(F)* as a function of time can be represented as Eq. (15):

$$xylose \xrightarrow{k_4} furfural \qquad (14)$$

$$F = F_0(1 - e^{-k_4 t}) \qquad (15)$$

where F_0 is the potential furfural concentration calculated by regression analysis and k_4 is also the furfural production rate (min^{-1}).

Acetic acid is a decomposition product obtained by acetyl group's degradation from hemicelluloses. The model for acetic acid generation can be represented as Eq. (16):

$$acetyl\,groups \xrightarrow{k_5} aceticacid \qquad (16)$$

Acetic acid concentration (A) in the hydrolysates as a function of time can be represented as Eq. (17):

$$A = A_0(1 - e^{-k_5 t}) \qquad (17)$$

where A_0 is the potential acetic acid concentration calculated by regression analysis and k_5 is the acetic acid production rate (min^{-1}).

Eqs. (10), (13), (15) and (17) were applied to sulfuric acid hydrolysis of delignified PPF. Non-linear regression analyses were performed to obtain the kinetic parameters and constants. The results were statistically evaluated by using the statistical one-tailed *F*-test to the variances. Coefficient of determination (R^2) and *F*-test probability were obtained to establish the significance of the models (Rahman *et al*., 2006; Téllez-Luis *et al*., 2002).

For predicting all products, the modified model was developed by relating kinetic parameters with sulfuric acid concentration by empirical equation (Eq. 18) (Rahman *et al*., 2006).

$$k_m \bullet k_0 C_a^n \qquad (18)$$

where m is in the range of 1 to 5; k_0 and n are the regression parameters; C_a is sulfuric acid concentration expressed in % v/v.

3.6 Cellulosic hydrolysate production

3.6.1 Cellulosic hydrolysate production by using enzymatic hydrolysis

The solids, cellulose obtained from hemicellulose extraction, were hydrolyzed with the cellulase (Accellerase 1000, activity 2500 U/ml). Cellulosic hydrolysate was determined total reducing sugars by the Somogyi-Nelson method using glucose as standard (Somogyi, 1952; Nelson, 1944). The cellulosic hydrolysate was then used as a source for bio-ethanol production. Parameters affecting cellulose hydrolysis, which were pH, temperature, substrate concentration, cellulose dosage and incubation time were optimized.

3.6.1.1 Effect of pH on cellulase activity

Cellulase (0.1 ml/25ml or 500 U/g substrate) was prepared in sodium acetate buffer (0.05 M) with different pH ranging from 3.6 to 6.0. Substrate or cellulose (2 g/l) was added in 25 ml of total volume. The incubation condition was at 50°C for 24 h. The reducing sugar was determined by the Somogyi-Nelson method.

3.6.1.2 Effect of temperature on cellulase activity

Cellulase (0.1 ml/25ml or 500 U/g substrate) was prepared in sodium acetate buffer (0.05 M with the optimal pH). Substrate concentration of 2 g/l was added in 25 ml of total volume and then incubated for 24 h at various temperatures ranging from 35°C to 70°C. The reducing sugar produced was determined by the Somogyi-Nelson method.

3.6.1.3 Effect of substrate concentration on cellulase activity

Cellulase (0.1 ml/25ml or 500 U/g substrate) was prepared in sodium acetate buffer (0.05 M with the optimal pH). The various substrate concentrations

ranging from 0.4 g/l to 20 g/l was added into 25 ml of enzyme solution, and then incubated for 24 h at the optimal temperature. The reducing sugar produced was determined by the Somogyi-Nelson method.

3.6.1.4 Effect of of cellulase dosage

Cellulase varied from 50 ↘l/25 ml to 1000 ↘l/25 ml (or 416 U/g substrate to 8,333 U/g substrate) was prepared in sodium acetate buffer (0.05 M with the optimal pH). The optimal substrate concentration was added into 25 ml of enzyme solution, and then incubated for 24 h at the optimal temperature. The reducing sugar produced was determined by the Somogyi-Nelson method.

3.6.1.5 Effect of incubation time and saccharification

The optimal cellulase concentration was prepared in sodium acetate buffer (0.05 M with the optimal pH). The optimal substrate concentration was added into 25 ml of enzyme solution and then incubated at the optimal temperature. The sample was taken from 5.0 min to 4320 min (72 h). The reducing sugar produced was determined by the Somogyi-Nelson method.

The saccharification values were calculated by using Eq. 19 (Chen *et al*., 2008). All experiments were done in duplicate.

$$Saccharification\ value\ (\%) = \frac{Reducing\ sugar\ (g) \times 0.9}{Polysaccharides\ in\ substrate\ (g)} \times 100 \quad \ldots\ldots\ldots (19)$$

3.6.2 Cellulosic hydrolysate production by using concentrated sulfuric acid hydrolysis

Cellulose of PPF, obtained from PPF delignification process, was mixed with concentrated sulfuric acid (72%) at various solid-liquid ratios (1:10-1:20 g/ml). The reaction was carried out at room temperature (30ºC) for 90 min. After that, cellulose of PPF hydrolysates was then diluted with distilled water in the range of 1-5% and then boiled. Reaction time was controlled in the range of 60-180 min. After boiling, the cellulose of PPF hydrolysates were cooled immediately on ices and adjusted to the final volume of 720 ml. Reducing sugars, xylose, furfural and acetic acid were determined.

3.7 Ethanol production by *C. shehatae* TISTR5843 in synthetic medium

3.7.1 Optimization of ethanol production in synthetic medium

C. shehatae TISTR5843 was firstly cultured in YM medium and incubated at 30ºC for 24 h on a shaker with shaking speed of 180 rpm. Starter was prepared by adjusting to obtain OD_{600} of 0.5. After that, 10% (v/v) (20 ml) of starter culture was transferred to the 180 ml working volume of modified YM broth (Lebeau *et al*., 2007) consisting of glucose or xylose 15 g/l; KH_2PO_4 10 g/l; $(NH_4)_2SO_4$ 5.0 g/l; malt extract 3.0 g/l; yeast extract 3.0 g/l. pH was adjusted to 5.0 by using 1 N NaOH. The routine cultivation was incubated at 30ºC on a rotary shaker (180 rpm). 4.0 ml of samples were taken every 6 h to determine the ethanol yield, residual glucose, pH, acetic acid, furfural and dry cell weight (DCW).

3.7.1.1 Effect of glucose concentration

C. shehatae TISTR5843 was cultured in the modified YM medium containing various glucose concentrations of 4, 7, 12, 24, 45 and 75 g/l and cultured under the same condition.

3.7.1.2 Effect of xylose concentration

C. shehatae TISTR5843 was cultured in the modified YM medium containing various xylose concentrations of 4, 8, 20, 40, 60 and 90 g/l and cultured under the same condition.

3.7.1.3 Effect of glucose to xylose ratio

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of glucose to xylose ratio of 10:0, 8:2, 6:4, 5:5, 4:6, 2:8 and 0:10 (w/w). and incubated as described in Section 3.3.7.1.1.

3.7.1.4 Effect of temperature

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of the optimum glucose to xylose ratio obtained from Section 3.3.7.1.3. To study the effect of temperature, the cells were incubated at room temperature (30ºC) and 35ºC.

3.7.1.5 Effect of initial pH

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of the optimum glucose to xylose ratio with various initial pH of 3.0, 4.0,

4.5, 5.0 and 6.0. The cells were cultured at the optimum temperature obtained from Section 3.3.7.1.4.

3.7.1.6 Effect of shaking speed

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of the optimum glucose to xylose ratio with the optimum pH from Section 3.3.7.1.5. The cells were cultured at the optimum temperature. The shaking speed was various at 60, 120, 180 and 240 rpm.

3.8 Ethanol production in cellulosic hydrolysate

3.8.1 Selection of ethanolic producing yeasts and bacteria

Starter culture of *C. shehatae* TISTR5843 and *S. cerevisiae* TISTR5017 were prepared by cultivating in YM medium and incubated at 30ºC for 24 h with the optimum shaking obtained from Section 3.7.1.6 (Lebeau *et al*., 2007). After that, 10% (v/v) of starter (OD_{600}=0.5) was transferred to the cellulosic hydrolysate with 200 ml working volume. The cellulosic hydrolysate containing 7.8 g/l glucose was supplemented with; KH_2PO_4 10 g/l; $(NH_4)_2SO_4$ 5.0 g/l; malt extract 3.0 g/l; yeast extract 3.0 g/l and pH 5.0. The yeast cells were cultured at 30ºC for 72 h with the optimum shaking obtained from Section 3.7.1.6. During fermentation, samples (2 ml) were taken every 6 h to determine the ethanol concentration and residual sugars.

Seed culture of *Z. mobilis* TISTR405 was prepared by cultivating in the medium (pH 5.0) containing the following components: 100 g/l glucose, 10 g/1 yeast extract, 1 g/1 KH_2PO_4, 1 g/1 $(NH_4)_2SO_4$ and 0.5 g/1 $MgSO_4.7H_2O$ under stationary incubation at 30➡C (Ruanglek *et al*., 2006; Bandaru *et al*., 2006). Fermentation was started with 10% (v/v) inoculum after 12 h cultivation and at an absorbance of approximately 0.5 at 660 nm which indicated cells in the exponential growth phase (Davis *et al*., 2006). After that, 10% (v/v) of starter (OD_{660}=0.5) was transferred to the 200 ml working volume of cellulosic hydrolysate (7.8 g/l glucose) supplemented with; 10 g/l yeast extract and 10 g/l bacteriological peptone and cultivated at 30ºC for 72 h on a shaker (180 rpm). During fermentation, samples (2 ml) were taken every 6 h to determine the ethanol concentration and residual sugars.

The strain either yeast or bacteria giving the highest ethanol concentration, ethanol yield and ethanol productivity was selected to further investigate.

3.8.2 Optimization of ethanol production from cellulosic hydrolysate by the selected stain

The selected strain was cultivated in the cellulosic hydrolysate (200 ml working volume) supplemented with nutrients as described in section 3.8.1. The optimization was performed by RSM. The initial pH (X_4) was tested in the range of 4.0 to 6.0, and cultured at 30 ºC for 72 h. Shaking speed (X_5) was varied in the range of 120 to 240 rpm and inoculum size with cell concentration (X_6) of 0.725 to 1.20 g/l corresponded to OD_{600} of 0.5-1.0. During fermentation, samples (2 ml) were taken every 6 h until 72 h cultivation to determine the ethanol concentration, residual sugars, residual acetate, pH and dry cell weight.

20 batch experiments were also conducted by central composite designs (CCD). The variables were also coded according to Eq. (4), and a quadratic model (Eq. (5)) was also used to evaluate the optimization of environmental parameters and stepwise regression analysis, as described above. The expected response values predicted from RSM consist of ethanol concentration (g/l), ethanol yield (g ethanol/g sugar) and ethanol productivity (g/l/h). Regression analysis of ethanol production was illustrated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA). The control condition contained the initial pH of 5.0, shaking speed of 180 rpm, initial cell concentration of 0.725 g/l without any nutrient supplementation.

3.9 Effect of inhibitory compounds presented in PPF hydrolysate on ethanol production by *C. shehatae* TISTR5843

Acetate, furfural and vanillin (derivative of lignin) are the main inhibitors present in lignocellulosic hydrolysate treated by dilute acid (Olsson and Hahn-Hägerdal, 1996; Delgenes *et al*., 1996). Acetate concentration was ranged from 0-10 g/l, furfural concentration was ranged from 0-2 g/l and vanillin concentration was ranged from 0-2 g/l (Delgenes *et al*., 1996). The fermentations were controlled at the optimum initial pH obtained from Section 3.7.1.5, the optimum shaking obtained from Section 3.7.1.6, and cell concentration of 0.725 g/l (OD_{600}=0.5). During

fermentation, samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al*., 2006), residual xylose (Sreenath *et al*., 2001), residual acetic acid (Suwansaard *et al*., 2009), furfural (Mansilla *et al*., 1998), furfuryl alcohol, pH and dry cell weight (DCW). The control treatment was 180 ml PPF hydrolysate without any inhibitor supplementation.

3.10 Ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate

3.10.1 Effect of nitrogen source, nitrogen concentration and C/N ratio on ethanol production from hydrolysate of PPF by *C. shehatae* TISTR5843

Starter culture of *C. shehatae* TISTR5843 was prepared by cultured in the YM broth and incubated at 30ºC for 24 h with shaking at 180 rpm (Lebeau *et al*., 2007). After that, 10% (v/v) (20 ml) of initial cell concentration of 0.725 g/l was transferred to the PPF hydrolysate with 180 ml working volume. The PPF hydrolysate containing 10 g/l xylose was supplemented with; KH_2PO_4 10 g/l; $(NH_4)_2SO_4$ 5.0 g/l; malt extract 3.0 g/l; and pH 5.0 (obtained from section 3.7) otherwise indicated. The yeast cells were cultured at 30ºC for 72 h with shaking speed of 180 rpm (obtained from Section 3.7).

Three parameters consisted of nitrogen source (NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3PO_4$, urea, yeast extract, peptone and tryptone), nitrogen concentration of 0-10 g/l and C/N ratio varied from 2.8/1-9.3/1 (w/w) were studied with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l).

3.10.1.1 Effect of nitrogen source

NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3PO_4$, urea, yeast extract, peptone and tryptone were tested as a nitrogen source to obtain the maximum ethanol production by *C. shehatae* TISTR5843. 3 g/l (based on nitrogen content) of all nitrogen sources were added into the PPF hydrolysate medium. During fermentation, samples (2 ml) were taken at 24 h and 48 h to determine the ethanol concentration (Zhu *et al*., 2006).

3.10.1.2 Effect of nitrogen concentration

The selected nitrogen source which was peptone was studied the concentration in the ranged of 0-10 g/l in the PPF hydrolysate. During fermentation,

samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al*., 2006).

3.10.1.3 Effect of C/N ratio

C/N ratio was studied in the ranged from 2.8/1-9.3/1. During fermentation, samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al*., 2006).

3.10.2 Effect of dilution of PPF hydrolysate on ethanol production by *C. shehatae* TISTR5843

PPF hydrolysates were diluted with distill water in the range of no dilution, 1/2 dilution, 1/3 dilution and 1/5 dilution (Suwansaard, 2010) with trace elements supplementation as described section 3.10.1. During fermentation, samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al*., 2006), residual xylose (Sreenath *et al*., 2001), pH and dry cell weight (DCW).

3.10.3 Optimization of factors affecting on cells growth and ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate

Due to the complex composition of PPF hydrolysate, three parameters consisted of initial pH in the range of 4.0 to 6.0, shaking speed of 60 to 180 rpm and inoculum size or cells concentration varied from 0.9 to 1.5 g/l were studied by RSM. During fermentation, samples (2 ml) were taken every 6 h to determine the ethanol concentration (Zhu *et al*., 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al*., 2009) and furfural (modified from Suwansaard *et al*., 2009). Ethanol yield was calculated from ethanol concentration divided by xylose consumption (Kim *et al*., 2008).

In order to describe the effects of initial pH, shaking speed and initial cells concentration (as the independent variables) on ethanol production (as the dependent variables), 20 batch experiments were conducted by central composite designs (CCD) (Box *et al*., 1978; Sangkharak and Prasertsan, 2007; O-Thong *et al*., 2008). The variables are coded according to Eq. (4), and a quadratic model (Eq. (5)) was also used to evaluate the optimization of environmental parameters and stepwise regression analysis, as described above. The expected response values predicted from RSM consist of ethanol concentration (g/l), ethanol yield (g ethanol/g sugar) and ethanol productivity (g/l/h). The response variable was fitted using a predictive

polynomial quadratic equation (5) in order to correlate the response variable to the independent variables (O-Thong *et al*., 2008). Regression analysis of ethanol production was illustrated by Design Expert v. (Stat-Ease. Inc., MN, USA). The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface plots. The quality of the fit of quadratic model was expressed by the coefficient of determination R^2 and its statistical significance was checked by the *F*-test (O-Thong *et al*., 2008).

3.11 Ethanol production from PPF hydrolysate in 3 L reactor by *C. shehatae* TISTR5843

3.11.1 Batch fermentation

To produce large quantity of ethanol and compare the predict yield, scaling-up from Erlenmeyer flask (250 ml) to reactor (3 L) with different geometry was studied under controlled agitation condition. The optimal condition of ethanol production achieved from Erlenmeyer flask (section 3.10) was used as a control condition. During fermentation, samples (2 ml) were taken every 12 h to determine the ethanol concentration (Zhu *et al*., 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al*., 2009), furfural (modified from Suwansaard *et al*., 2009) and dry cell weight (DCW).

3.11.2 Fed-batch fermentation

This work was carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium. The initial working volume was 450 ml fresh medium combined with 10% starter culture. Fresh medium of the second and the third cycles were approximately 500 and 1000 ml at 48 and 96 h cultivation time, respectively. During fermentation, samples (2 ml) were taken every 12 h until 144 h to determine the ethanol concentration (Zhu *et al*., 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al*., 2009), furfural (modified from Suwansaard *et al*., 2009) and dry cell weight (DCW).

3.11.3 Semi-continuous fermentation

This work was also carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium. The initial working volume was 1,820 ml fresh medium combined with 10% starter culture (2,000 ml total volume). Fresh

medium of the second and the third cycles were approximately 1000 ml at 48 and 96 h cultivation time, respectively. During fermentation, samples (2 ml) were taken every 12 h until 144 h to determine the ethanol concentration (Zhu *et al*., 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al*., 2009), furfural (modified from Suwansaard *et al*., 2009) and dry cell weight (DCW).

3.12 Bioethanol production by immobilized *C. shehatae* TISTR5843 using palm pressed fiber as a support

PPF was delignified (dPPF) by method described in section 3.2. The sizes of natural supports were < 5.0 mm for small PPF particles (sPPF) and small delignified PPF particles (sDPPF), and 5-20 mm for large PPF particles (lPPF).

Starter culture of immobilized *C. shehatae* TISTR5843 on PPF and DPPF was prepared by adding 100 g/l supports into a liquid yeast cell (0.725 g/l) medium containing 25 g/l glucose, 10 g/l KH_2PO_4, 5 g/l $(NH_4)_2SO_4$, 3 g/l yeast extract and 3 g/l malt extract (initial pH 5) with working volume of 200 ml in the Erlenmeyer flasks. The routine experiments were incubated on an orbital shaker (150 rpm) for 18 h at room temperature (30˚C). The 10% (v/v) of immobilized starter culture was introduced to a fresh medium with 200 ml working volume. Samples were taken to determine dried cell weight (DCW) (Fujii *et al*., 1999), ethanol production by GC-flame ionized detector using a column and the operational condition as described in section 2.3, and residual glucose by HPLC as described in Section 2.1.2.1. The immobilized system was detected by a scanning electron microscope as described in section 2.5.

Repeated batches fermentation (4 cycles) was carried out as described in Kopsahelis et al. 2007. Briefly, the fermented liquids were decanted after the end of each fermentation batch, and then fresh medium was added for the next fermentation batch. Samples of the fermented liquids were collected and analyzed for ethanol, residual sugar and volatile-by products.

CHAPTER 3

RESULTS AND DISCUSSIONS

Many valuable products could be produced from palm press fiber (PPF) using either by chemical or biological methods. PPF was firstly removed lignin to obtain delignified PPF (dPPF). Hemicellulose was then extracted from dPPF and used as a biomaterial for furfural production via one and two stage process; and xylose production by dilute acid hydrolysis. Cellulose is another product after KOH extraction of dPPF. It is used as a source for glucose, which is a substrate for ethanol production. The PPF is not only used directly as a substrate for ethanol production by *Candida shehatae* TISTR5843 but also used as a carrier in cells immobilization.

3.1 PPF composition

PPF consists of cellulose, hemicellulose and lignin which is a complex structure acting as a protective physical barrier (Taniguchi *et al*., 2005) and can not be removed by steam pretreatment (Öhgren *et al*., 2007). In this study, PPF was delignified by alkali (KOH) method and its composition was compared to that of PPF (Table 9). The cellulose, hemicellulose and lignin contents of the PPF in this study (32.06%, 25.83% and 17.28%, respectively) were within the range of the content of cellulose (24-40%), hemicelluloses (14-26%) and lignin (12-27%) of the PPF in other studies (Aziz *et al*., 2002; Kelly-Yong *et al*., 2007; Gutiérrez *et al*., 2009).

3.2 Delignification of PPF

In this study, delignification process by sodium chlorite and acetic acid could remove more than half (57.7%) of total lignin content (from 17.28% to 7.31%) (Table 9). This was lower than those reported by using the same method (60%) (Ahlgren and Goring, 1971) and sodium hydroxide (NaOH) at 120°C (77.2%) (Koba and Ishizaki, 1990). NaOH pretreatment was not used in this study as it required higher reaction temperature and hemicellulose content decreased (~13% of the initial content) (Koba and Ishizaki, 1990). In this study there was no loss of the

hemicellulose during the delignification process. In addition, delignification by using NaOH and microwave-assisted NaOH pretreatment resulted in 81% and 86% lignin removal, respectively, with the high loss of hemicellulose (76-84%) compared to the initial content (Zhu *et al*., 2006). This means that the substrate for value added products was decreased simultaneously. In this study, lignin was removed due to the attack of ClO_2 (generated during delignification process) directly to the aromatic ring of lignin to decompose quickly and completely to form chlorous acid such as fumaric acid, oxalic acid and monochloroacetic acid (Collings *et al*., 1978).

Table 9. Compositions of palm pressed fiber (PPF) and delignified PPF (dPPF).

Parameters	Composition (%)		
	PPF[a]	dPPF[b]	dPPF[c]
Crude fiber	81.49 ± 1.86	76.82	86.31 ± 2.02
Cellulose	32.06 ± 0.64	37.70	42.36 ± 1.07
Hemicellulose	25.83 ± 1.12	34.67	38.96 ± 0.67
Lignin	17.28 ± 0.18	7.31	8.21 ± 0.37
Protein	17.10 ± 0.20	12.04	13.53 ± 0.59
Lipid	12.89 ± 1.28	2.68	3.01 ± 0.10
Moisture	5.04 ± 0.48	5.70	6.41 ± 0.29
Ash	8.30 ± 0.02	7.58	8.52 ± 0.38

[a] based on 1 g PPF
[b] based on 1 g PPF:1 g PPF converted to 0.89 g dPPF
$dPPF^b = dPPF^c \times 0.89$
[c] based on 1 g dPPF

The benefits of lignin removal are the increase of hemicelluloses (from 25.83% to 34.67%) and cellulose (from 32.06 to 37.70%) as well as reduce lignin content (from 17.28% to 7.31%) which is the inhibitor in downstream process for ethanol production (Delgenes *et al*., 1996; Limtong *et al*., 2000; Taniguchi *et al*., 2005; Karimi *et al*., 2006). Nevertheless, this sodium chlorite delignification process caused the reduction of the minor components of PPF such as protein from 17.10 to 12.04% and lipid from 12.89 to 2.68%. Loss of the protein content was similar to that

using acid chlorite delignification of Alfalfa silage (from 17.8% to 13.8%) (Ely *et al*., 1956). Therefore, the native hemicellulosic polysaccharide-protein and polysaccharide-protein-polyphenol complexes might be partially modified by this deligification method (Hedley, 2001).

3.3 Optimization of hemicellulose extraction by alkaline hydrolysis

3.3.1 Experimental design by using Response Surface Methodology (RSM)

Experimental ranges and levels of independent process variables; KOH concentration (X_1; 10-50% w/v), the dPPF to KOH ratio (X_2; 1:20-1:50 w/v) and reaction time (X_3; 20-60 min) as well as dependent process variables (responses); hemicellulose (Y_1) concentration, are given in Table 10. The hemicelluloses in the range of 20.98-42.93% and percentage of extraction in the range of 53.85-110.19% were generated. However, 110.19% extraction was resulted from the mixture of hemicellulose and cellulose. High level of hemicellulose (33.96-42.93%) and percentage of extraction (87.17-110.19%) were obtained by using the moderate (30% w/v) and high (50% w/v) KOH concentrations (33.96-38.88% hemicellulose and 87.17-99.79% extraction in trials 6-15, and 38.72-42.93% hemicellulose and 99.38-110.19% extraction in trials 16-20, respectively). While KOH concentration had a profound effect on the hemicellulose production, the reaction time had much less effect. For example, at 30% KOH and the dPPF to KOH ratio of 1:35 w/v (trials 8 and 9); the hemicellulose yield and percentage of extraction increased only 4.84% (from 35.97% to 37.71%) and 4.83% (from 92.33% to 96.79%), respectively, with 3-folds increase of reaction time (from 20 min to 60 min). To evaluate the results, the data in Table 10 were subjected to regression analysis, using the following quadratic equation (20):

$$Y_1 = 20.22 + 1.24 X_1 - 460.56 X_2 - 0.10 X_3 - 0.015 X_1^2 + 6545.454 X_2^2 + 0.002 X_3^2 + 2.456 X_1 X_2 + 0.001 X_1 X_3 + 0.819 X_2 X_3 \quad \text{..... ..... (20)}$$

where X_1, X_2 and X_3 are the actual values of KOH concentration, the dPPF to KOH ratio and reaction time, respectively (Table 10). The models illustrated the high determination coefficients (R^2=*0.97*) (Table 11) explaining 97% of variability in the

responses of hemicellulose. The high adjusted determination coefficients (adjusted R^2=*0.93*) indicated high significance of the model (O-Thong *et al*., 2008). In addition, the ANOVA quadratic regression demonstrated that the model was significant, as evidenced from a very low probability (*P<0.0001*) while the lack of fit of the model was not significant (*P=0.7578*). Low variation coefficient value (C.V.=5.23%) indicated a high precision and reliability of the experiments (O-Thong *et al*., 2008). The significance of each coefficient was determined by probability values (Table 11). Linear term of X_1 and quadratic term of X_1^2 were significant (*P<0.05*), demonstrated that maximizing for hemicellulose production required an optimum value of KOH concentration. To find the optimum values, estimation of hemicellulose yield over the three independent variables (X_1, X_2 and X_3) in terms of response surfaces were conducted. For hemicelluloses production (Fig. 13A-13C), results indicated that the KOH concentration (Fig. 13A and 13B) had a significant effect while the dPPF to KOH ratio (w/v) (Fig. 13A and 13C) and reaction time (Fig. 13B and 13C) gave no significant difference (*P>0.05*) on hemicellulose production. The maximum hemicellulose yield of 42.93% giving the maximum percentage of extraction of 110.19% were obtained by operating at 50% KOH concentration with the dPPF to KOH ratio of 1:50 (w/v) for 60 min reaction time. It could be implied that some cellulose were also extracted under this condition. The reaction time had no effect on hemicellulose extraction which agreed to the results of the extracted hemicelluloses (6.04-6.51%) from palm cake using 20 min to 8 h reaction time at 80°C (Prasertsan and Oi, 2001).

The advantages of alkali extraction are that there are no any by-products (furan derivatives and acetic acid), low cost, and high yield (Carrillo *et al*., 2005). Mechanism of alkaline extraction is a saponification of intermolecular ester bonds cross-linking hemicellulose and other components, for example, hemicellulose linked by lignin and hemicelluloses linked by itself (Sun and Cheng, 2002). In the process of hemicellulose extraction by using potassium hydroxide (KOH), it can be reacted with acetic acid to form potassium acetate (CH_3COOK). In the step of hemicellulose precipitation by ethanol, CH_3COOK is soluble in ethanol and the pellet of hemicelluloses can be separated easily.

Table 10. Central composite experimental design matrix defining potassium hydroxide (KOH) concentration (%, w/v) (X_1), the PPF to KOH ratio (w/v) (X_2), and reaction time (min) (X_3) and results on hemicelllulose concentration.

Trials	Parameters			Response (Y_1)	% extraction
	X_1	X_2	X_3	Hemicellulose (%)	
1	10	1:50	20	24.74 ± 0.36	63.850
2	10	1:20	20	21.72 ± 3.28	55.75
3	10	1:50	60	24.48 ± 1.90	62.83
4	10	1:20	60	24.08 ± 1.21	61.81
5	10	1:35	40	20.98 ± 0.40	53.85
6	30	1:50	40	36.66 ± 1.37	94.10
7	30	1:20	40	38.88 ± 2.91	99.79
8	30	1:35	20	35.97 ± 0.67	92.33
9	30	1:35	60	37.71 ± 0.47	96.79
10	30	1:35	40	37.51 ± 0.61	96.28
11	30	1:35	40	35.97 ± 0.46	92.32
12	30	1:35	40	37.04 ± 3.04	95.07
13	30	1:35	40	36.88 ± 4.03	94.66
14	30	1:35	40	36.68 ± 0.81	94.15
15	30	1:35	40	33.96 ± 1.49	87.17
16	50	1:50	20	38.72 ± 0.71	99.38
17	50	1:20	20	40.94 ± 1.78	105.08
18	50	1:50	60	42.93 ± 1.78	110.19
19	50	1:20	60	41.22 ± 1.22	105.80
20	50	1:35	40	39.12 ± 2.24	100.41

Table 11. Model coefficient and analysis of variance estimated by ANOVA for hemicellulose production from dPPF.

Parameter	Hemicellulose production	
	Coefficient estimate	Probability (*P*)
Intercept	36.86	-
X_1	8.69	<0.0001*
X_2	-0.27	0.6478
X_3	0.83	0.1754
X_1X_2	0.49	0.4596
X_1X_3	0.30	0.6500
X_2X_3	-0.16	0.8028
X_1^2	-6.07	0.0002*
X_2^2	0.65	0.5613
X_3^2	0.72	0.5210
Model	-	< 0.0001
Lack of fit	-	0.7578
R^2	0.97	-
Adjusted R^2	0.93	-
C.V.	5.23	-

*Significant level at 95%

C.V. = Coefficient of variation

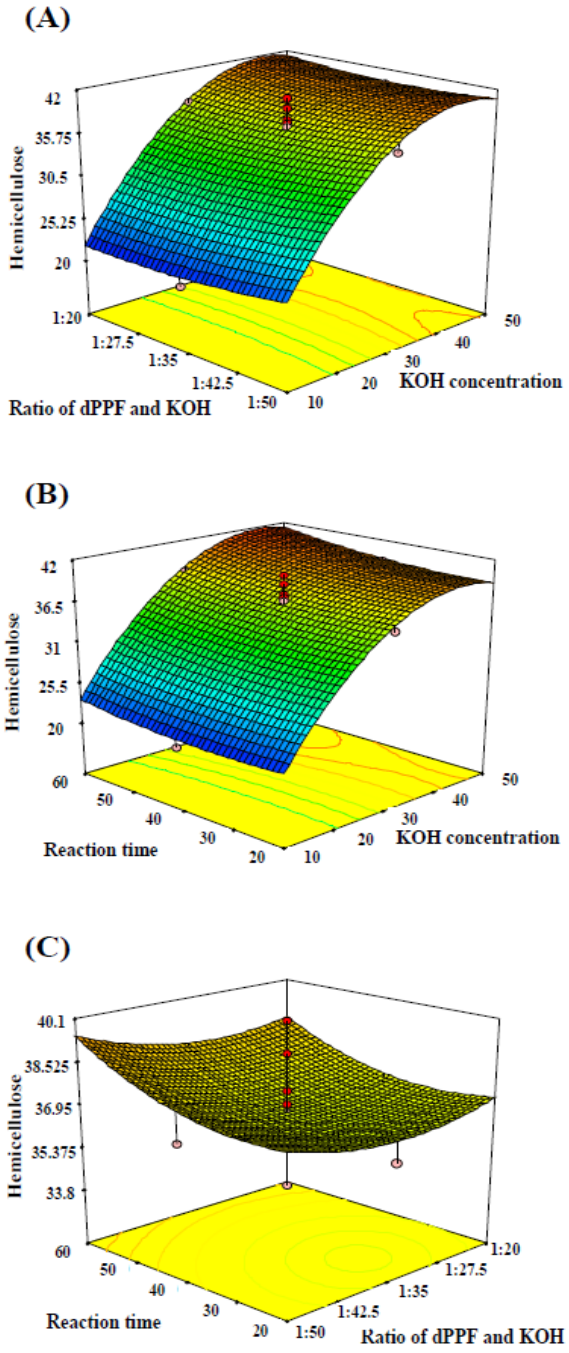


Figure 13. Three-dimensional graphs of the quadratic model for hemicellulose yield (%) (A-C) within the central composite design (CCD). Experiments A fixed reaction time at centre point of 40 minutes; Experiments B fixed the PPF: KOH ratio at centre point of 1:35 (w/v); and Experiments C fixed KOH concentration at centre point of 30 % (w/v).

3.3.2 Confirmation experiments and adequacy of the model of hemicellulose production

The optimal condition, close to the original content, calculated by RSM contained 28.88% (w/v) KOH concentration, the dPPF to KOH ratio of 1:20 (w/v) and reaction time of 20 min. To confirm the validity of the statistical experimental strategies of hemicellulose extraction, three replicates of batch experiments were performed under the optimal condition compared to the control and the central parameters (Table 12). Results from confirmation experiments indicated that the experimental values of hemicellulose yield (38.67±1.21%) was close to its predicted values (36.78%) with 99.25% extraction and low deviation of 5.14%. There was no significant difference of both hemicellulose yield between the experimental values and the predicted value *(P<0.05)*. After optimization, hemicellulose extracted from dPPF increased 1.60 fold, compared with the control condition. Furthermore, the efficiency of hemicellulose production was 99.25% (Table 12). The results suggested that the model could be used as a tool for hemicellulose production.

Table 12. The confirmation experiments for hemicellulose contents after extraction at the optimal condition.

Trials	Conditions	X_1	X_2	X_3	Hemicellulose (%)		% extraction
					Predicted	Measured	
-	Optimal[a]	28.88	1 : 20	20	36.78[b]	38.67 ± 1.21[b] (5.14%[c])	99.25
10	Central	30	1 : 35	40	39.56[d]	37.51 ± 0.61	96.28
-	Selected	24	1 : 50	30	35.94[d]	24.11 ± 3.13	61.88

X_1: KOH concentration (%, w/v), X_2: the PPF to KOH ratio (w/v) and X_3: reaction time (min).

[a]: based on hemicellulose extraction, [b]: not significant at level *P<0.05*

[c] Deviation (%) = [(Measured value – predicted value) x 100]/predicted value.

[d] calculated by Eq. 20

3.4 Composition of the extracted hemicellulose

The hemicellulose was digested to monomeric sugars by 5% sulfuric acid at 120°C for 30 min and analyzed by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

3.4.1 Characterizations of sugars in hemicellulose hydrolysate using TLC (qualitative method)

The types of monomeric sugar in PPF could be separated based on their polarities by thin layer chromatography (TLC, normal phase silica gel 60 F254 (Merck)). Mobile phase was isopropyl alcohol, ethyl acetate and water in the ratio of 3:3:1, and N-(1-napthyl)-ethylenediamine as sprayed dye. The results of experiments are shown in Fig. 14.

The retention factor (R_f) of unknown samples (No. 8-11) were calculated and compared to the various standard sugars (Fig. 14). The R_f values of standard sugars consisting of arabinose, rhamnose, xylose, fructose, galactose, glucose and mannose were 0.53, 0.71, 0.64, 0.52, 0.46, 0.53 and 0.57, respectively, meanwhile the unknown samples were observed to consist of two bands with the R_f values of 0.54 and 0.65. It was illustrated that the first band of hemicellulose hydrolysate might be arabinose, fructose and glucose because of their R_f value compared to standard sugar. The second band (0.65-0.66) was xylose (0.64). The carbohydrates in the fiber was consisted of 56.4% glucose, 36.0% xylose, 5.9% arabinose, and 1.7% mannose (Koba and Ishizaki, 1990). Since the result of the first lane was unclear, thus HPLC was used to identify these components.

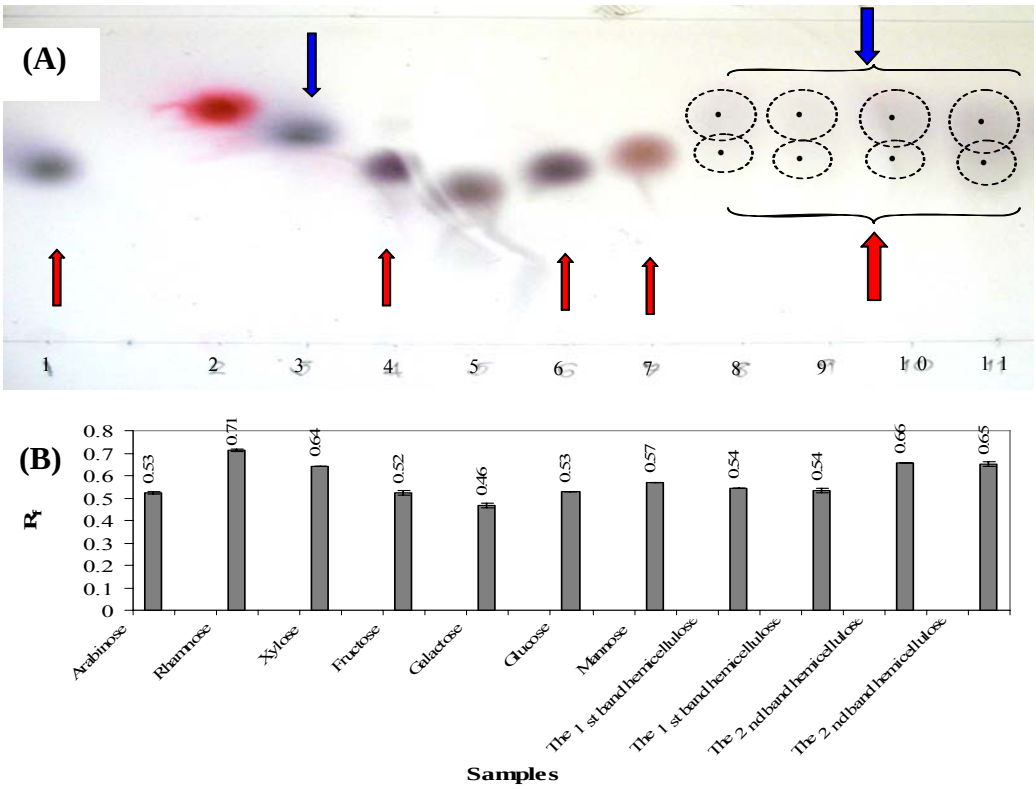


Figure 14. (A); TLC chromatogram of digested PPF by 2 N TFA at 120°C for 90 min; 1: standard arabinose, 2: standard rhamnose, 3: standard xylose, 4: standard fructose, 5: standard galactose, 6: standard glucose, 7: standard mannose, 8 and 9: PPF, 10 and 11: extracted hemicellulose from PPF. (B); the retention factor (R_f) values of various standard sugars and hydrolysate samples of PPF.

3.4.2 Characterizations of sugars in hemicellulose hydrolysate using HPLC (quantitative method)

The composition of hemicellulose was determined by HPLC. Hemicellulose was digested to monomeric sugars by sulfuric acid and xylose (80%) was found to be the main sugar in the extracted hemicelluloses of PPF and glucose (15%) was the second compound without of any other sugars. In general, the inhibitory compounds generated during hydrolysis were acetic acid and furfural whereby acetic acid was from the hydrolysis of the acetyl groups bound to the hemicellulosic monomers (Herrera *et al*., 2003; Rahman *et al*., 2006), while furfural,

is a derivative product from xylose hydrolysis (Herrera *et al*., 2003; Rahman *et al*., 2006). While 3.16% of acetic acid was detected, there was no detection of furfural. Therefore, the hydrolytic condition in this study may be suitable for xylose production as it gave low concentration of both acetic acid and furfural.

3.5 Furfural production from the extracted hemicellulose

3.5.1 Furfural production using one-stage process

The operational conditions, assayed and experimental results are illustrated in Table 13. Furfural was produced in the range of 0.06-0.86 g/l. The maximum furfural production (0.86 g/l) was achieved using high temperature (150°C), low LSR (8 ml/g), low sulfuric acid concentration (5% v/v) for 90 min reaction time. In this study, it was found that the requirement for giving high furfural production (0.80-0.86 g/l) was achieved under high reaction temperature in all experiments together with more than 60 min reaction time. It is similar to the suggestion of Parajó and Santos (1995) that the requirement of furfural production could be under high temperature (>110°C), high acid concentration (> 0.2%) and low L/S ratio (< 10/1 g/g). On the other hand, the acid hydrolysis had the disadvantages that pentosan removal was lower and reaction period was longer (90 min) (Punsuvon *et al*., 2008). Comparison of furfural yields generated from different lignocellulosic materials is given in Table 14. The yield expressed as grams furfural/g initial dry substrate. Moderate temperature (100°C-134°C) produced furfural in the range of 3.34-13.36 wt% (Abad *et al*., 1997; Mansilla *et al*., 1998; Vázquez *et al*., 2007). When compared the furfural yield of this study (3.44 wt%) to other one stage processes (3.34 wt%), the amount of furfural yields was the same as that obtained at 5% H_2SO_4, 150°C for 90 min (Mansilla *et al*., 1998). Using the moderate temperature combined with pretreated substrate gave the advantages of lower equipment, simple and easy. However, using moderate temperature gave lower furfural yield than the high temperature condition (Table 14). The highest furfural yield of 70% can be obtained at high temperature (240°C) with short time condition (Montané *et al*., 2002), but many disadvantages on cost of equipment and high energy usage. To achieve higher furfural yields with lower cost, two-stage process (hydrolysis followed by dehydration process) (Dias *et al*., 2005) will be employed for further investigation.

Table 13. Operational conditions and experimental data on the furfural production from the hemicellulose extracted from dPPF.

Exper.	T (°C)	LSR (ml/g)	H_2SO_4 (% v/v)	Time (min)	XC (g/l)	FC (g/l)
1	120	10	5	0	0	0
				30	0.59	0.06
				60	1.00	0.11
				90	2.40	0.22
				120	5.92	0.31
2	120	10	10	0	0	0
				30	3.44	0.13
				60	6.18	0.45
				90	6.09	0.58
				120	6.17	0.11
3	120	8	5	0	0	0
				30	0.75	0.06
				60	1.46	0.17
				90	2.53	0.29
				120	6.34	0.41
4	120	8	10	0	0	0
				30	5.26	0.16
				60	5.81	0.52
				90	8.56	0.68
				120	8.53	0.71
5	150	10	5	0	0	0
				30	2.17	0.06
				60	3.30	0.61
				90	4.56	0.81
				120	4.20	0.85
6	150	10	10	0	0	0
				30	4.22	0.34
				60	3.70	0.84
				90	2.48	0.80
				120	0.96	0.55
7	150	8	5	0	0	0
				30	1.71	0.15
				60	3.70	0.78
				90	4.60	0.86
				120	6.01	0.82
8	150	8	10	0	0	0
				30	4.92	0.61
				60	7.35	0.81
				90	3.31	0.61
				120	2.15	0.42

LSR = liquid/solid ratio (ml/g), XC = xylose content in hemicellulose (g/l),
FC = furfural concentration (g/l)

Table 14. Comparison of furfural yield produced from various lignocellulosic materials using one-stage process.

Type of raw material	Condition	Furfural yield (%)[a]	References
Sorghum straw	6% phosphoric acid at 134°C for 300 min	13.36%	Vázquez *et al*., 2007
Eucalyptus globulus wood	0.4 g conc. HCl/100 g at 130°C for 45 min	4.48 g/l (3.8%)	Abad *et al*., 1997
Rice hull	10.5% H_2SO_4	5.55%	Gladkova,
Rice hull	One stage: 20% (w/w) H_2SO_4 at 125°C, 1.5 atm for 30 min	3.34%	Mansilla *et al*., 1998
Corn cubs	ND	10%	Jaeggle,
Bagasse	ND	8-9%	1975 cited
Cotton husks	ND	8-9%	by Mansilla
Hard wood	ND	6-8%	*et al*., 1998
Beech bark	ND	5-6%	
Rice husk	ND	6%	
Sunflower hull	ND	8-9%	
Hemicellulose of dPPF	One stage: 5% H_2SO_4 at 150°C for 90 min, liquid/solid ratio of 8 ml/g	3.44%[b] (0.86 g/l)	This study

ND = no detail

* % (grams furfural/g initial dry substrate)

** Transformation unit of g/l to %, calculated by = $\left(\frac{0.86 \times A}{1000}\right)\left(\frac{100}{0.2}\right)$,

where A = adjusted volume after hydrolysis (10 ml)

0.2 = initial weight of extracted hemicellulose (g)

3.5.2 Furfural production using two-stage process

3.5.2.1 Optimization of hydrolysis process by RSM

Response surface methodology is an efficient tool to establish the relationship of the interesting variables (at least two variables) with the obtained responses. The data analysis was developed by fitting the experimental data in a smooth curve, which is plotted by calculation of specific predicted response (Khanna and Srivastava, 2005). Therefore, response surface analysis establishes a relationship between variables and responses more professionally than the traditional design (Launen *et al*., 1999). The effective variables in the hydrolysis stage were optimized and the pentose sugars in the extracted hemicellulose of PPF would be used as substrate for furfural production. Results of the thirty experiments (Table 15)

indicated that xylose was generated in the range of 0.43-12.58 g/l. High xylose concentrations (10.21-12.58 g/l) were achieved at the reaction temperature of 100-125 °C, the acid concentration of 5.5-10 % v/v, L/S ratio of 8-10 ml/g and reaction time of 30-75 min (trial 5, 7, 10, 11, 13, 15 and 16-21). Higher values of reaction temperature (150 °C) and reaction time (120 min) tremendously reduced the xylose yield to 0.43-1.87 g/l (average 0.99 g/l) (trials 23 and 28-30), which was nearly 92 % lower yield compared to the high xylose concentration (average 12.36 g/l). This was due to the degradation of xylose to furfural (Rahman *et al*., 2006; Rahman *et al*., 2007; Punsuvon *et al*., 2008). The maximum xylose production (12.58 g/l) was achieved under 125 °C, 5.5 % sulfuric acid, L/S ratio of 9 ml/g for 30 min (trial 16). In addition, small amount of by-products were also formed; 0.16 to 1.18 g/l furfural, 1.21-8.22 g/l acetate and 0.02-5.88 g/l glucose. To evaluate the influence of these variables on xylose yield (g/l), the design matrix of experimental conditions with the corresponding xylose yield values (Table 15) were subjected to regression analysis, generating the following quadratic equation (21):

$$\begin{aligned} Xylose\,(g/l) = {} & -130.88 + 2.27X_4 + 2.26X_5 + 1.03X_6 - 0.09X_7 - 0.01X_4X_5 \\ & - 0.01X_4X_6 - 0.0005X_4X_7 + 0.003X_5X_6 - 0.004X_5X_7 + 0.016X_6X_7 \\ & - 0.009X_4^2 - 0.078X_5^2 - 0.038X_6^2 + 0.00008X_7^2 \quad \text{.....(21)} \end{aligned}$$

where X_4, X_5, X_6 and X_7 are the actual values of reaction temperature, sulfuric acid concentration, L/S ratio and reaction time, respectively. The model presented a high value of regression coefficient (R^2 = 0.90) explaining 90% of variability in the response. The value of the adjusted determination coefficient (adjusted R^2 = 0.81) is quite high, indicating a high significance of the model (Tanyildizi *et al*., 2005; O-Thong *et al*., 2008). The ANOVA quadratic regression model demonstrated that the model was highly significant, as evidenced from the Fisher's F-test with a very low probability ($P<0.0001$). Moreover, a lower of variation coefficient value (CV=15.15%) indicated a high precision and reliability of the experiments (O-Thong *et al*., 2008). The optimum conditions for maximizing xylose production yield, calculated by setting the partial derivatives of Eq. 21 to zero with respect to the corresponding variables, were a reaction temperature of 120°C, a sulfuric acid

concentration of 5.7% (v/v), an L/S ratio of 8.5 ml/g, and a reaction time of 31 min. The maximum response value for xylose production yield was estimated as 13.01 g/l. A dimensional and contour plot was based on Eq. 20 with varying the four variables within the experimental range (Fig. 15). The main goal of response surface analysis is to unravel the optimum combination of variables in order to maximize the response.

The response surface of xylose production indicated that xylose yield increased with increasing reaction temperature (up to 120 °C) (Fig. 15(a) and 15(b)) and sulfuric acid concentration (up to 5.5 %, v/v) (Fig. 15(a), 15(d) and 15(e)). This is in agreement with the suggestion of other researchers that acid concentration is an important parameter for release of sugars (Rahman *et al*., 2006; Rahman *et al*., 2007). The maximum xylose yield was achieved after 30 min of reaction (Fig. 15(c), 15(e) and 15(f)) and decreased with prolonged degradation to furfural (Rahman *et al*., 2006; Rahman *et al*., 2007) especially at longer reaction time (Table 15 trials 16, 15 and 14 for 30, 75 and 120 min, respectively). The hydrolysis process for release of sugars should therefore employ higher acid concentration with lower reaction time to minimize the formation of furfural in the resulting hydrolysate (Rahman *et al*., 2006).

However, reaction temperature (X_4) and reaction time (X_7) had an individual significant influence on xylose production. The significance of each coefficient was calculated by probability values which are listed in Table 16. The variables with a significant effect on xylose production were the reaction temperature (X_4) and time elapsed (X_7) ($P<0.05$). It also showed that linear terms of X_4 and X_7, interaction term of X_4X_5, and quadratic term of X_4^2 are significant ($P<0.05$), demonstrating that the xylose production required a suitable reaction temperature, reaction time and sulfuric acid concentration for the highest xylose production.

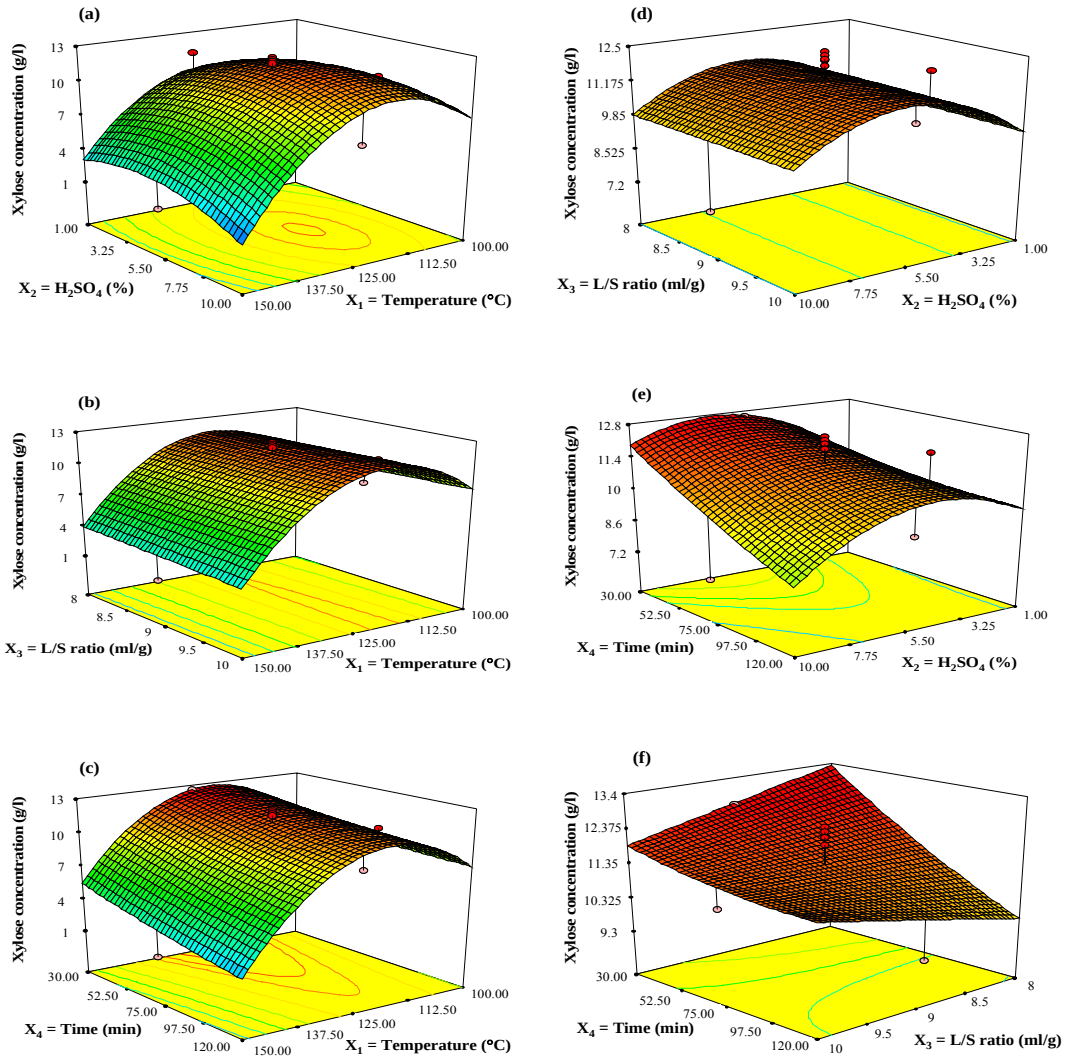


Figure 15. Three-dimensional graphs of the quadratic model for xylose yield (g/l) (a-f) within the central composite design (CCD): (a) fixed L/S ratio and reaction time at centre point of 9 ml/g and 75 min; (b) fixed H_2SO_4 and reaction time at centre point of 5.5% and 75 min; (c) fixed H_2SO_4 and L/S ratio at centre point of 5.5 % and 9 ml/g; (d) fixed reaction temperature and time at centre point of 120°C and 75 min; (e) fixed reaction temperature and L/S ratio at centre point of 120°C and 9 ml/g; (f) fixed reaction temperature and H_2SO_4 at centre point of 120°C and 5.5%.

Table 15. Central composite experimental design matrix defining reaction temperature (°C) (X_4), sulfuric acid concentration (% v/v) (X_5), L/S ratio (ml/g) (X_6), and reaction time (min) (X_7) and results on productions of xylose (Y_2), glucose (Y_3), furfural (Y_4) and acetate (Y_5) from the hemicellulose extracted from dPPF.

Trial	Variables				Responses			
	X_4	X_5	X_6	X_7	Xylose (g/l)	Glucose (g/l)	Furfural (g/l)	Acetate (g/l)
1	100	1	8	30	4.11	0.05	0.42	1.32
2	100	10	10	120	6.21	3.76	0.98	5.48
3	100	1	10	30	3.67	0.02	0.38	1.21
4	100	1	10	120	8.34	0.40	0.44	1.75
5	100	10	10	30	10.21	1.89	0.96	5.02
6	100	1	8	120	5.89	0.67	0.52	1.88
7	100	10	8	30	10.97	2.08	1.07	5.32
8	100	5.5	9	75	9.48	1.67	0.82	4.89
9	100	10	8	120	5.12	3.89	1.05	5.72
10	125	1	9	75	11.18	0.68	0.48	2.32
11	125	5.5	9	75	12.49	3.27	0.92	7.21
12	125	10	9	75	7.21	3.92	0.99	7.97
13	125	5.5	9	75	12.19	3.34	0.87	7.42
14	125	5.5	9	120	9.31	3.67	1.11	7.52
15	125	5.5	9	75	10.87	3.29	0.94	7.61
16	125	5.5	9	30	12.58	2.12	0.88	7.25
17	125	5.5	9	75	12.17	3.44	0.96	7.47
18	125	5.5	10	75	10.75	3.21	0.82	7.32
19	125	5.5	9	75	12.35	3.50	0.90	7.56
20	125	5.5	9	75	11.97	3.35	0.99	7.39
21	125	5.5	8	75	10.75	3.62	1.02	7.69
22	150	5.5	9	75	1.27	4.17	1.18	8.07
23	150	1	8	120	0.58	1.34	0.58	6.12
24	150	10	10	30	2.46	3.68	0.82	7.96
25	150	10	8	30	4.47	3.81	0.97	8.12
26	150	1	8	30	6.98	0.56	0.50	2.22
27	150	1	10	30	3.47	0.41	0.40	1.95
28	150	1	10	120	1.87	1.29	0.56	2.92
29	150	10	8	120	0.43	5.88	0.23	8.22
30	150	10	10	120	1.09	5.52	0.16	8.01

Table 16. Model coefficient and analysis of variance estimated by ANOVA for xylose production.

Parameter	Xylose production	
	Coefficient estimate	Probability (*P*)
Intercept	11.48	-
X_4	-2.35	<0.0001*
X_5	0.06	0.8923
X_6	-0.01	0.9770
X_7	-1.06	0.0279*
X_4X_5	-1.00	0.0475*
X_4X_6	-0.31	0.5166
X_4X_7	-0.56	0.2416
X_5X_6	0.01	0.9798
X_5X_7	-0.79	0.1060
X_6X_7	0.70	0.1501
X_4^2	-5.41	0.0003*
X_5^2	-1.59	0.1854
X_6^2	-0.04	0.9739
X_7^2	0.16	0.8932
Model	-	< 0.0001
R^2	0.90	-
Adjusted R^2	0.81	-
C.V.	15.15	-

*Significant level at 95%

R^2 = Regression coefficient

C.V. = Coefficient of variation

For better understanding of xylose production, three main by-products (furfural, acetate and glucose) from acid hydrolysis of xylose (Rahman *et al*., 2006; Herrera *et al*., 2003; Garrote *et al*., 2001) were illustrated in the response surface plots (Fig. 16). Furfural concentration increased with increasing sulfuric acid concentration from 0 to 5.5 %, (v/v) (Fig. 16(a), 16(d) and 16(g)) and decreasing L/S ratio from 10 to 8 ml/g (Fig. 16(d)). In addition, reaction temperature and reaction time had profound effect on furfural formation (Fig. 16(a) and 16(g), respectively), which increased with either increasing reaction temperature and shorter reaction time, or decreasing reaction temperature with longer reaction time. Acetate is generated from degradation of acetyl groups of hemicellulose (Rahman *et al*., 2006; Herrera *et al*., 2003; Garrote *et al*., 2001). Acetate concentration increased with increasing sulfuric acid concentration (in the range of 0-10 %, v/v) (Fig. 16(b), 16(e) and 16(h)) and increasing reaction temperature (100-137 °C) (Fig. 16(b)). Meanwhile, both L/S ratio (Fig. 16(e)) and reaction time (Fig. 16(h)) had no influence on acetate production. This demonstrated that 30 min reaction time was optimum for production of xylose from hemicellulose as no acetate was detected at prolonged reaction time. Glucose was also generated from hemicellulose hydrolysis and its concentration increased with the increase of sulfuric acid concentration (0-10 %, v/v) (Fig. 16(c), 16(f) and 16(i)), reaction temperature (100-150 °C) (Fig. 16(c)), and reaction time (30-120 min) (Fig. 16(i)). Glucose could not been produced so much in the diluted acid hydrolysis because of a little glucose content in hemicellulose (Rahman *et al*., 2006).

3.5.2.2 Confirmation experiments and adequacy of the model of hydrolysis process

Three replicates of batch experiments were performed under the optimal condition calculated by RSM (Table 17). Results from confirmation experiments indicated that the experimental value of xylose yield (12.32 ± 2.42 g/l) was no significant difference *($P<0.05$)* from its predicted value (13.01 g/l). After optimization, xylose production from dPPF hemicellulose increased 5.4 and 1.4 folds, compared with the control and the central conditions, respectively.

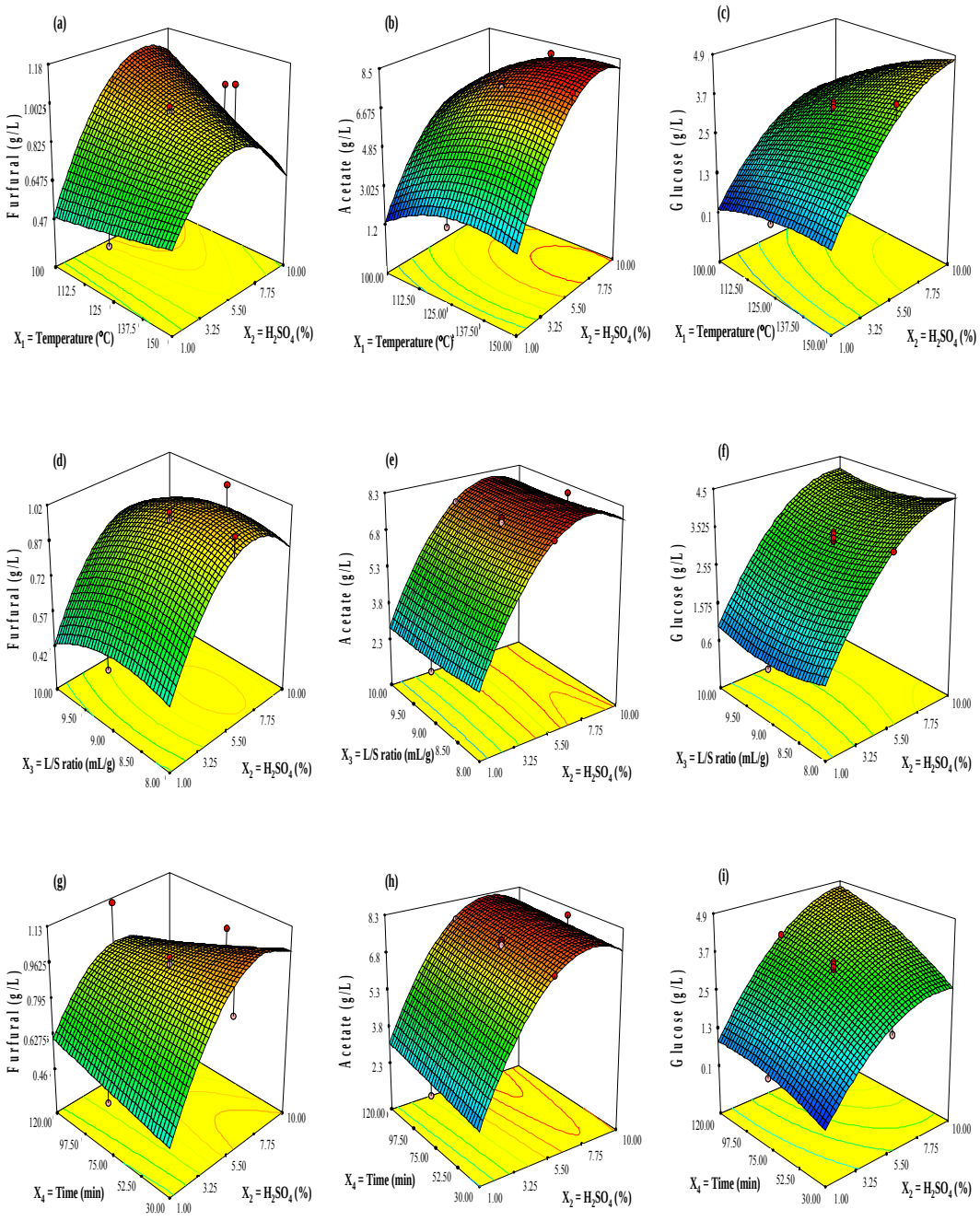


Figure 16. Three-dimensional graphs showing the effect of reaction temperature, H_2SO_4 concentration, L/S ratio and reaction time on furfural (a, d, g), acetate (b, e, h) and glucose (c, f, i) productions.

Table 17. The confirmation experiments for xylose contents after hydrolysis using the optimal condition.

Trials	Conditions	X_4	X_5	X_6	X_7	Xylose (g/l)	
						Predicted	Measured
-	Optimal[a]	120	5.70	8.5	31	13.01[b]	12.32 ± 2.42 [b] (5.30[c]%)
11, 13, 15, 17, 19, and 20	Central	125	5.50	9	75	8.70[d]	12.01 ± 0.58
-	Selected	100	1	10	15	4.21[d]	3.21 ± 1.23

X_4: reaction temperature (°C), X_5: H_2SO_4 concentration (%, v/v), X_6: L/S ratio (ml/g) and X_7: reaction time (min).

[a]: based on xylose production

[b]: not significant at level $P<0.05$

[c] Deviation (%) = [(Measured value – predicted value) x 100]/predicted value.

[d] calculated by Eq. 21

3.5.2.3 Optimization of dehydration process by RSM

Many plant materials contain the polysaccharide hemicellulose, a polymer of sugars containing five carbon atoms each. When heated with sulfuric acid, hemicellulose undergoes hydrolysis to yield these sugars, principally xylose. Under the same conditions of heat and acid, xylose and other five carbon sugars undergo dehydration, losing three water molecules to become furfural (Dias *et al*., 2005). In this study, the optimum hydrolysis process was used to give the substrate for dehydration process. Two variables (reaction temperature and time) were optimized as it was suggested that reaction time needs to be reduced and temperature should be increased to enhance furfural production (Carrasco *et al*., 1991; Vedernikov *et al*., 1993). The results of all thirteen experiments were summarized (Table 18) with the furfural production in the range of 0-8.52 g/l. The maximum furfural production (8.52 g/l) was achieved under reaction temperature of 140°C for 90 min reaction time.

Table 18. Central composite experimental design matrix of dehydration process defining reaction temperature (°C) (X_8) and reaction time (min) (X_9) and results on production of furfural in one stage process.

Trials	Variables		Response
	X_8	X_9	Furfural (g/l)
1	120	150	4.92
2	120	30	2.34
3	120	90	6.42
4	140	90	8.30
5	140	150	3.83
6	140	90	8.20
7	140	30	5.24
8	140	90	8.43
9	140	90	8.40
10	140	90	8.52
11	160	30	3.00
12	160	150	0
13	160	90	1.42

Results indicated that high furfural production (4.92-8.52 g/l) could be achieved and higher temperature with shorter time (140°C/90 min) gave higher furfural yield than lower temperature with longer time (120°C/150 min). To evaluate the influence of both variables on furfural yield, the design matrix of experimental conditions with the corresponding furfural yield values (Table 18) were subjected to regression analysis, generating the equation (22):

$$Furfural\,(g/l) = -160.68 + 2.30X_8 + 0.29X_9 - 0.001X_8X_9 - 0.008X_8^2 - 0.001X_9^2 \quad \text{.....(22)}$$

where X_8 and X_9 are the actual values of reaction temperature and reaction time, respectively (Table 18). The model presented a high value of regression coefficient

(R^2 = 0.93). The value of the adjusted determination coefficient (adjusted R^2 = 0.88) is quite high, indicating a high significance of the model (Tanyildizi *et al*., 2005; O-Thong *et al*., 2008). The ANOVA quadratic regression model demonstrated that the model was highly significant, as evidenced from the Fisher's *F-test* with a very low probability (*P=0.0006*). Moreover, a smaller coefficient of variation (C.V. = 9.53 %) indicated a high precision and reliability of the experiments (O-Thong *et al*., 2008). The optimum conditions for maximizing furfural production were the reaction temperature of 135.2 °C and a reaction time of 90.3 min. The maximum response value for furfural production yield was estimated as 8.21 g/l.

A dimensional and contour plot based on Eq. 22 with varying the two variables within the experimental range was illustrated in Fig. 17. The response surface of furfural production indicated that furfural yield increased with increasing reaction temperature in the range of 120-135 °C. The optimum reaction time was 90 min. However, reaction temperature (X_8) and reaction time (X_9) had an individual significant influence on furfural production. The significance of each coefficient was calculated by probability values which are listed in Table 19. It is clear that the variable with a significant effect on furfural production was the term of reaction temperature (X_8) ($P<0.05$). Linear term of X_8, interaction term of X_8X_9, and quadratic terms of X_8^2 and X_9^2 are significant ($P<0.05$), demonstrating that the furfural production required a suitable reaction temperature and reaction time for the highest furfural production.

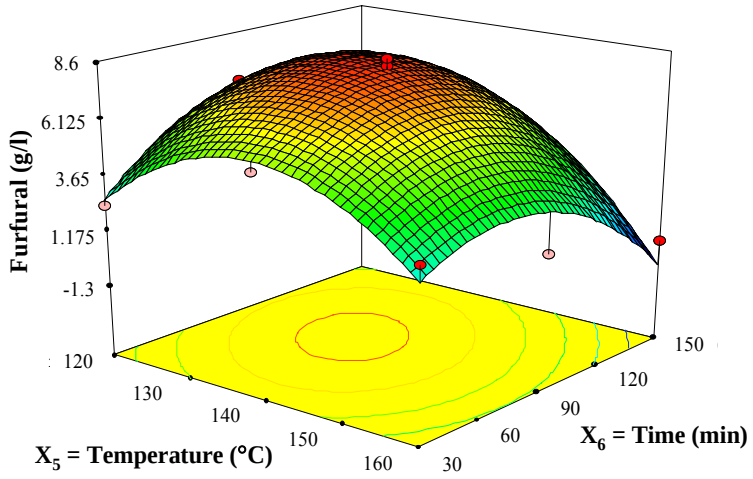


Figure 17. Graph of the quadratic model for furfural production (g/l) within the central composite design (CCD) of reaction temperature and reaction time.

Table 19. Model coefficient of furfural production estimated by ANOVA.

Parameter	Furfural production	
	Coefficient estimate	Probability (*P*)
Intercept	8.03	-
X_8	-1.54	0.0082*
X_9	-0.31	0.4946
X_8X_9	-1.39	0.0311*
X_8^2	-3.25	0.0012*
X_9^2	-2.64	0.0039*
Model	-	0.0006
R^2	0.93	-
Adjusted R^2	0.88	-
C.V.	9.53	-

*Significant level at 95%

R^2 = Regression coefficient

C.V. = Coefficient of variation

3.5.2.4 Confirmation experiments and adequacy of the model of furfural production

Results of confirmation experiments (Table 20) and commercial pure xylose experiments (Fig. 18) were done in three replicates under the optimal conditions calculated by RSM. It was indicated that the experimental values of furfural yield (8.67 g/l) was very similar to its predicted value (8.21 g/l) with no significantly difference *(P<0.05)*. However, furfural yield from xylose in the hydrolysate (8.67 ± 0.62 g/l) was 41 % higher than that from commercial pure xylose (5.11±0.29 g/l) under the similar conditions. As shown in Fig. 18, commercial pure xylose was easily converted to furfural (8.75 ± 0.17 g/l) within 30 min and slightly decreased thereafter because of its degradation to form formic acid and levulinic acid (Jing and Lü, 2007). After optimization, furfural production increased 5.4 and 1.4 folds, compared to the control and the central conditions, respectively. Comparison of furfural yields generated from various lignocellulosic materials is given in Table 21. Results indicated that using the moderate temperature (100°C-135°C) produced furfural in the range of 10.3-17.3 % (Punsuvan *et al*., 2008; Mansilla *et al*., 1998). The furfural yield from two-stage process was 5.04 folds higher than that from one-stage process (from 3.44 wt% (0.86 g/l) to 17.34 wt% (8.67 g/l)). After optimizations of hydrolysis and dehydration processes, the furfural yield of 17.34 % was obtained at reaction temperature of <200 °C which is economic prospects of operation and equipment costs reduction. Besides the optimization process, a high furfural yield could be obtained from substrate pretreatment (delignification) and optimization processes (hydrolysis and dehydration steps).

Table 20. The confirmation experiments for furfural yield after hydrolysis and dehydration processes using the optimal condition predicted by RSM.

Trials	Conditions	X_8	X_9	Furfural (g/l)		
				Predicted	Measured	Deviation[c] (%)
-	Optimal[a]	135.24	90.34	8.21[b]	8.67±0.62 [b]	5.60
4, 6, 8, 9, and 10	Medium	140	90	9.92[d]	8.37 ± 0.12	15.62
-	Selected 1	120	30	2.32[d]	1.81 ± 0.23	21.98

X_8: reaction temperature (°C) and X_9: reaction time (min).
[a]: based on furfural production
[b]: not significant at level $P<0.05$
[c] [(Measured value – predicted value) x 100]/predicted value.
[d] calculated by Eq. 22

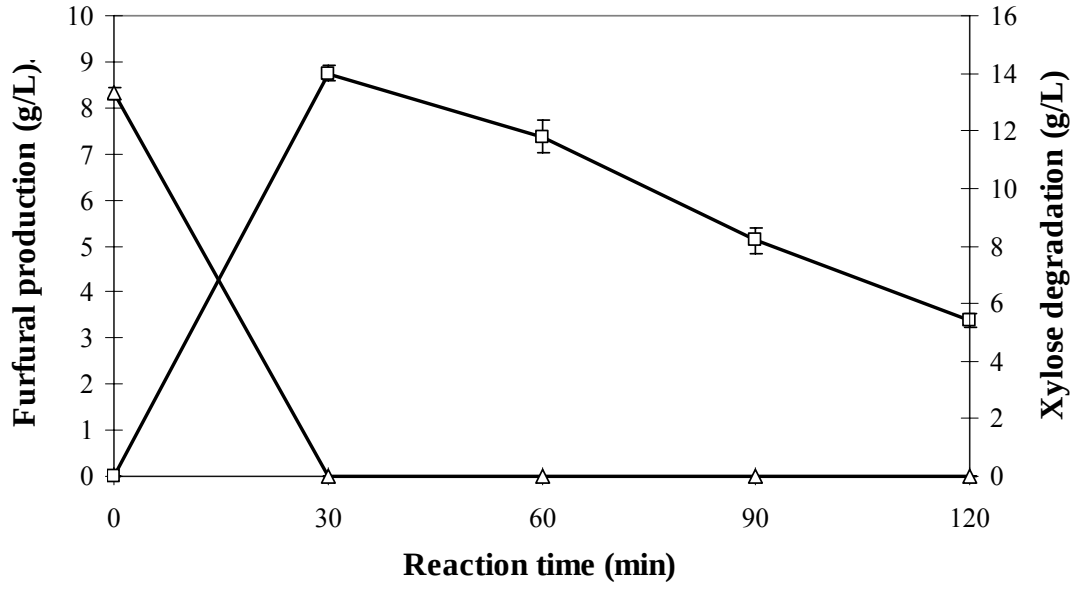


Figure 18. Time course of furfural production (□) and pure xylose degradation (Δ) at 135.2 °C for 90.3 min.

Table 21. Comparison of furfural yield produced from various lignocellulosic materials using two-stage process.

Type of raw material	Conditions	Furfural yield (%)[a]	References
Rice hull	Two stage: (i) Hydrolysis: 3 % H_2SO_4 for refluxing 30 min, (ii) Dehydration: 15 % H_2SO_4 and taking 250 mL of steam distillation	~10.5 %	Mansilla *et al.* (1998)
Hemicellulose (xylose) of Bagasse	Two stage: (i) Hydrolysis: steam explosion and concentrated 6 folds, (ii) Dehydration: 3 % H_2SO_4 at 121 °C for 1 h	~10.3 %	Punsuvon *et al.*(2008)
Hemicellulose of dPPF	Two stage: (i) Hydrolysis: 5.7 % H_2SO_4, L/S ratio 9 mL/g, at 120 °C for 31 min, (ii) Dehydration: 5.7 % H_2SO_4 at 135 °C for 90 min	17.34 %[b] (8.67 g/L)	This study
	- One stage: 5% H_2SO_4, L/S ratio of 8 mL/g, at 150 °C for 90 min	3.44 % (0.86 g/L)	This study

[a] % (grams furfural/g initial dry substrate)

[b] Transformation unit of g/l to %, calculated by = $\left(\frac{8.67 \times A}{1000}\right)\left(\frac{100}{0.5}\right)$

where A = adjusted volume after hydrolysis (10 ml)

0.5 = initial weight of extracted hemicellulose (g) for two-stage process of furfural production

3.6 Cellulosic hydrolysate production

3.6.1 Cellulosic hydrolysate production by enzymatic hydrolysis

3.6.1.1 Effect of pH on cellulase activity

Cellulose of PPF was used as a substrate for glucose production by enzymatic hydrolysis. To determine the effect of pH on cellulase on this material, the pH was varied from 3.6 to 6.0. The reaction temperature was controlled at 50°C for 24 h. The results are shown in Fig. 19. The optimal pH giving the highest yield of

reducing sugar (2.1 g/l) was 4.8. However, the optimal pH of saccharification of rice straw was 5.0 by cellulase (*Trichoderma reesei*) (Kaur *et al*., 1998).

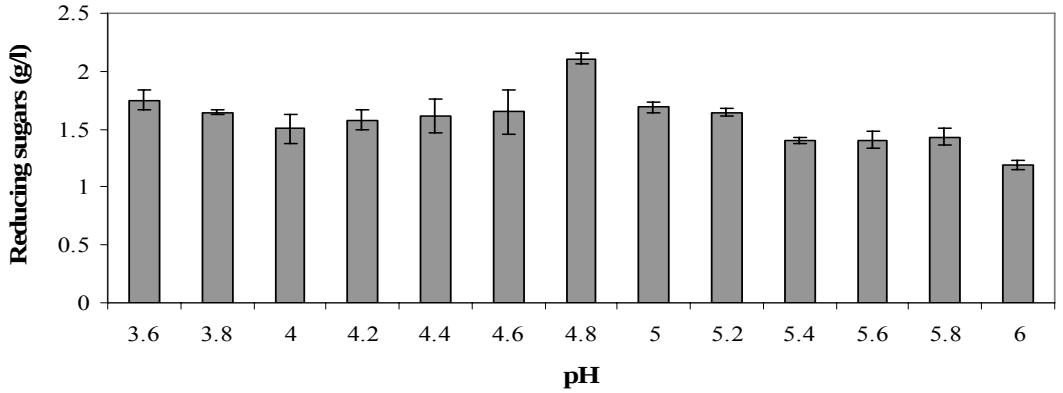


Figure 19. Effect of pH of cellulase on reducing sugar production from cellulose of PPF at substrate concentration of 2 g/l with cellulase dosage of 500 U/g substrate under 50°C for 24 h.

3.6.1.2 Effect of temperature on cellulase activity

Reaction temperature of enzymatic hydrolysis was varied from 35°C to 70°C. The pH of reaction was controlled at 4.8 (section 3.6.1.1). The results of effect of reaction temperature are shown in Fig. 20. The optimal reaction temperature was 50°C giving the highest reducing sugar of 1.59 g/l. This optimal reaction temperature was similar to the result of Kaur *et al*. (1998). The cellulase activity would decrease in the temperature condition more than 50°C due to denature of the enzyme.

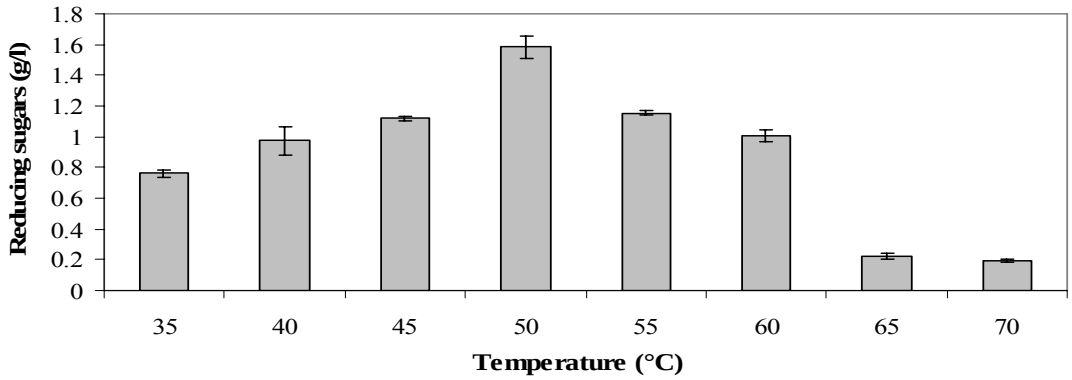


Figure 20. Effect of reaction temperature on reducing sugar production from cellulose of PPF at substrate concentration of 2 g/l with cellulase dosage of 500 U/g substrate under pH 4.8 for 24 h.

3.6.1.3 Effect of substrate concentration on cellulase activity

This study was conducted by varying the concentration of cellulose from 0.4 g/l to 20 g/l. The reaction was controlled at pH 4.8 and 50°C (section 3.6.1.1 and 3.6.1.2), respectively for 24 h. These experimental results are shown in Fig. 21. The optimal substrate concentration of cellulose was 12 g/l (833 U/g substrate) giving the maximum reducing sugar yield of 6.1 g/l. When the substrate concentration was higher than 12 g/l, reducing sugars concentration did not increase because cellulase activity was inhibited by product inhibition.

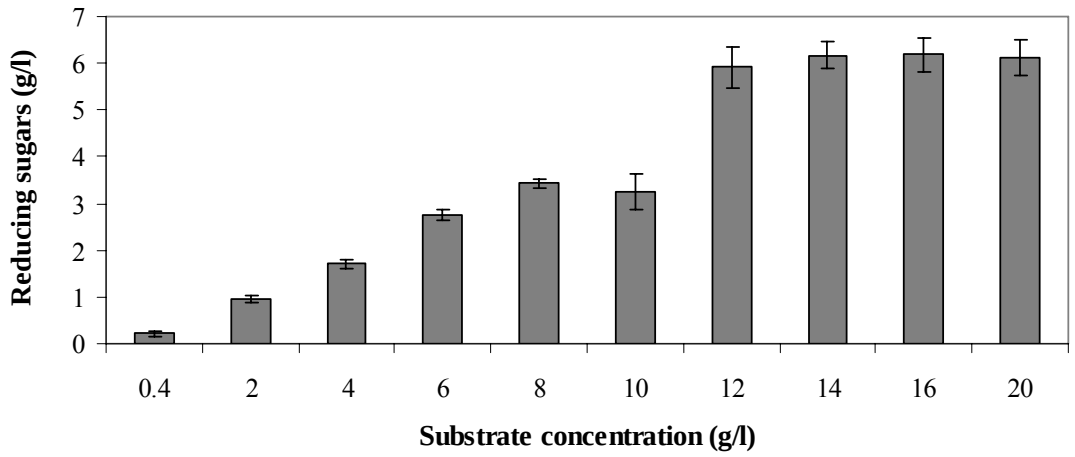


Figure 21. Effect of substrate concentration (cellulose) on reducing sugar production by enzymatic hydrolysis at cellulase dosage of 500 U/g substrate under 50°C, pH 4.8 for 24 h.

3.6.1.4 Effect of cellulase dosage

To determine the effect of cellulase dosage on reducing sugar production, the cellulase concentration was ranged from 416 U/g substrate to 8,333 U/g substrate. The reaction was controlled at pH 4.8, 50°C, and cellulase concentration of 12 g/l obtained from section 3.6.1.1, 3.6.1.2 and 3.6.1.3, respectively, for 24 h. The optimum cellulase concentration was 4,166 U/g substrate (Fig. 22) giving the highest reducing sugar yield of 7.4 g/l. The decrease cellulase loadings from 4,166 to 416 U/g substrate significantly decreased reducing sugars yields from 7 to 1 g/l which is similar with the results of Sathitsuksanoh *et al*. (2010). Meanwhile, percent of enzymatic hydrolysis of steam-exploded corn stover increased from 65 to

80% with increasing cellulase dosage from 10 to 25 IU/g glucan (Fang *et al*., 2010). Moreover, incubation time was an important factor affecting on release of reducing sugars. Therefore, incubation time would be studied in the next work.

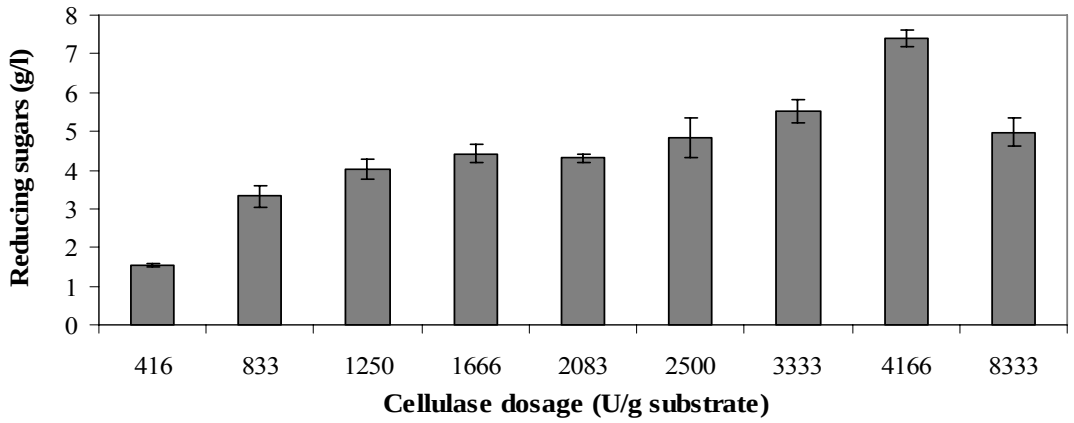


Figure 22. Effect of cellulase dosage on reducing sugar production from cellulose of PPF at substrate concentration of 12 g/l under 50°C, pH 4.8 for 24 h.

3.6.1.5 Effect of incubation time and saccharification value

To study the incubation time of reducing sugar production from cellulose by enzymatic hydrolysis, the hydrolysates were taken from 5 min to 4,320 min. The reaction was controlled at pH 4.8, 50°C, cellulose concentration of 12 g/l, and cellulase dosage of 4,166 U/g substrate obtained from section 3.6.1.1, 3.6.1.2, 3.6.1.3, and 3.6.1.4, respectively. The optimal incubation time for cellulose hydrolysis was 900 min (15 h) giving the highest reducing sugar yield of 7.9 g/l. In addition, the saccharification of this material was 60% (Fig. 23). Glucan digestibility of delignified PPF was much greater for 15 h, whereas 12-24 h and 48 h were the suitable glucan digestibility of Bamboo (Sathitsuksanoh *et al*., 2010) and steam-exploded corn stover (Fang *et al*., 2010), respectively. These results illustrated that cellobiose and glucose may affect the hydrolysis rate after 15-24 h incubation time (Yang *et al*., 2010). The fast initial rate at the beginning of hydrolysis was due to preferential hydrolysis of the amorphous region and then the rate decreased as the enzyme encountered the more recalcitrant crystalline region (Laureano-Perez *et al*., 2005; Yang *et al*., 2010). It was observed, with the crystallinity index measured by X-ray assay, that the crystalline

cellulose had been shown to be more recalcitrant than amorphous portions because the crystallinity index had increased after enzymatic hydrolysis meaning the amorphous portion was more readily hydrolyzed than the crystalline portion (Cao and Tan, 2005; Zhu *et al*., 2008; Yang *et al*., 2010). The optimal values of these parameters and the highest reducing sugar yield are summarized in Table 22.

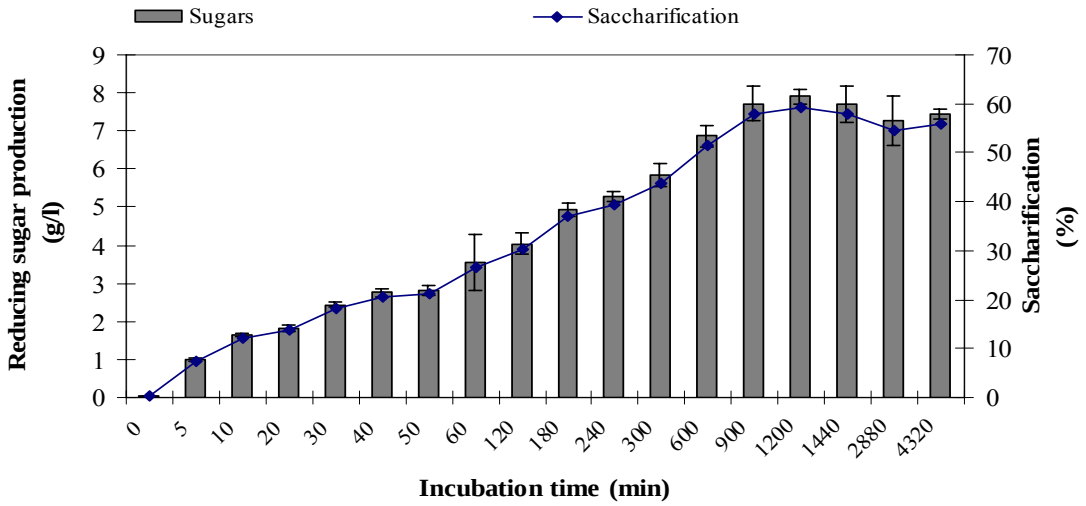


Figure 23. Effect of incubation time on reducing sugar production and saccharification values from cellulose by using cellulase hydrolysis at substrate concentration of 12 g/l with cellulase dosage of 4,166 U/g substrate under 50°C, pH 4.8 for 24 h.

Table 22. Summary of the optimal value of each parameter for the highest reducing sugar yield production.

Parameters	Optimal point
1. pH	4.8
2. Temperature (°C)	50
3. Substrate concentration (g/l)	12
4. Enzyme dosage (U/g substrate)	4,166
5. Incubation time (min)	900
6. Saccharification (%)	60
7. Reducing sugar (g/l)	7.9

3.6.2 Cellulosic hydrolysate production by concentrated sulfuric acid hydrolysis

3.6.2.1 Optimization for reducing sugars production by concentrated sulfuric acid using RSM

To understand the nature of the α-cellulose hydrolysis reaction, these experiments were conducted based on the effect of the solid (cellulose)-liquid (sulfuric acid) ratio (X_{10}), the dilute H_2SO_4 concentration in the hydrolysis (X_{11}) and reaction time (X_{12}) combined with statistical design. Levels of solid-liquid ratio, sulfuric acid concentration and reaction time were not only important factors for glucose production by chemical process, also imperative response-dependent variables for by-products formation (Table 23). Fischer's *F*-test demonstrated that the model applied was significant ($P<0.05$). The significant terms were calculated using *t*-test and the responses under different combinations were analyzed by analysis of variance (ANOVA). Only significant terms were selected to receive the maximum value of coefficient of determination (R^2) (Box *et al*., 1978) as shown in Eqs. (23)-(27). In this study, the values of R^2 varied from 0.83-0.93, which suggested that the model gave quite good fit. Equations obtained for reducing sugars (Eq. 23) and xylose production (Eq. 24) by sulfuric acid hydrolysis were:

$$\text{Reducing sugars}, Y_7\ (g/l) = -14.65 + 0.45x_{11} - 0.03x_{11}^2 \quad \text{.....(23)}$$

$$\text{Xylose}, Y_8\ (g/l) = -(8.19\times10^{-4}) - (4.33\times10^{-6})x_{11}^2 \quad \text{.....(24)}$$

In the pretreatment step, sulfuric acid (72% v/v) hydrolysis at room temperature for 90 min and then diluted to various acid concentrations at 120°C produced reducing sugars (0.25-0.55 g/l) and xylose (<0.0002 g/l). The lowest value of reducing sugars (0.25 g/l) was obtained from 2 conditions consisting of; (i) 1:15 (w/v) solid-liquid ratio, 1.0% (v/v) sulfuric acid concentration for 180 minutes (treatment number 4), and (ii) 1:10 (w/v) solid-liquid ratio, 1.0% (v/v) sulfuric acid concentration for 120 minutes (treatment number 8). Therefore, 1.0% (v/v) sulfuric acid concentration is not suitable for reducing sugars production due to the complex structure of cellulose (Herrera *et al*., 2003).

Table 23. Results of the experimental design for response surface analysis for producing reducing sugars and by-products formation.

Run	Real value			Product response		By-products response		
	X_{10}	X_{11}	X_{12}	Reducing sugars, Y_7 (g/l)	Xylose, Y_8 (g/l)	Furfural, Y_9 (g/l)	Acetic acid, Y_{10} (g/l)	5-HMF, Y_{11} (g/l)
1	1:20	3	60	0.42	0.000127	1.05	1.32	0.22
2	1:20	1	120	0.27	0.000126	0.99	0.00	0.00
3	1:15	3	120	0.50	0.000135	0.00	1.47	0.43
4	1:15	1	180	0.25	0.000113	0.20	1.62	0.03
5	1:15	3	120	0.50	0.000146	0.00	1.49	0.35
6	1:15	3	120	0.48	0.000146	0.00	1.49	0.40
7	1:15	3	120	0.49	0.000146	0.00	1.49	0.41
8	1:10	1	120	0.25	0.000131	1.03	0.40	0.04
9	1:10	5	120	0.46	0.000131	2.05	1.33	0.53
10	1:20	5	120	0.42	0.000112	1.93	0.68	0.31
11	1:20	3	180	0.50	0.000139	1.02	1.66	0.45
12	1:15	5	60	0.55	0.000123	1.22	1.24	0.40
13	1:15	3	120	0.49	0.000149	0.00	0.95	0.38
14	1:15	5	180	0.30	0.000101	3.74	1.84	0.52
15	1:10	3	180	0.47	0.000129	1.03	1.40	0.47
16	1:10	3	60	0.34	0.000138	1.03	0.80	0.15
17	1:15	1	60	0.26	0.000135	0.00	0.00	0.00

The lowest value of xylose, however, is normal because these substrates have been used for xylose production prior to this study (Section 3.7). In treatment number 12, the hydrolysis conditions required the highest level of sulfuric acid concentration (5%, v/v), the moderate level of solid-liquid ratio (1:15, w/v), and the lowest level of reaction time (60 min) to give the highest concentration of reducing sugars (0.55 g/l). Equations representing by-products formation from sulfuric acid hydrolysis were equations 25-27:

$$Furfural,\ Y_9\ (g/l) = 331.47 - 1.25x_{11} + 0.02x_{10}^2 + 0.22x_{11}^2 \quad \text{.....(25)}$$

$$Acetic\ acid,\ Y_{10}\ (g/l) = -178.53 + 1.84x_{11} + 0.02x_{12} - 0.11x_{11}^2 \quad \text{.....(26)}$$

$$5 - HMF,\ Y_{11}\ (g/l) = -25.95 + 0.79x_{11} + 0.01x_{12} - 0.03x_{11}^2 \quad \text{.....(27)}$$

where x_{10}, x_{11} and x_{12} represent the actual values of solid-liquid ratio, sulfuric acid concentration and reaction time, respectively.

The sulfuric acid hydrolysis of cellulose of PPF also gave the formation of by-products (furfural, acetic acid and 5-HMF) (Table 23). Furfural and acetic acid are by-products from xylose degradation (Dias *et al*., 2005); however, 5-HMF is a by-product from degradation of glucose (Larsson *et al*., 1999; Karimi *et al*., 2006). Sulfuric acid (72% v/v) hydrolysis produced furfural (0-3.74 g/l), acetic acid (0-1.84 g/l) and 5-HMF (0-0.53 g/l) in the same range of various factors. The less or no 5-HMF formation was observed from treatment number 2, 4, 8 and 17, the lowest of sulfuric acid concentration (1.0%, v/v) was used in these treatments. Therefore, the factor of sulfuric acid concentration is an important factor affecting on 5-HMF formation. The generation of furfural and acetic acid was very low due to the low xylose content in the substrate.

The effects of solid-liquid ratio, sulfuric acid concentration and reaction time on needed product and by-products productions were analyzed by three dimension (3D) graph obtained from RSM. Plus (+) and minus (-) symbol represented the positive and negative effects on the response as shown in Eqs. (23)-(27). However, interaction of the factors had a pronounced effect on reducing sugars

optimization indicating the importance of these factors for increasing of reducing sugars yield and decreasing of by-products yields.

Interaction of solid-liquid ratio, sulfuric acid concentration and reaction time on reducing sugars production using cellulose as a substrate revealed the response surface of reducing sugars production (Fig. 24A-24C) in which one variable kept at the optimal level and the other two variables varied within the experimental ranges. These results show the interaction between three parameters influencing significantly on reducing sugars production. The optimal conditions for reducing sugars production as mainly glucose from cellulose were 1:15 (w/v) of solid-liquid ratio, 5.0% (v/v) of sulfuric acid concentration and 60 minutes reaction time giving the maximum reducing sugars concentration of 0.55 g/l (Fig. 24A-24C). Reducing sugars produced by sulfuric acid hydrolysis increased with the increase of sulfuric acid concentration up to a value of 4% (v/v) (Fig. 24A and 24C). Similar with the effect of solid-liquid ratio and reaction time, the reducing sugars decreased when solid-liquid ratio and reaction time increase rather than 1:15 (w/v) and 90 minutes, respectively (Fig. 24B and 24C). However, the reducing sugars obtained from this study were very low. It might be low reaction time (90 min) and low reaction temperature (room temperature, 28-30°C) for decrystallization of cellulose. Xiang et al. (2003) reported that the α-cellulose form treated by concentrated sulfuric acid of 65% at high temperature (more than 200°C) can be changed from fibrous form to gelatinous form within 4 hours. The results was successfully hydrolyzed α-cellulose around 95% after carrying out at 120°C, 4% H_2SO_4 for 90 min.

At the optimal level of reducing sugars production (0.55 g/l), furfural (1.22 g/l), acetic acid (1.24 g/l) and 5-HMF (0.40 g/l) were also produced by reducing sugars degradation processes. The results (Fig. 25A-25I) demonstrated that furfural, acetic acid and 5-HMF increased when sulfuric acid concentration and reaction time increased while solid-liquid ratio had quite no effect on all by-products formation.

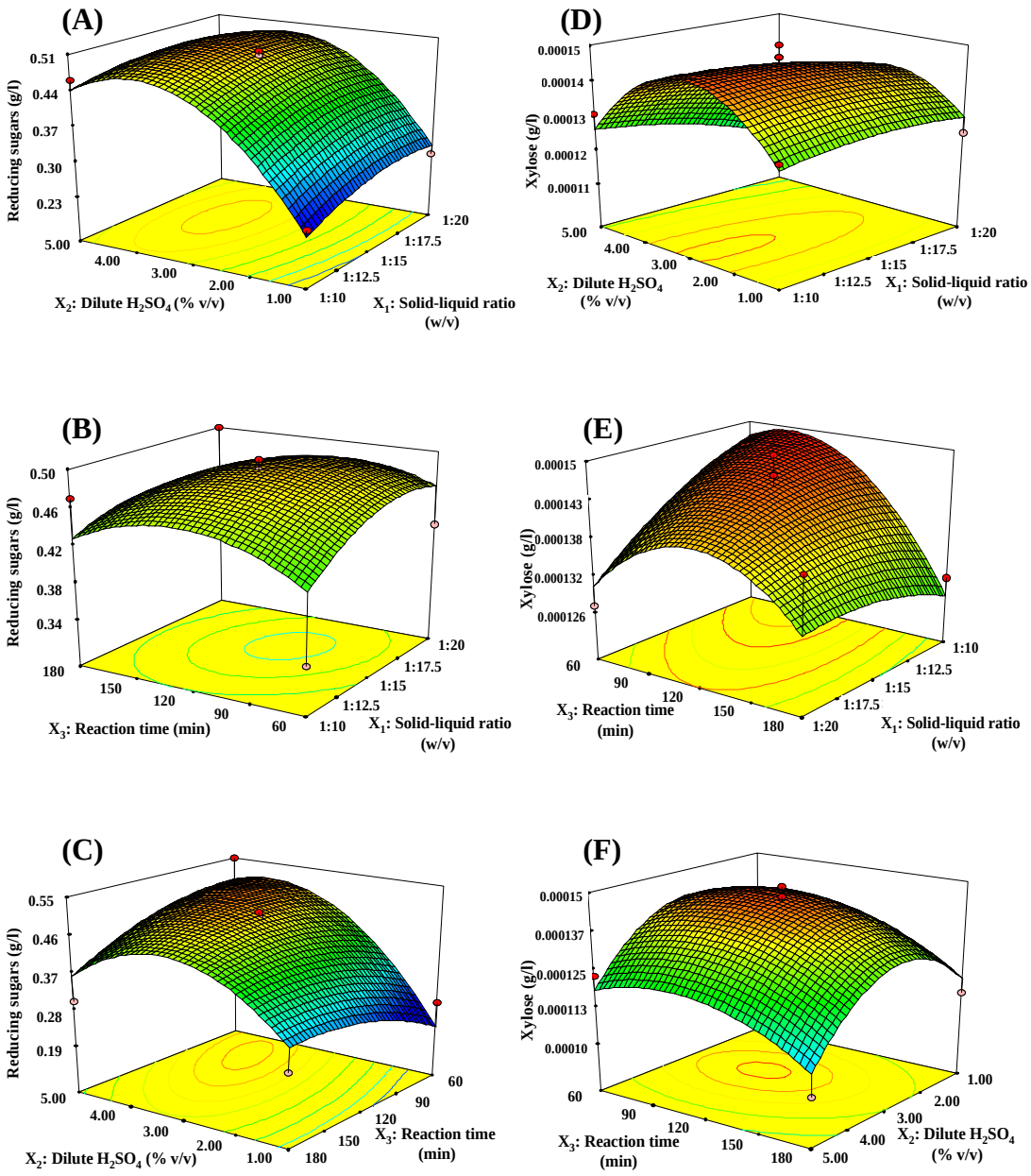


Figure 24. Three-dimensional graphs of the quadratic model for reducing sugars yield (g/l) (A-C) and xylose yield (g/l) (D-F) by using Box-Behnken design: (A and D); fixed reaction time at centre point of 120 minutes, (B and E); fixed the dilute sulfuric acid at centre point of 3% (v/v), and (C and F); fixed the solid-liquid ratio at centre point of 1:15 (w/v).

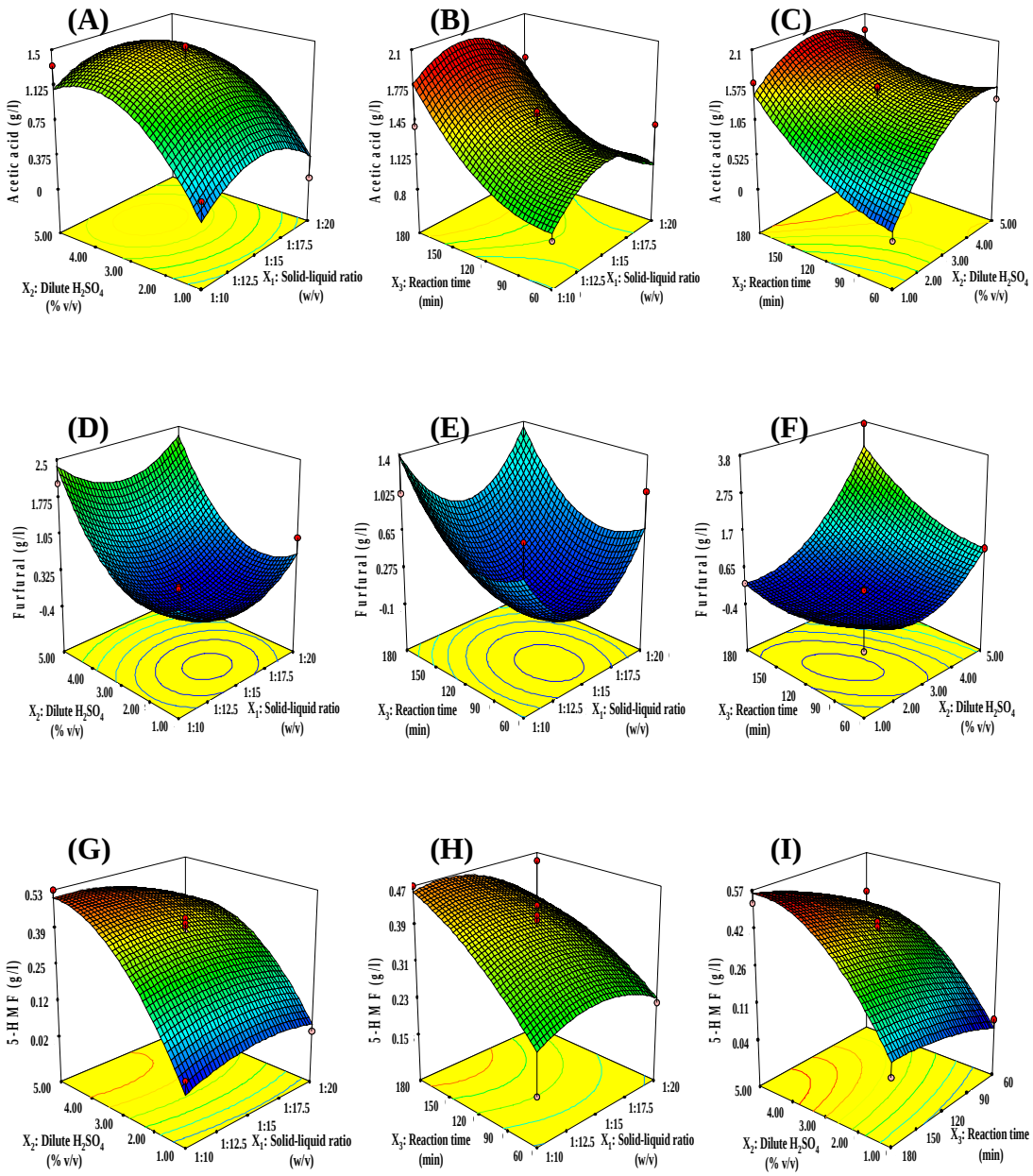


Figure 25. Three-dimensional graphs of the quadratic model for Acetic acid (g/l) (A-C) furfural (g/l) (D-F) and 5-HMF (g/l) (G-I) by using Box-Behnken design: (A, D and G); fixed reaction time at centre point of 120 minutes, (B, E and H); fixed the dilute sulfuric acid at centre point of 3% (v/v), and (C, F and I); fixed the solid-liquid ratio at centre point of 1:15 (w/v).

3.6.2.2 Model validation and confirmation

Verification experiments performed at the predicted conditions was selected to confirm the model. The experiment was conducted in triplication. The results (Table 24) demonstrated that the experiment values were similar to the predicted values and gave the percentage of deviation from predicted value at 3.68-13.33%, indicating the validity and adequacy of the predicted model.

Table 24. Comparison of predicted and experimental values of five responses (Y_7-Y_{11}) at the optimal levels predicted from model using response surface method (RSM).

Conditions	Response	Predicted value[a]	Experimental value[b]	Deviation[c] (%)
X_{10}=1:16 (w/v)	Y_7= Reducing sugars	0.52	0.54 ± 0.04	3.85
X_{11}=4.0%(v/v)	(g/l)			
X_{12}= 86 (min)	Y_8= Xylose (g/l)	0.000136	0.000131 ± 0.00005	3.68
	Y_9= Furfural (g/l)	0.45	0.51 ± 0.06	13.33
	Y_{10}=Acetic acid (g/l)	1.36	1.42 ± 0.18	4.41
	Y_{11}= 5-HMF (g/l)	0.39	0.35 ± 0.09	10.26

Parameters: X_{10} = solid-liquid ratio (w/v), X_{11} = sulfuric acid concentration (%, v/v), X_{12} = reaction time (min).

[a] Predicted value obtained from RSM model.

[b] Observed value determined from experiments.

[c] [(Observed value – predicted value) x 100]/predicted value.

3.6.2.3 Comparison of reducing sugar produced between enzymatic hydrolysis and concentrated sulfuric acid

In order to select the better method for producing reducing sugar from cellulose of PPF, concentrated sulfuric acid hydrolysis and enzymatic hydrolysis were studied. Enzymatic hydrolysis method is a better method than concentrated sulfuric acid hydrolysis method for reducing sugar production (Table 25). Therefore,

enzymatic hydrolysis method was selected to produce reducing sugar as a substrate for producing ethanol in the further investigation. However, the optimal conditions of concentrated sulfuric acid hydrolysis (solid-liquid ratio (X_{10}) = 1.16 w/v, sulfuric acid concentration (X_{11}) 4.0 %, v/v, reaction time (X_{12}) 86 min) were unclear because very low reaction time and temperature were given in these studies. Moreover, the inhibitor compounds in both hydrolysates of enzymatic method and concentrated sulfuric acid should be studied.

Table 25. Comparison of reducing sugar and inhibitors between concentrated sulfuric acid (72% v/v) and enzymatic hydrolysis.

Parameters	Enzymatic hydrolysis*	Concentrated sulfuric acid hydrolysis**
1. Reducing sugar (g/l)	7.9	0.54
2. Furfural (g/l)	0	0.51
3. Acetate (g/l)	6.8	1.42
4. 5-HMF (g/l)	0	0.35

* pH 4.8, temperature 50°C, substrate concentration 12 g/l, enzyme dosage 80 U/g substrate, and 900 min incubation time

**Solid-liquid ratio (X_{10}) = 1.16 w/v, sulfuric acid concentration (X_{11}) 4.0 %, v/v, reaction time (X_{12}) 86 min

3.7 Production of delignified PPF (dPPF) hydrolysate

3.7.1 Delignified PPF (dPPF) hydrolysate

The component of the main fractions of dPPF was; cellulose 42.36 ± 1.07%, hemicellulose 38.96 ± 0.67%, and lignin 8.21 ± 0.37% (Table 9). The hemicellulose fraction of this material was mainly xylan 80.8% (w/w). If we assume that xylan is completely converted to xylose without formation of any decomposition products, then P_0 can be represented by equivalent amount of xylose by Eq. (28) and (29):

$$P_0 \ of \ dPPF = \left(\frac{X_{P0} \times 150 \times 10}{132 \times LSR} \right) = 35.77 \ g \ xylose/l \qquad (28)$$

$$P_0 \ of \ PPF = \left(\frac{X_{P0} \times 150 \times 10}{132 \times LSR} \right) = 29.35 \ g \ xylose/l \qquad (29)$$

where X_{P0} is the initial xylan polymer presented in the PPF or dPPF on dry basis (25.83 g xylan/100g PPF or 31.48 g xylan/100g dPPF; calculated from (38.96x80.8)/100), 150/132 is the stoichiometric factor (Rahman *et al*., 2006) and LSR is liquid solid ratio (10 g liquid/g dPPF).

The maximum release of xylose from hemicellulose was 30.67 g/l under dilute sulfuric acid (5% v/v), giving 85.74% of potential concentration of xylose. Reaction temperature and reaction time of xylose production were important parameters affecting on release of xylose (Fig. 26a). In the autohydrolysis at 75-148°C, the maximum of xylose production (9.10 g/l) required higher reaction temperature (120-148°C) and reaction time (180 min). Moreover, under condition of 10% sulfuric acid gave the maximum xylose released of 23.54 g/l. It was observed that with increase in acid concentration, concentration of xylose in the dPPF hydrolysate was decreased (Fig. 26b and 26c). Experimental results illustrated that probably there are some decomposition reaction leading to dehydration of xylose to furfural (Rahman *et al*., 2006).

Glucose was also released during acid hydrolysis but the concentration was low (0.20-6.05 g/l) (Fig. 27). The maximum of glucose released in dPPF hydrolysate was 6.05 g/l under 10% sulfuric acid concentrations at 148°C for 180 min (Fig. 27c). On the other hand, with 0 and 5% sulfuric acid concentration, the maximum glucose released was 1.51 and 5.33 g/l, respectively. Normally, the release of glucose could be either from hemicellulose or cellulose chain (Téllez-Luis *et al*., 2002; Rahman *et al*., 2006). Thus, the glucose released in this study is from both hemicellulose and cellulose; however, it comes basically from hemicelluloses by using dilute acid (Téllez-Luis *et al*., 2002).

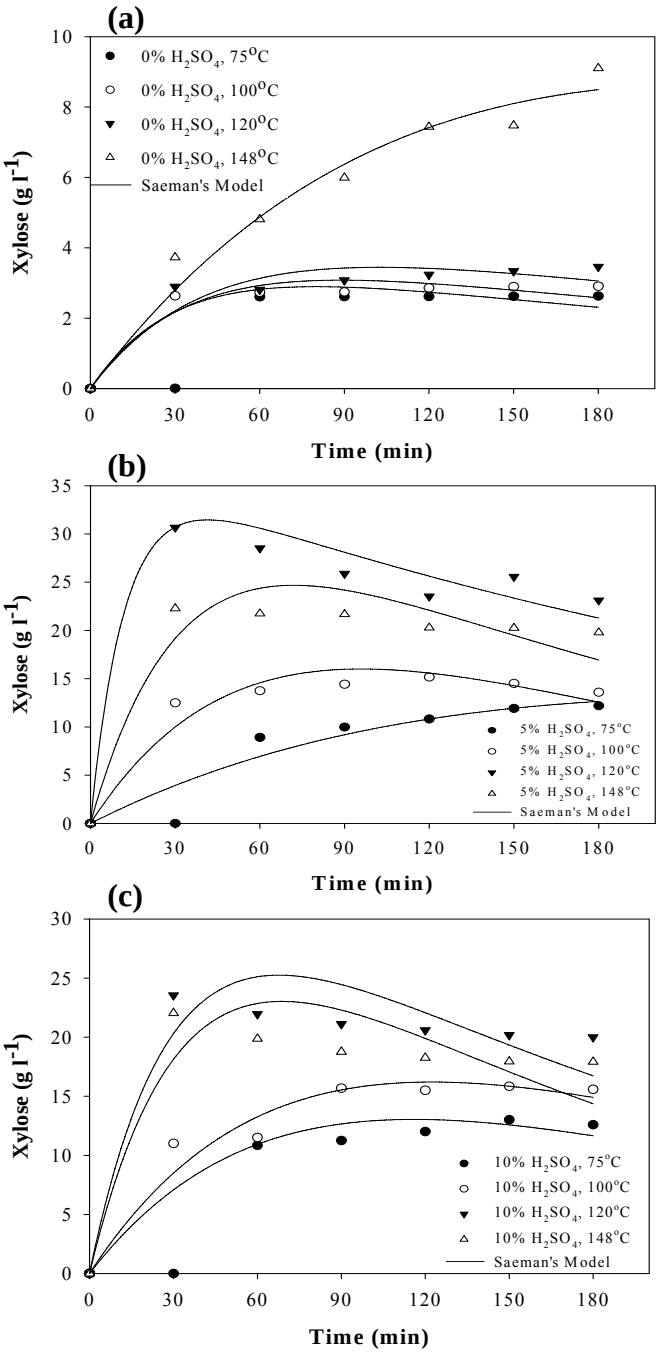


Figure 26. Experimental (dot) and predicted (line) concentrations of xylose released from dPPF at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

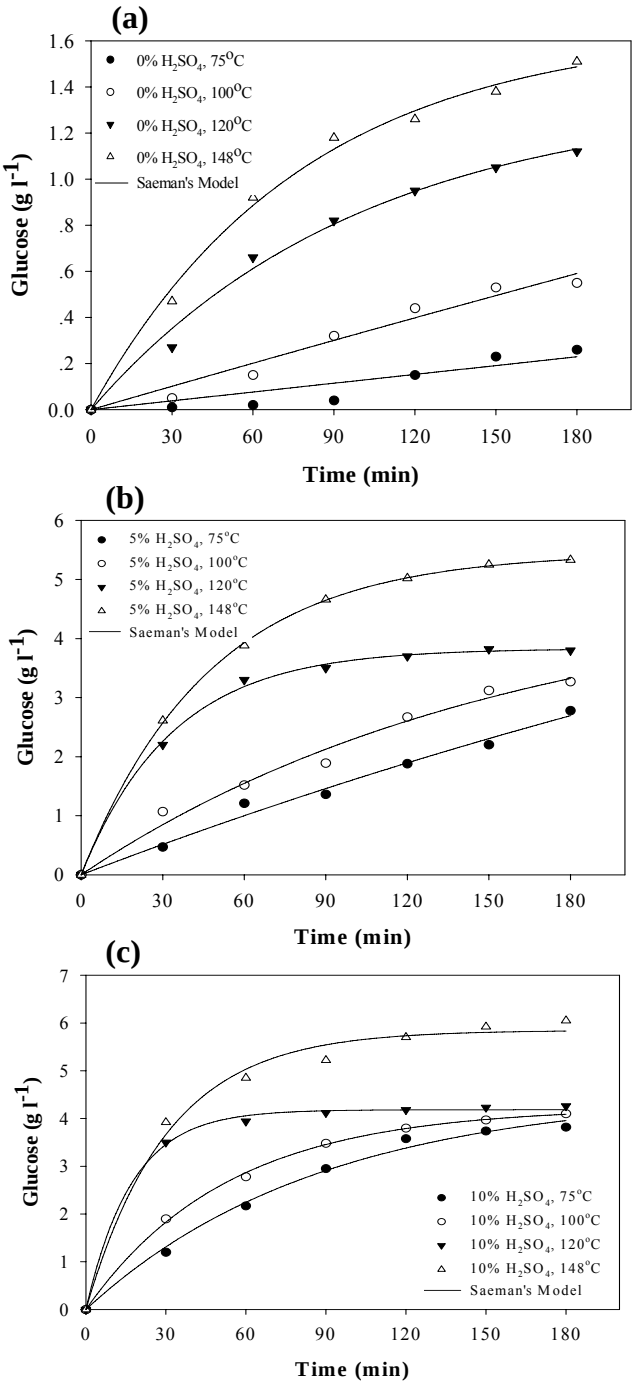


Figure 27. Experimental (dot) and predicted (line) concentrations of glucose released from dPPF at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

The furfural formation as a decomposition product from xylose in the dPPF hydrolysate is shown in Fig. 28. It was demonstrated that when sulfuric acid concentration and reaction temperature were increased from 0 to 5% (Fig. 28a and 28b) and 75 to 148°C (Fig. 28a), furfural concentration was increased in the hydrolysate. The highest concentration of furfural (Fig. 28b) was 1.16 g/l when sulfuric acid concentration and reaction time were 5% and 60 min, respectively.

During acid hydrolysis, acetic acid is generated from acetyl groups of hemicellulose (Rahman *et al*., 2006). The maximum and minimum generations of acetic acid in the dPPF hydrolysate were 8.02 and 0.65 g/l when sulfuric acid concentrations were 10 and 0%, respectively, and reaction time of 90 and 30 min, respectively (Fig. 29).

3.7.1.1 Kinetic model of xylose production

Kinetic and statistical parameters obtained from dPPF hydrolysis at various reaction temperatures (75-148°C) with various sulfuric acid concentrations (0-10%) is shown in Table 26. Experimental and predicted data for xylose production with various acid concentrations, reaction temperatures and reaction times are shown in Fig. 26. It was demonstrated that with both sulfuric acid concentrations (5% and 10%, v/v) and ≥100°C of reaction temperature, xylose production rate (k_1) was higher than the decomposition rate (k_2) which were 0.0102-0.0821 min^{-1} and 0.0044-0.0510 min^{-1}, respectively. When the reaction temperature was 75°C, xylose production rate (k_1) and the decomposition rate (k_2) under 0%, 5% and 10% sulfuric acid were the same values of 0.0033-0.0031 min^{-1}, 0.0042 min^{-1} and 0.085-0.0087 min^{-1}, respectively. Therefore, the 75°C of reaction temperature had no effected on xylose production. The determination of coefficient R^2 showed a good agreement between experimental and predicted data for all regressions. It was also demonstrated that with increase in acid concentration, the values of k_1 and k_2 were also increased. It can be implied that the optimum reaction time to obtain maximum release of xylose and minimum release of furfural and acetic acid in the dPPF hydrolysate is essential.

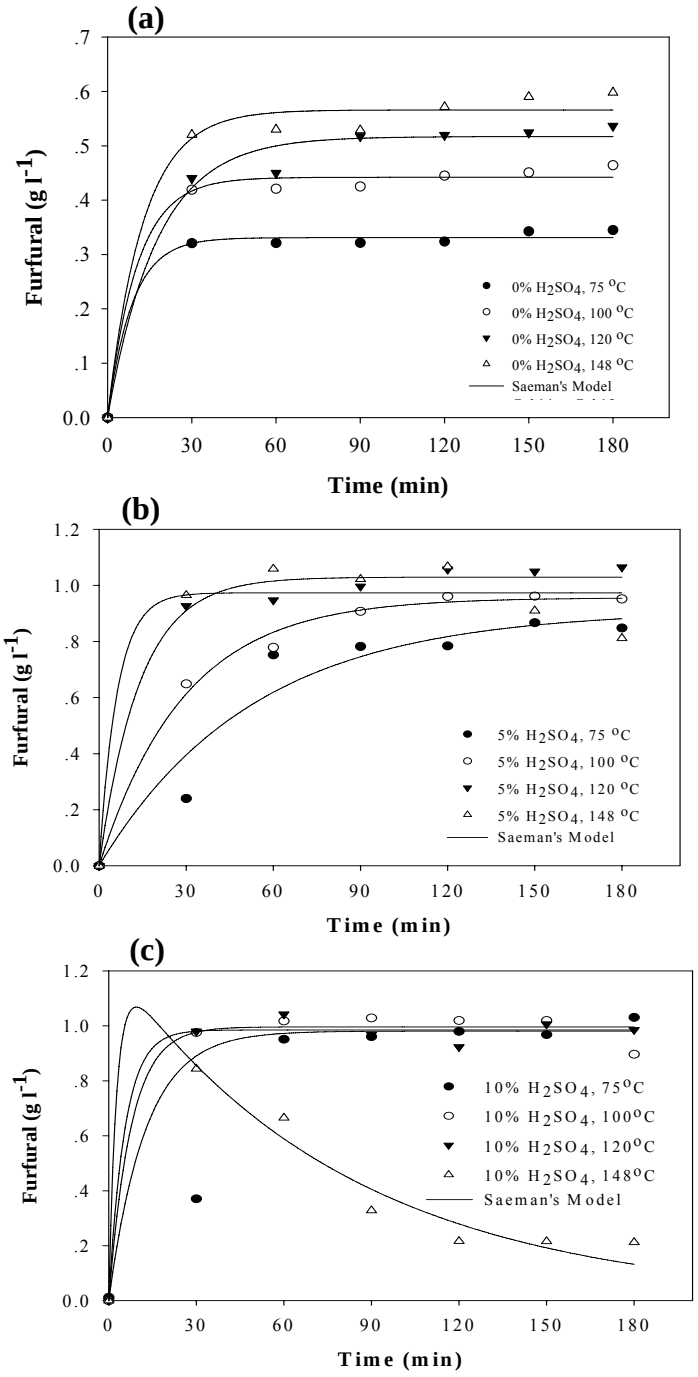


Figure 28. Experimental (dot) and predicted (line) concentrations of furfural generated in dPPF hydrolysate at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

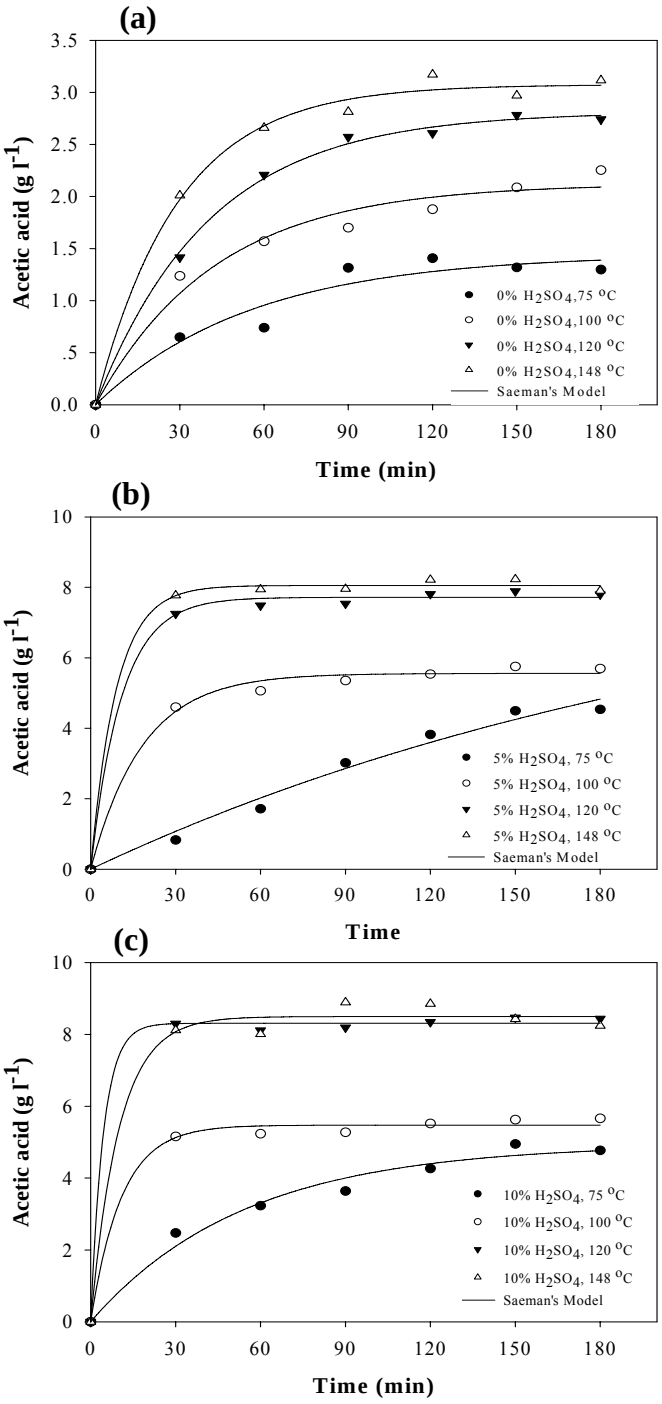


Figure 29. Experimental (dot) and predicted (line) concentrations of acetic acid generated in dPPF hydrolysate at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

Table 26. Kinetics and statistical parameters of xylose, glucose, furfural and acetic acid released during dilute sulfuric acid hydrolysis of delignified palm pressed fiber (dPPF) at various reaction temperatures.

	0% H_2SO_4				5% H_2SO_4				10%H_2SO_4			
	75°C	100°C	120°C	148°C	75°C	100°C	120°C	148°C	75°C	100°C	120°C	148°C
Xylose												
k_1 (min^{-1})	0.0033	0.0031	0.0030	0.0030	0.0042	0.0128	0.0821	0.0293	0.0085	0.0102	0.0922	0.0277
k_2 (min^{-1})	0.0031	0.0276	0.0228	0.0063	0.0042	0.0044	0.0310	0.0052	0.0087	0.0065	0.0510	0.0064
R^2	0.96	0.96	0.95	0.98	0.94	0.97	0.98	0.95	0.96	0.97	0.94	0.92
Glucose												
k_3 (min^{-1})	0.0051	0.0004	0.0100	0.0128	0.0019	0.0064	0.0297	0.0212	0.0114	0.0189	0.0582	0.0328
G_0	1.32	1.70	1.35	1.65	4.45	4.87	3.83	5.45	4.54	4.23	4.18	5.84
R^2	0.91	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Furfural												
k_4 (min^{-1})	0.1141	0.0954	0.0286	0.0794	0.0189	0.0341	0.0717	0.1612	0.0794	0.1312	0.1806	0.3706
F_0	0.33	0.44	0.52	0.57	0.91	0.96	1.03	0.97	0.98	0.99	0.98	0.01
R^2	0.99	0.99	0.99	0.99	0.97	0.99	0.99	0.97	0.99	0.99	0.99	0.98
Acetic acid												
k_5 (min^{-1})	0.0180	0.0234	0.0247	0.0342	0.0041	0.0546	0.0918	0.1108	0.0185	0.0919	0.2213	0.0998
A_0	1.44	2.12	2.81	3.07	9.18	5.56	7.72	8.05	4.94	5.47	8.31	8.50
R^2	0.97	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99

k_1 is the rate of xylose production (min^{-1}), k_2 is the rate of xylose decomposed (min^{-1}), k_3 is the rate of glucose production (min^{-1}), k_4 is the furfural production rate (min^{-1}), and k_5 is the acetic acid production rate (min^{-1})

G_0 is the potential glucose concentration, F_0 is the potential furfural concentration, and A_0 is the potential acetic acid concentration

Table 27. Generalized models for kinetic parameters prediction of delignified palm pressed fiber (dPPF) hydrolysis with dilute sulfuric acid at 120°C.

Products	Models	R^2
Xylose	$k_1 = 0.063C_a^{0.1660}$ (30)	0.99
	$k_2 = 0.0092C_a^{0.7370}$ (31)	0.99
Glucose	$k_3 = 0.0065C_a^{0.9511}$ (32)	0.99
Furfural	$k_4 = 0.0173C_a^{1.0011}$ (33)	0.95
Acetic acid	$k_5 = 0.0214C_a^{1.0017}$ (34)	0.98

The generalized model for prediction of xylose production rate is calculated by Eq. (30) and represented by empirical Eq. (18) where k_1 is represented with acid concentration (C_a). Similarly, xylose degradation rate (k_2) is represented by empirical Eq. (31). The determination coefficients R^2 for both parameters were in good agreement which is shown in Table 27. Combination of Eq. (30) and (31) with the model of xylose production and degradation, it is possible to predict xylose concentration at any time and acid concentration within the time period (0-180 min) and acid concentration (0-10%). The generalized model predicted that maximum xylose concentration of more than 25 g/l could be achieved by treated dPPF with 5% sulfuric acid at reaction temperature of 120°C for 30 min. The dependence of xylose concentration with various acid concentrations and internal range of time at fixed reaction temperature of 120°C are shown by response surface in Fig. 30(a). Therefore, reaction time should be 30 min to obtain the maximum release of xylose with the minimum decomposition products in the hydrolysate.

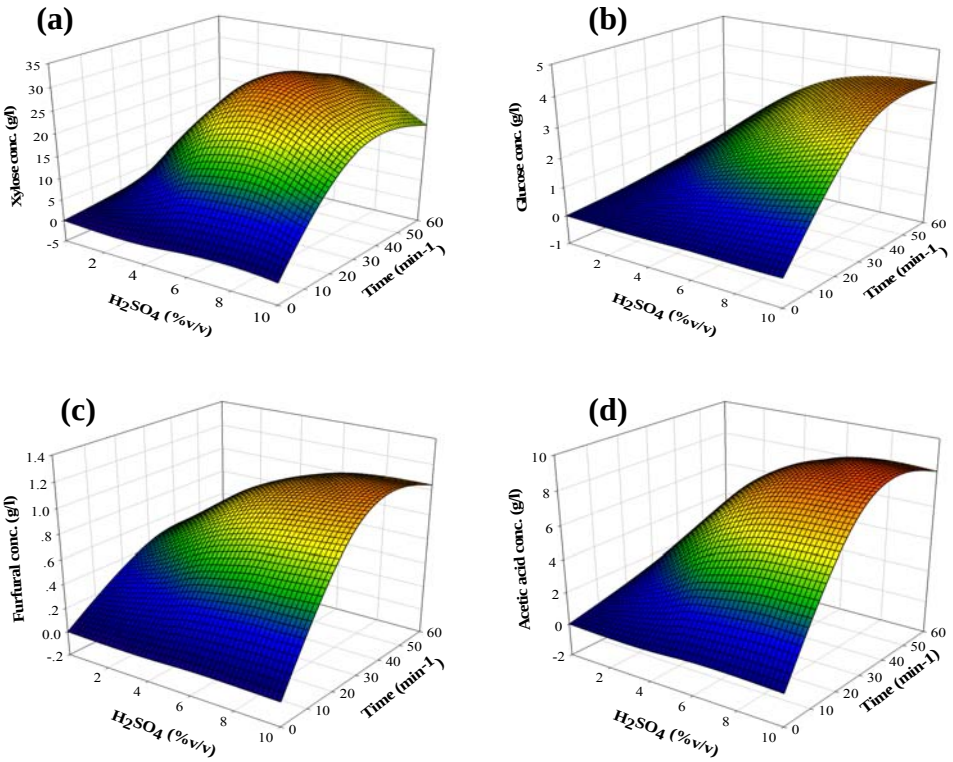


Figure 30. Effect of sulfuric acid concentration and reaction time on generalized model for prediction of; (a) xylose production, (b) glucose production, (c) furfural formation and (d) acetic acid release.

3.7.1.2 Kinetic model of glucose production

Glucose is a secondary product in many hydrolysis of biomasses, i.e. oil palm empty fruit bunch (OPEFB) (Rahman *et al*., 2006), sorghum straw (Téllez-Luis *et al*., 2002) and dPPF as well. It is difficult to determine how much glucose released either from cellulose or hemicellulose (Téllez-Luis *et al*., 2002). In the model of glucose production (Eq. 13), G_0 and kinetic parameter k_3 were obtained by regression. The determination coefficient (R^2) was very high agreement with experimental and predicted data. The G_0 (potential glucose concentration) were in the range of 1.32-5.84 g/l, corresponding to 3.14-13.90% of total cellulose in dPPF (see Appendix F). The values of k_3 were obtained in the range of 0.0004-0.0582 min^{-1} (Table 26). The values of k_3 increased with the increase in both acid concentration and reaction temperature. Therefore, the model for prediction of glucose production was developed correlated with acid concentration, reaction temperature and kinetic

parameter k_3. The value of k_3 was lower than that of corresponding value of k_1 (xylose production) because the structure of hemicellulose is amorphous which is easier hydrolysis than crystalline of cellulose (Rahman *et al*., 2006).

The hydrolysis of cellulose is dependent on degree of crystallinity (Alvira *et al*., 2010) and swelling state of cellulose (Rahman *et al*., 2006). Experimental and predicted data of glucose production in the resulting dPPF hydrolysate is given in Fig. 27. Therefore, the combination of Eq. (32) and glucose production model was feasible to predict release of glucose within the experimental ranges. The highest glucose production predicted by the models was 5.84 g/l at 10%v/v (0.1 M) sulfuric acid concentration, 148°C for 180 min. The k_3 is presented by empirical Eq. (32). The determinant coefficient R^2 was well fitted which was given in Table 27. The response surface plot of the generalized model for glucose production with increase in acid concentration and reaction time is shown in Fig. 35(b). From the response surface plot, there was no decomposition reaction occurred during the hydrolysis process because the degradation of glucose to HMF requires high temperature (180-230°C) and pressure (1.5-2.0 MPa) (Karimi *et al*., 2006). To obtain maximum xylose and glucose concentration in this hydrolysate, it is necessary to keep the reaction time at 30 min where glucose concentration is very low.

3.7.1.3 Kinetic model of furfural formation

Furfural is the principle degradation product of xylose in the acid hydrolysis of dPPF. Kinetic and statistical parameters for furfural are given in Table 26. The values of F_0 (potential furfural concentration) and kinetic parameter k_4 were within the range of 0.01-1.03 g/l and 0.0286-0.3706 min^{-1}, respectively. The determinant coefficients R^2 were well fitted with furfural formation model. The experimental and predicted data is shown in Fig. 28. The k_4 was increased with the increase in both sulfuric acid concentration and reaction temperature. A generalized model of furfural production was modified to correlate k_4 with acid concentration and reaction temperature for prediction of furfural concentration at any acid concentration, reaction temperature and time within the operating range (Rahman *et al*., 2006).

The empirical Eq. (33) represents the generalized model which is shown in Table 27. The response surface graph of generalized model for furfural

formation is given in Fig. 30c. From this response surface graph, furfural production increased with increase in acid concentration and reaction time. Furfural is well known as an inhibitory compound to fermentation process and thus its concentration must be minimized to facilitate optimum use of dPPF hydrolysate for ethanol (Sun and Cheng, 2002) and xyliyol productions (Rahman *et al*., 2006). Therefore, resulted from response surface graphs of xylose (Fig. 30a) and furfural (Fig. 30c) production, the hydrolysis process for the highest xylose production and the lowest furfural formation should be conducted with higher acid concentration and lower reaction time. In this study, the generalized model of furfural formation can facilitate to predict concentration of furfural. Hence, the selection of acid concentration and reaction time can be carried out in order to obtain the maximum xylose concentration in the dPPF hydrolysate while keeping concentration of furfural at minimum level.

3.7.1.4 Kinetic model of acetic acid production

Acetic acid is principle product released from acetyl group degradations of hemicellulose in acid hydrolysis of dPPF and other lignocellulosic materials (Rahman *et al*., 2006; Herrera *et al*., 2003; Téllez-Luis *et al*., 2002; Garrote *et al*., 2001). Kinetic and statistical parameters for acetic acid are given in Table 26. The values of A_0 (potential acetic acid concentration) and kinetic parameter k_5 were within the range of 1.44-9.18 g/l and 0.0041-0.2213 min^{-1}, respectively. The determinant coefficients R^2 were well fitted with acetic acid production model. The experimental and predicted data is shown in Fig. 29. The value of kinetic parameter k_5 was increased with increase in acid concentration and reaction temperature. A generalized model for prediction of acetic acid production was conducted to correlate k_5. This is obtained by Eq. (34) as shown in Table 27. The value of regression parameter n for acetic acid production k_5 was higher than that of regression parameter n for xylose production k_1 in all experiments (Table 26). These phenomenon shows that the effect of acid on acetyl removal from hemicellulose was higher compared to that of effect of acid on xylose generation. In another word, xylose was easily produced from dPPF observed by the degradation of acetyl group of hemicellulose. However, the value of regression parameter n for acetic acid production k_5 obtained in some studies was lower than that of regression parameter n for xylose production k_1

(Rahman *et al*., 2006). There was a big different thing between this work and Rahman *et al*. (2006) work which is raw material pretreatment, delignification process. Due to the lignin removal from the surface of PPF, the effect of acid on acetyl groups degradation was increased which was the reason for obtaining the higher value of k_5.

The response surface plot of generalized model for acetic acid released in the dPPF hydrolysis is shown in Fig. 30d. It was observed that acetic acid concentration was increased with increase in sulfuric acid concentration and reaction time which is similar to the experiments of Rahman *et al*. (2006) and Téllez-Luis *et al*. (2002). Therefore, to maximize xylose concentration in the resulting of dPPF hydrolysate should be conducted the experiments at high sulfuric acid concentration and low reaction time for keeping the concentration of acetic acid at minimum level.

3.7.1.5 Comparison of xylose production rate with other lignocellulosic materials under acid hydrolysis

Xylose production rate (k_1) would be higher resulted from not only acid concentration, but also type of acid. Table 28 clearly shows that a higher k_1 value was obtained from 6% sulfuric acid (0.0798 min^{-1}) than 6% hydrochloric acid (0.0333 min^{-1}). Moreover, sulfuric acid gave lower xylose decomposition (k_2) and acetic acid generation (k_5). However, a higher furfural formation (k_4) would be obtained from sulfuric acid than hydrochloric acid.

Pretreatment process, delignification, might increase furfural and acetic acid production. When compared the k_5 value between dPPF (0.0918 min^{-1}) and OPEFB (0.0189 min^{-1}), a higher k_5 value was obtained from dPPF because of lower content of lignin resulted to increase acid hydrolysis on hemicellulose (Table 28).

Table 28. Comparison of xylose production rate (k_1), xylose decomposition rate (k_2), glucose production rate (k_3), furfural formation rate (k_4), and acetic acid generation rate (k_5) on various lignocellulosic materials by acid hydrolysis.

Sample	Conditions	k_1 (min^{-1})	k_2 (min^{-1})	k_3 (min^{-1})	k_4 (min^{-1})	k_5 (min^{-1})	References
Sorghum straw	6% HCl, 122°C for 70 min	0.0333	0.0047	0.0204	0.0047	0.1992	Herrera *et al.*, 2003
Sorghum straw	6% H_2SO_4, 100°C for 60 min	0.0798	0.0001	0.0626	0.0129	0.0971	Téllez-Luis *et al.*, 2002
OPEFB*	6% H_2SO_4, 120°C for 15 min	0.1695	0.0057	0.0518	0.0118	0.0189	Rahman *et al.*, 2006
dPPF	5% H_2SO_4, 120°C for 30 min	0.0821	0.0310	0.0297	0.0717	0.0918	This study

* Oil palm empty fruit bunch

3.7.2 Comparison of dPPF hydrolysate and PPF hydrolysate

In order to reduce a cost of raw material pretreatment, xylose yields from both dPPF and PPF were compared. If the xylose concentrations obtained from above materials were not significantly difference, PPF would be selected and used as a substrate for producing xylose. However, amounts of by-products (acetic acid and furfural) have to be considered as well. The experiments were designed by conventional method. Reaction times were controlled at 0, 30 and 60 min. Sulfuric acid was diluted in the range of 1-6% (v/v). The ratio of solid and liquid (PPF or dPPF and diluted sulfuric acid) and reaction temperature were fixed at 1:10 (w/v) and 120°C, respectively. Results are shown in Fig. 31.

For xylose production, the optimal condition for xylose production from both PPF and dPPF was 2% H_2SO_4 and 30 min reaction time at 120°C giving the highest xylose concentration of 27.23 g/l and 28.7 g/l, respectively (Fig. 31a) with the percentage of xylose extraction of 92.78% and 80.23%, respectively. The concentration of sulfuric acid had significantly effected on xylose production ($P \leq 0.05$), but there are no significantly differences between sample (PPF and dPPF), and reaction time (30 min and 60 min) ($P > 0.05$) as shown in Fig. 31a. However, the optimal condition gave the glucose concentration of 2-4 g/l (Fig. 31b).

Analysis of acetic acid, the selected condition of PPF and dPPF hydrolysis gave acetic acid concentrations of 5.99 g/l and 10.56 g/l, respectively, (Table 29 and Fig. 32a). For furfural analysis, the optimal condition of PPF and dPPF hydrolysis also gave furfural concentrations of 0.42 g/l and 0.55 g/l, respectively.

Therefore, PPF was more suitable material than dPPF (Table 29) due to (i): giving the same xylose yield (Fig. 31a); (ii): lower of acetic acid and furfural (Fig. 32a and 32b); (iii): reducing the cost in delignification process and also decreasing environmental emission from the wastes of delignification process (ClO_2 gas and wastewater) (Collings *et al*., 1978).

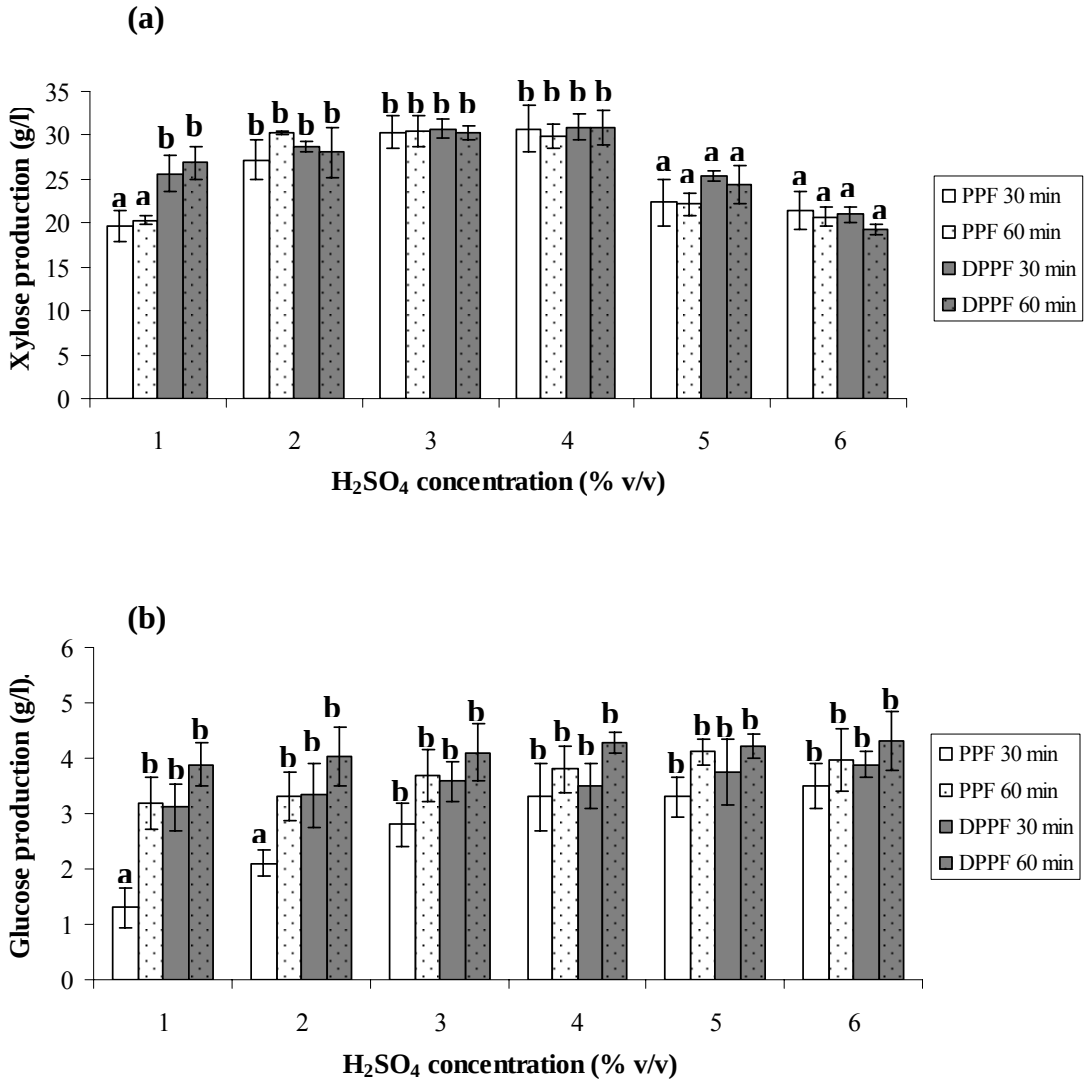


Figure 31. Comparative studies of xylose (a), and glucose productions (b) from PPF and dPPF using various diluted sulfuric acid at 30 and 60 min.
a, b are significantly differences of sugars concentration at $\alpha = 0.05$

Table 29. Comparison of reducing sugars and by-products formation during various acid hydrolysis conditions from lignocellulosic materials.

Raw materials	Conditions	Ratio of solid and liquid (g:g)	Products yields				References
			Xylose (g/l)	Glucose (g/l)	Furfural (g/l)	Acetic acid (g/l)	
1. Oil palm empty fruit bunch	H_2SO_4 4%, 115 °C, 60 min	1:8	30.81	2.2	0.8	2.0	Rahman, *et al.*, 2007
2. Oil palm empty fruit bunch	H_2SO_4 6%, 120 °C, 15 min	1:8	29.4	2.34	0.87	1.25	Rahman, *et al.*, 2006
3. Sorghum straw	HCl 6%, 122 °C, 70 min	1:10	16.2	3.8	2.0	1.9	Herrera, *et al.*, 2003
4. Sorghum straw	H_2SO_4 6%, 100 °C, 60 min	1:10	18.27	6.78	0.7	1.35	Téllez-Luis, *et al.*, 2002
5. Cardboard	H_2SO_4 3%, 130 °C, 180 min	1:10	10.7	9.2	nd	nd	Yáñez, *et al.*, 2004
6. DPPF	H_2SO_4 5%, 120 °C, 30 min	1:10	26.67	2.2	0.65	7.25	This study
7. DPPF	H_2SO_4 2%, 120 °C, 30 min	1:10	28.70	3.5	0.55	10.56	This study
7. PPF	H_2SO_4 2%, 120 °C, 30 min	1:10	27.23	2.3	0.42	5.99	This study

The xylose yields obtained from PPF (27.23 g/l) and dPPF (26.67-28.70 g/l) were similar with oil palm empty fruit bunch (OPEFB) (29.40-30.81 g/l) (Table 29). However, there were differences when compared to sorghum straw and cardboard because of the different composition of each material.

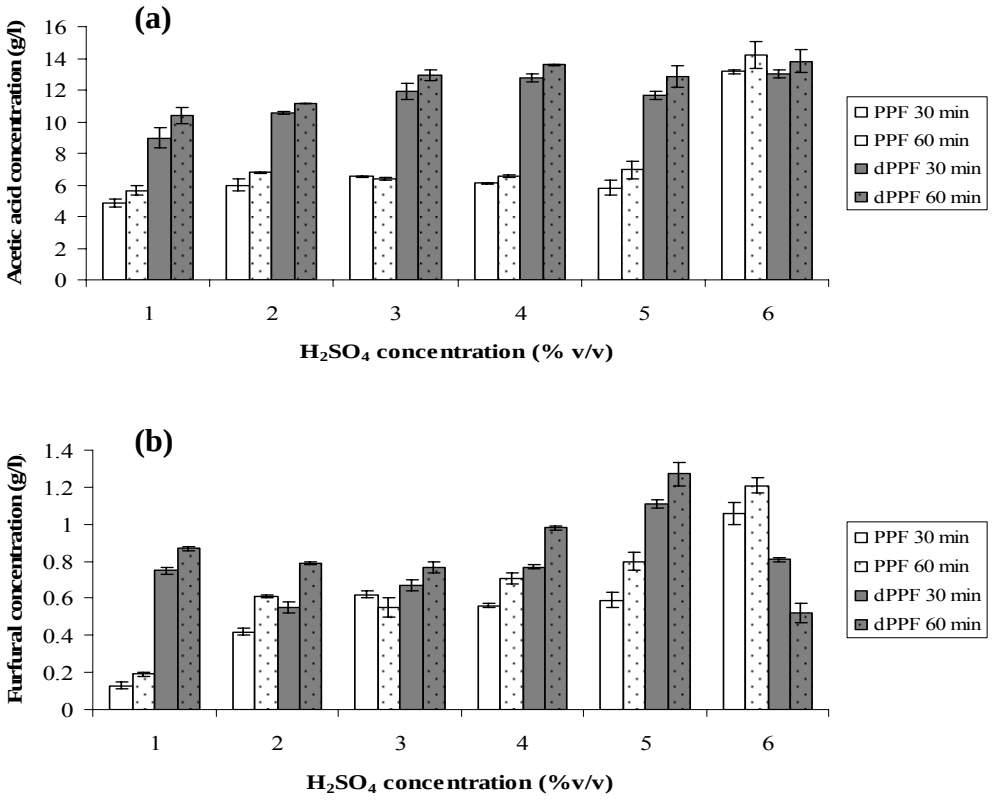


Figure 32. Comparative studies of acetic acid (a) and furfural productions (b) from PPF and dPPF using various diluted sulfuric acid.

3.8 Ethanol production in synthetic glucose or/and xylose medium by *Candida shehatae* TISTR5843

3.8.1 Effect of glucose concentration

Candida shehatae TISTR5843 is effective ethanolic producing yeast that is able to use directly glucose, xylose or glucose and xylose as substrates to produce ethanol. *Candida* species are known as sugar-tolerant yeasts (Petrovska *et al.*, 2000). In order to prevent substrate inhibition, the substrate concentrations were studied and ranged of 4, 7, 12, 24, 45 and 75 g/l, which usually found in cellulosic hydrolysates, and cultured for 96 hours at 30°C on the rotary shaker (180 rpm). The

considerate parameter for ethanol production is ethanol yield, which is a measurement of how much substrate is converted into ethanol. It is well known that 0.51 g ethanol is produced from 1 g glucose. However, the carbon flow in cells is also used for biomass production. Therefore, the theoretical ethanol yield is approximately 0.46-0.48 g ethanol/g glucose (Kopsahelis *et al*., 2007).

Glucose concentrations at 4, 7, 12, 24 and 45 g/l were consumed closely to zero by *C. shehatae* TISTR5843 within 12, 18, 24, 30 and 48 h (Fig. 33b), respectively, giving ethanol yields of 0.31, 0.37, 0.46, 0.45 and 0.37 g ethanol/g glucose and productivities of 0.054, 0.106, 0.222, 0.343 and 0.314 g ethanol/l/h, respectively. However, the glucose concentration of 75 g/l, glucose has been consumed to 22.19 g/l at 48 h cultivation giving ethanol yield and productivity of 0.24 g ethanol/g glucose and 0.290 g ethanol/l/h, respectively. It is demonstrated that the glucose concentrations of 12-24 g/l were suitable for ethanol production due to giving the highest ethanol yield (0.45-0.46 g/g) and productivity (0.343 g ethanol/l/h). However, the ethanol yield and productivity at the glucose concentration of 75 g/l were lower than those of glucose concentration of 45 g/l (Fig. 33a) because of substrate inhibition at the initial fermentation observing from the quirt longer lag phase and product inhibition at the cultivation of 48 h observing from the residual glucose of 22.19 g/l (Fig. 33a).

Acetate concentration of 5, 10 and 15 g/l decreased cell growth and ethanol production of *C. shehatae* ATCC22984 in the range of 5-20% and 20-40%, respectively (Delgenes *et al*., 1996). However, acetate concentration of 1.0 g/l presented at the initial cultivation might be from some of glucose degradation (Qian *et al*., 2005). During fermentation, the pH of cultured broth decreased from 5.0 to 3.5-4.5 because of many acid by-products production as well as carbon dioxide (CO_2) generation during yeast cell growth. The pH affects on alcohol dehydrogenase (ADH) activity (Nie *et al*., 2007). The optimal pH of ADH in this study was 4.5 at 12-18 h (Fig. 33a) (starting point of ethanol production) and Fig. 34b (pH changing to 4.5), which gave the maximum ethanol production (Fig. 33a). This result is similar to those results of Nie et al (2007), who reported the optimal pH of ADH by *Candida parapsilosis* was 4.5. However, the pH was dropped to 3-4 and changed back to closely pH 4.5 after 18 h cultivation time in the glucose concentrations of 4, 7, 12 and

23 g/l (Fig. 34b). After cultivation, the pH of broth at glucose concentrations of 45 and 75 g/l was 3.2 and 2.8, respectively. Cell growths of all glucose concentration experiments were in the stationary phase within 42-48 h (Fig. 34a) with the maximum DCW of 3.46-20.21 g/l.

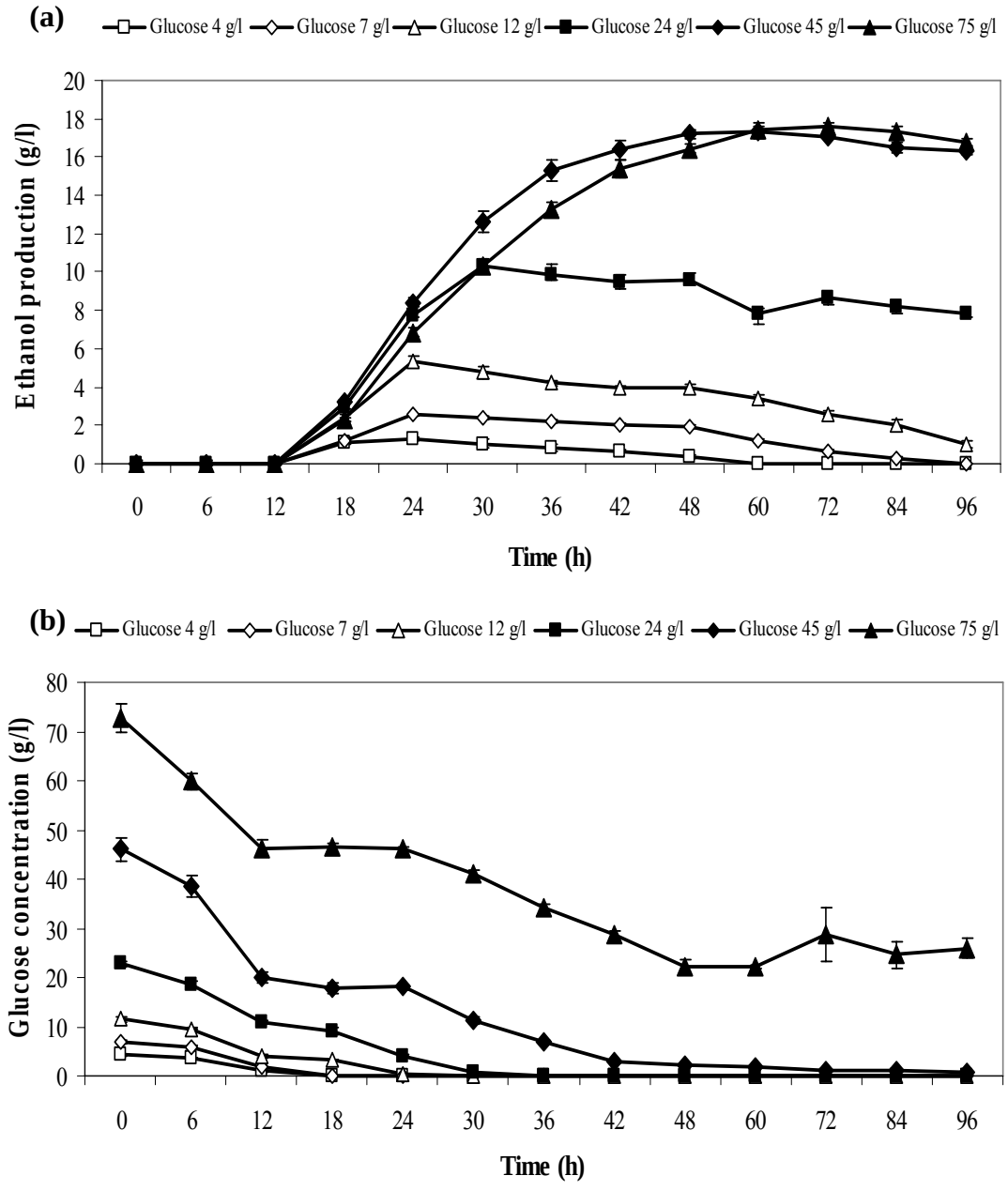


Figure 33. Time course of ethanol production (a) and glucose consumption (b) by *Candida shehatae* TISTR5843 in various glucose concentrations at 180 rpm, room temperature (28-30°C).

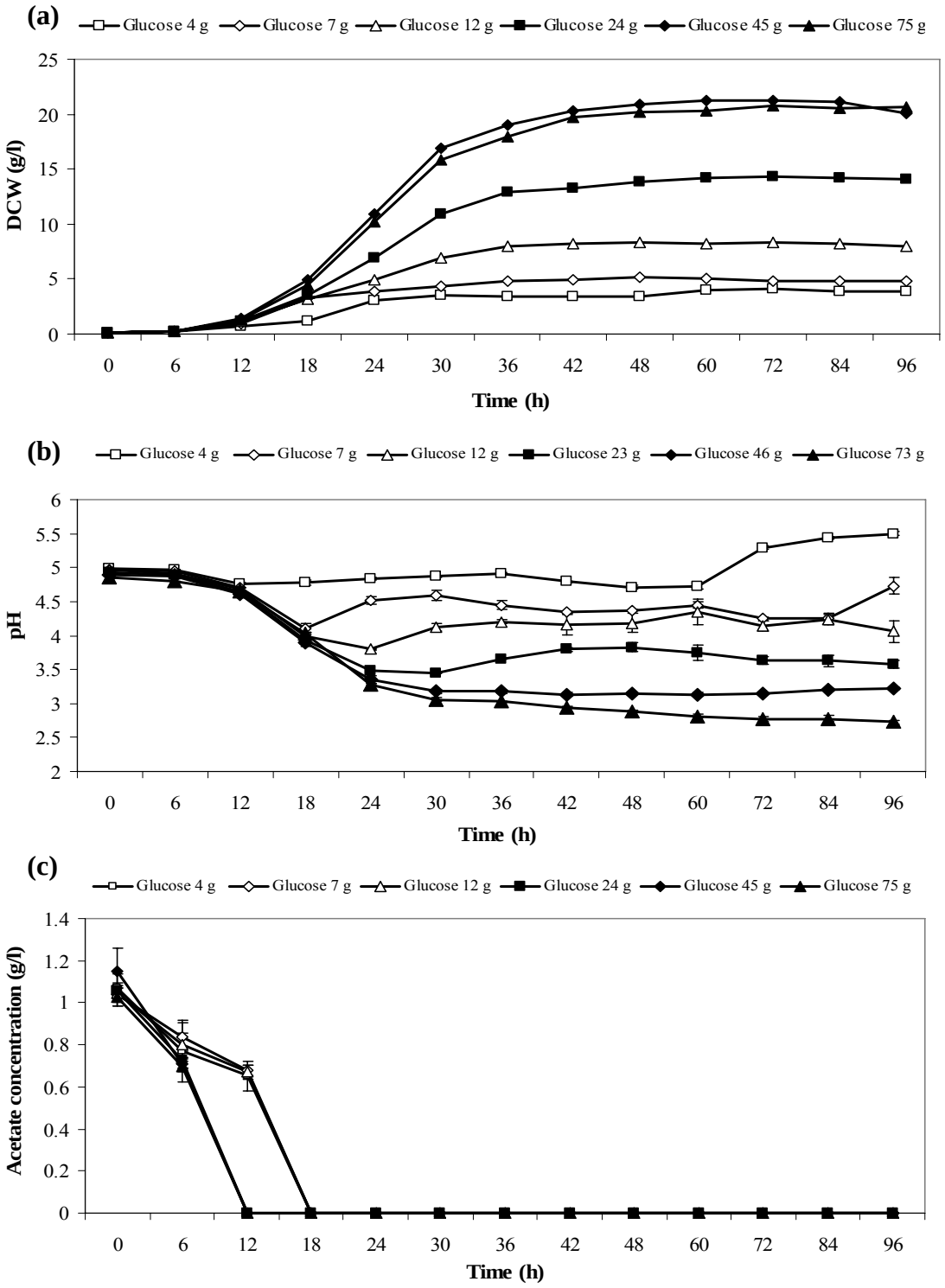


Figure 34. Time course of DCW (a), pH changes (b) and acetate residuals (c) during ethanol production by *Candida shehatae* TISTR5843 in various glucose concentrations at 180 rpm, room temperature (28-30°C).

3.8.2 Effect of xylose concentration

Effect of xylose concentrations on ethanol production was studied at 4, 8, 20, 40, 60 and 90 g/l and cultured with shaking of 180 rpm at room temperature (28-30°C) for 96 h without any furfural and acetate supplementation. Xylose concentrations of 4, 8, 20 and 40 g/l were consumed closely to zero by *C. shehatae* TISTR5843 within 18, 24, 36 and 60 h (Fig. 35b), respectively, giving ethanol yields and productivities of 0.33, 0.45, 0.42 and 0.29 g ethanol/g xylose and 0.058, 0.122, 0.194 and 0.186 g ethanol/l/h, respectively. It is demonstrated that, the xylose concentrations of 8 g/l gave the highest ethanol yield while the xylose concentration of 20 g/l gave the highest productivity. The xylose concentrations of 60 and 90 g/l could be also used to produce ethanol (13-14 g/l) as shown in Fig. 35a. However, they gave lower ethanol yield (0.22 and 0.16 g ethanol/g xylose, respectively) and lower productivity (0.138 and 0.146 g ethanol/l/h, respectively) than glucose concentration of 20 g/l because of (i) substrate inhibition at the initial fermentation, (ii) product inhibition at the cultivation time of 72 h observing from the residual glucose of 10 and 40 g/l (Fig. 35b), and (iii) by-products inhibition (Fig. 36a and 36b).

Furfural presented in this study could be produced from xylose degradation under autoclave (120°C for 10 min). Unfortunately, acetate also presented. Acetate was consumed within 18 h cultivation because it is less toxic than furfural (Fig. 36b). Furfural concentration less than 0.5 and 1.0 g/l was transformed completely to another form by using alcohol dehydrogenase-coupled with $NADH+H^+$ for furfuryl alcohol formation, and by using aldehyde dehydrogenase (AIDH)-coupled with NAD^+ for furoic acid formation (Modig *et al*., 2002; Zhao *et al*., 2005), within 18 and 84 h cultivation, respectively. While furfural concentration of 2.3 g/l decreased to 1.0 g/l after 84 h cultivation (Fig. 36a). This transformation is called "detoxification" (Keating *et al*., 2006). The equation of transformation of furfural to furoic acid and furfuryl alcohol was shown in equation (35) and (36), respectively (Modig *et al*., 2002);

$$Furfural + NAD^+ \xrightarrow{AIDH} furoic\ acid + NADH + H^+ \quad (35)$$

$$Furfural + NADH + H^+ \xrightarrow{ADH} furfuryl\ alcohol + NAD^+ \quad (36)$$

Moreover, the present of furfural inhibits the cell growth and ethanol production. Furfural concentrations of 0.5, 1.0 and 2.0 g/l could decrease the cell growth and ethanol production of *C. shehatae* ATCC22984 in the range of 20-90% (Delgenes *et al*., 1996). Furthermore, the transformation of furfural to the less toxic compounds is based on amount of air presented during fermentation. Horváth *et al* (2003) reported that under respiratory metabolism of *Saccharomyces cerevisiae* CBS8066 furfural is converted completely to furoic acid whereas furfural is converted to furfuryl alcohol (analyzed in section 3.10) under anaerobic condition.

For the pH change during fermentation, the pH dropped to 3-4 and changed to closely pH 4.5 after 24 h cultivation in the xylose concentrations of 4 and 8 g/l (Fig. 37b) whereas the pH of the xylose concentrations of 20-90 g/l dropped to 2.75-3.5 and no changing to pH 4.5 (Fig. 37b). These phenomena might be from many acid by-products production as well as carbon dioxide (CO_2) generation during yeast cell growth. For determination of DCW during cultivation, cell growth of among xylose concentrations of 4, 8 and 20 g/l was in the stationary phase within 42 h (Fig. 37a), while the stationary phase of cell growth of 40, 60 and 90 g/l xylose was 48 h cultivation, respectively (Fig. 37a).

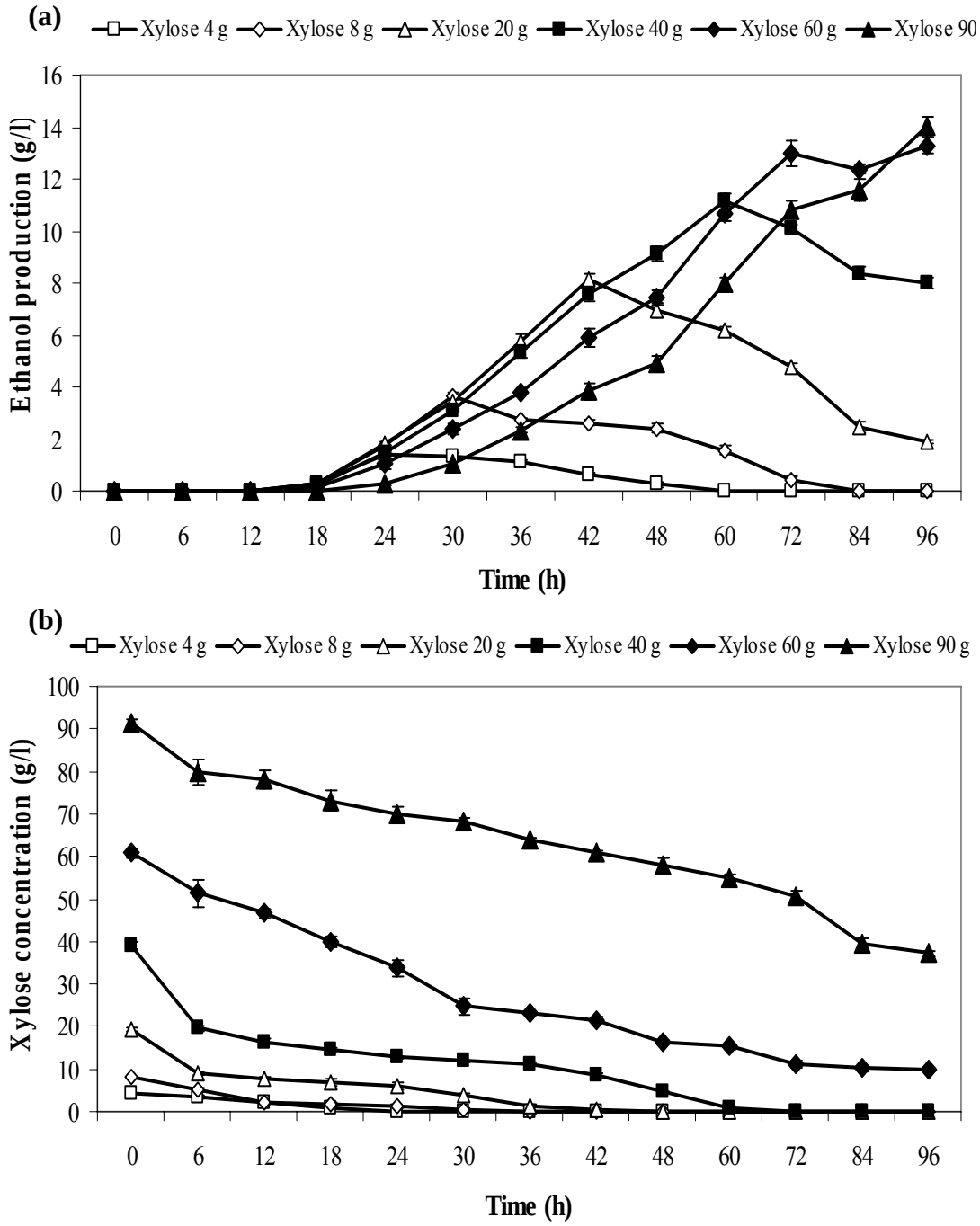


Figure 35. Time course of ethanol production (a) and xylose consumption (b) by *Candida shehatae* TISTR5843 in various xylose concentrations at 180 rpm, room temperature (28-30°C).

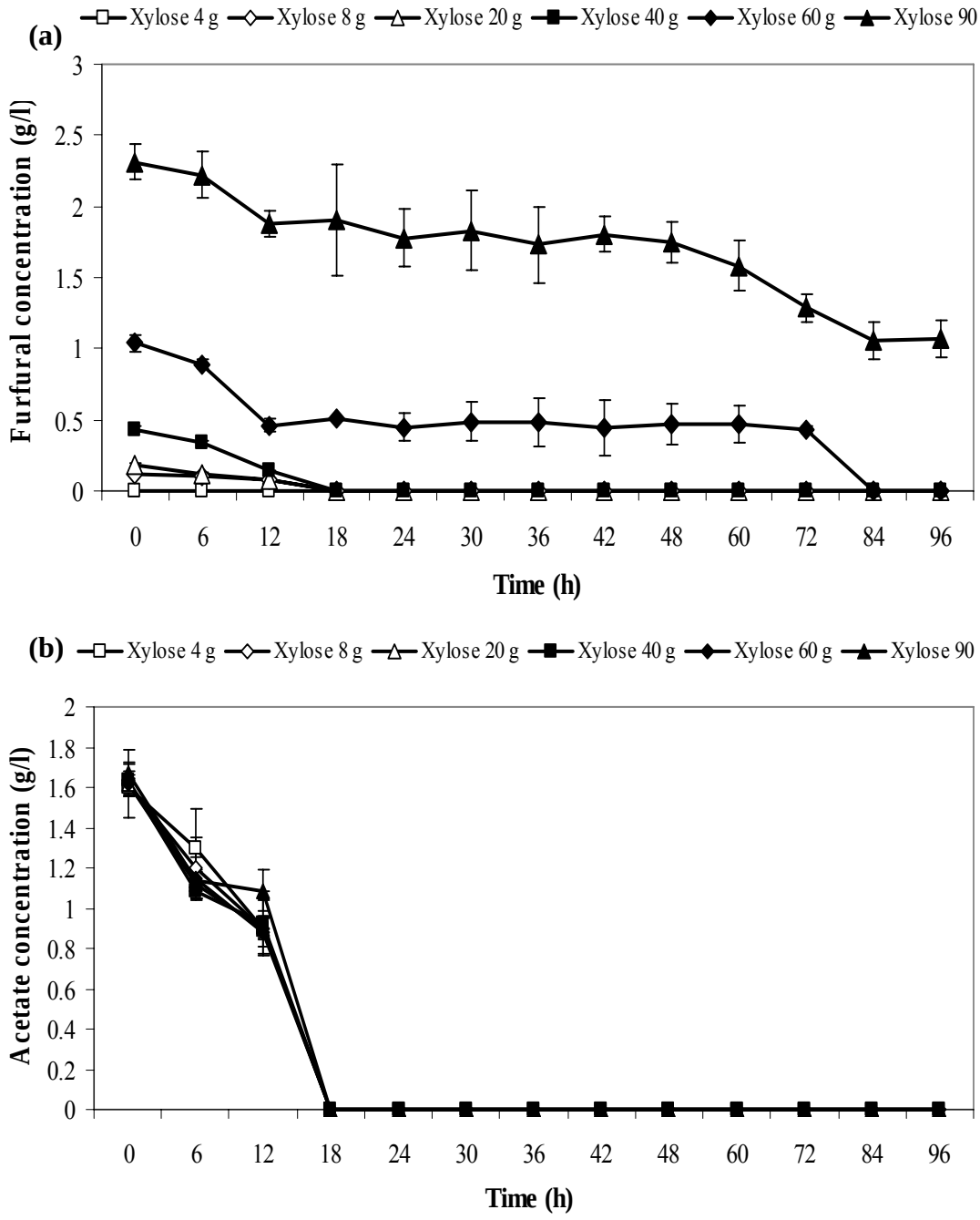


Figure 36. Time course of furfural residuals (a) and acetic acid residuals (b) in ethanol production by *Candida shehatae* TISTR5843 in various xylose concentrations at 180 rpm, room temperature (28-30°C).

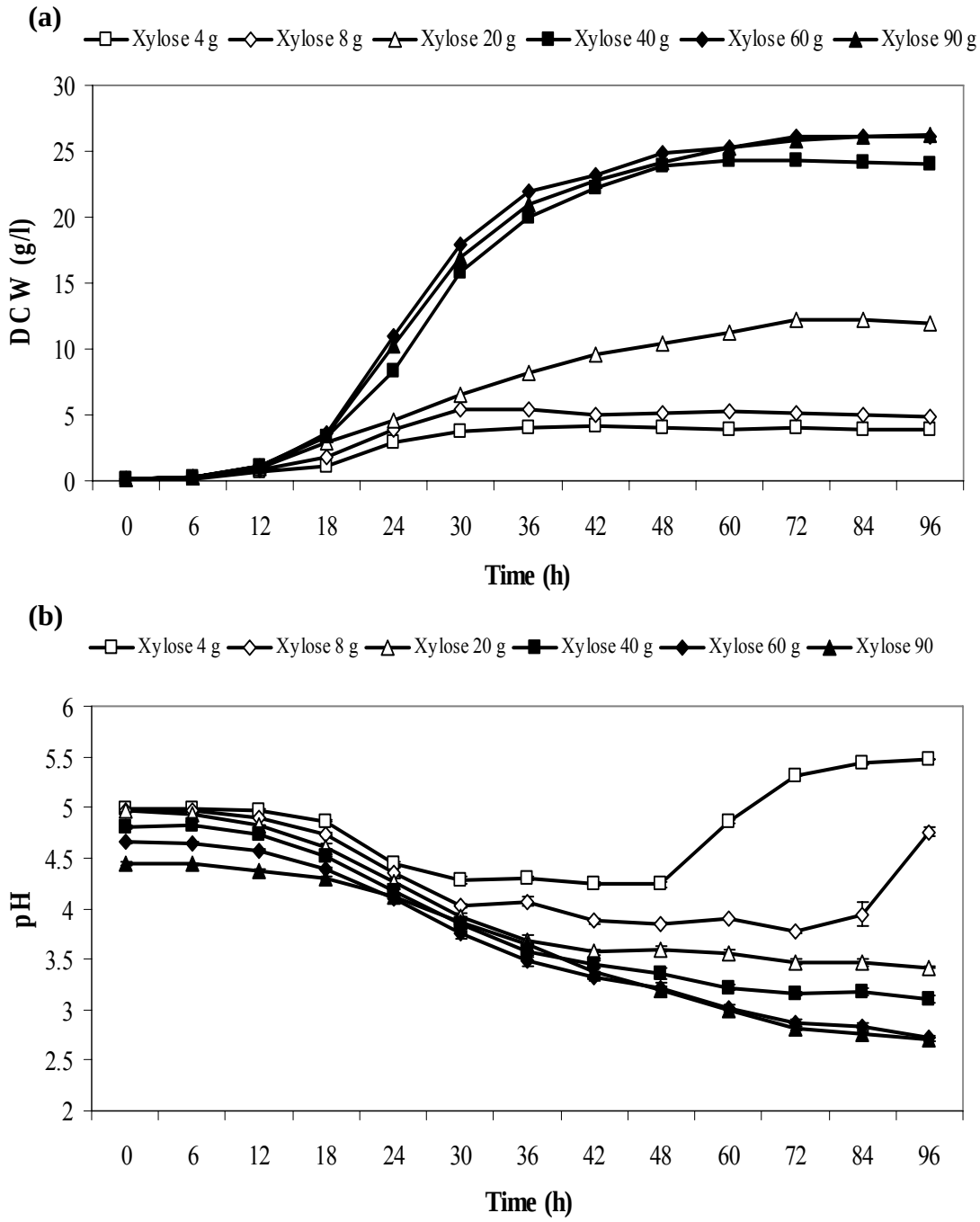


Figure 37. Time course of DCW (a) and pH changes (b) during ethanol production by *Candida shehatae* TISTR5843 in various xylose concentrations at 180 rpm, room temperature (28-30°C).

3.8.3 Effect of glucose to xylose ratio

C. shehatae TISTR5843 is ethanolic producing yeast that is able to use directly glucose and xylose as substrates to produce ethanol (Delgenes *et al*., 1996). Mixture of both monomerric sugars is an effective process to generate ethanol. The optimal glucose to xylose ratio, therefore, is a necessary parameter and need to be optimized. In these experiments, various glucose to xylose ratios were ranged from 10:0, 8:2, 6:4, 5:5, 4:6, 2:8 to 0:10 (w/w) (10 g/l total sugars) and cultured for 72 h at 30°C with shaking speed of 180 rpm. Results demonstrated that both sugars at all glucose to xylose ratios were consumed close to zero within 30 h (Fig. 38b), giving ethanol yields of 0.45, 0.43, 0.44, 0.44, 0.43, 0.43 and 0.42 g ethanol/g sugar while the productivities were 0.175, 0.178, 0.178, 0.174, 0.139, 0.111 and 0.109 g ethanol/l/h, respectively. It is illustrated that, the glucose to xylose ratios of 10:0, 8:2, 6:4, 5:5 and 4:6 g/l were the optimum ratios of glucose to xylose because of giving the highest ethanol yields (0.43-0.45 g ethanol/g sugar) and the highest ethanol productivities (0.139-0.178 g/l/h).

The ethanol yields and ethanol productivities decreased when the furfural generated after sterilization increased (Fig. 39a). However, furfural concentrations could be transformed to lower toxic compound within 24 h (Fig. 39a).The highest furfural generation was obtained from the glucose to xylose ratio of 0:10 (w/w). The acetate was consumed by *C. shehatae* TISTR5843 within 30 h cultivation time (Fig. 39b).

The pH of all glucose to xylose ratios dropped to 3.9-4.1 and then changed closely to pH 4.5 after 42 h cultivation. The ethanol production was maximum when pH changed to 4.5 due to its optimum pH for alcohol dehydrogenase (ADH) (Banerjee *et al*., 1981) (Fig. 40b). However, ethanol concentration of all experiments decreased after 42 h cultivation time because of alcohol dehydrogenaseII (ADH2) (Banerjee *et al*., 1981). ADH2 is an enzyme that is able to use ethanol as a substrate to covert back to acetyldehyde and then converted to acetate for producing energy and reducing power in the cell under limitation of substrate and higher ethanol concentration in broth (Banerjee *et al*., 1981). For DCW determination, cell growth of all glucose to xylose ratios would reach stationary phase within 42 h (Fig. 40a).

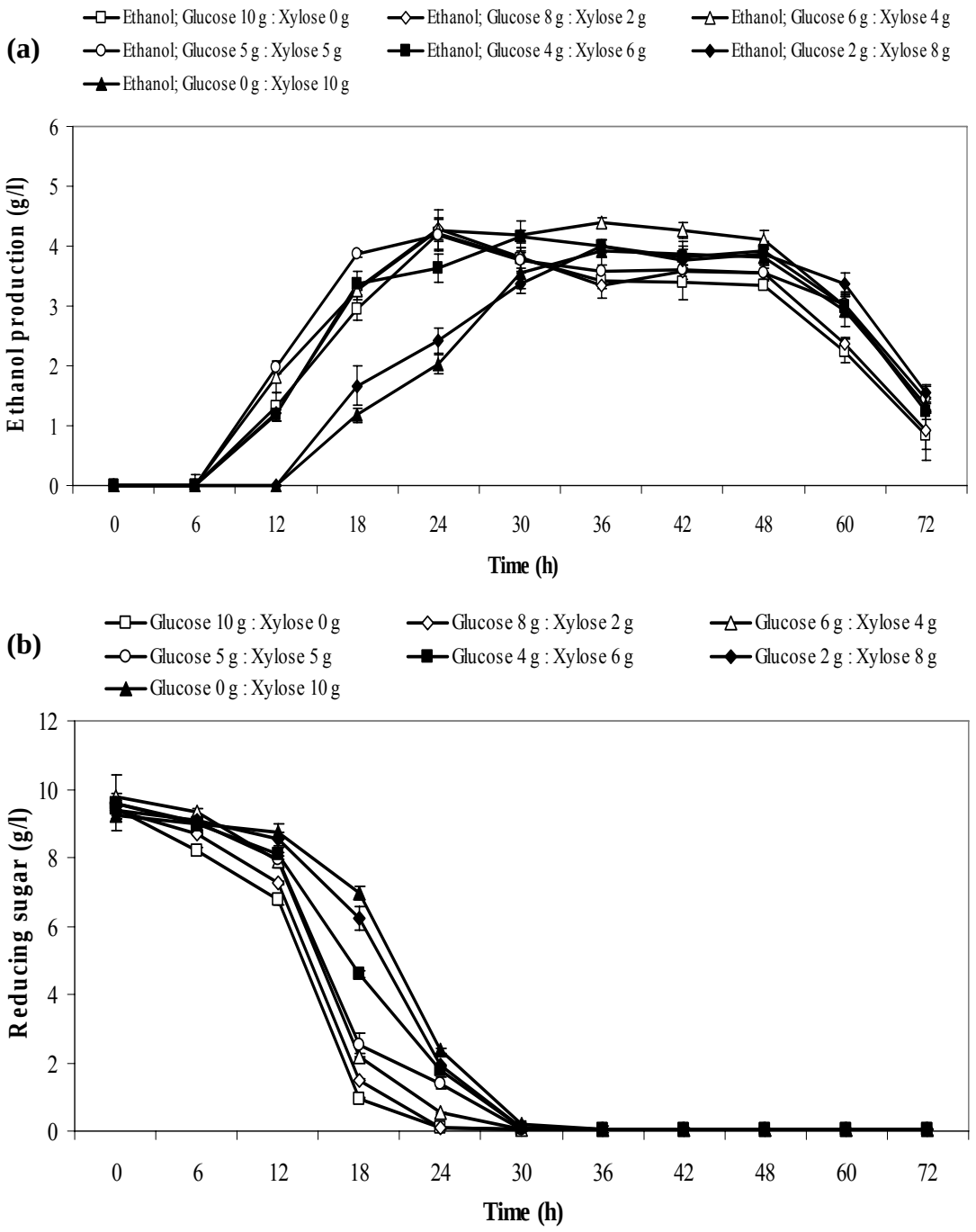


Figure 38. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in various glucose to xylose ratios at 180 rpm, room temperature (28-30°C).

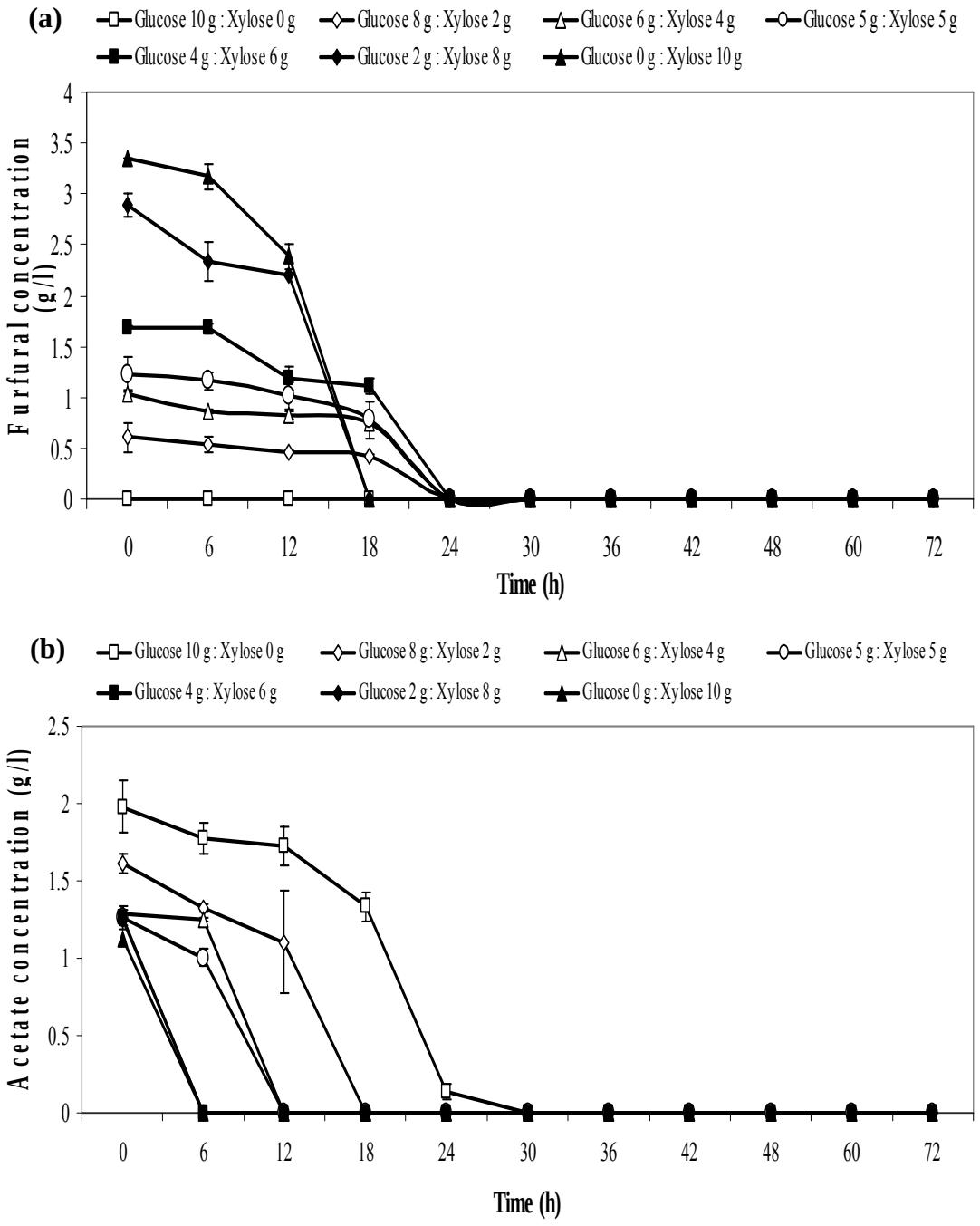


Figure 39. Time course of furfural (a) and acetic acid (b) concentration during ethanol production by *Candida shehatae* TISTR5843 in various glucose to xylose ratios at 180 rpm, room temperature (28-30°C).

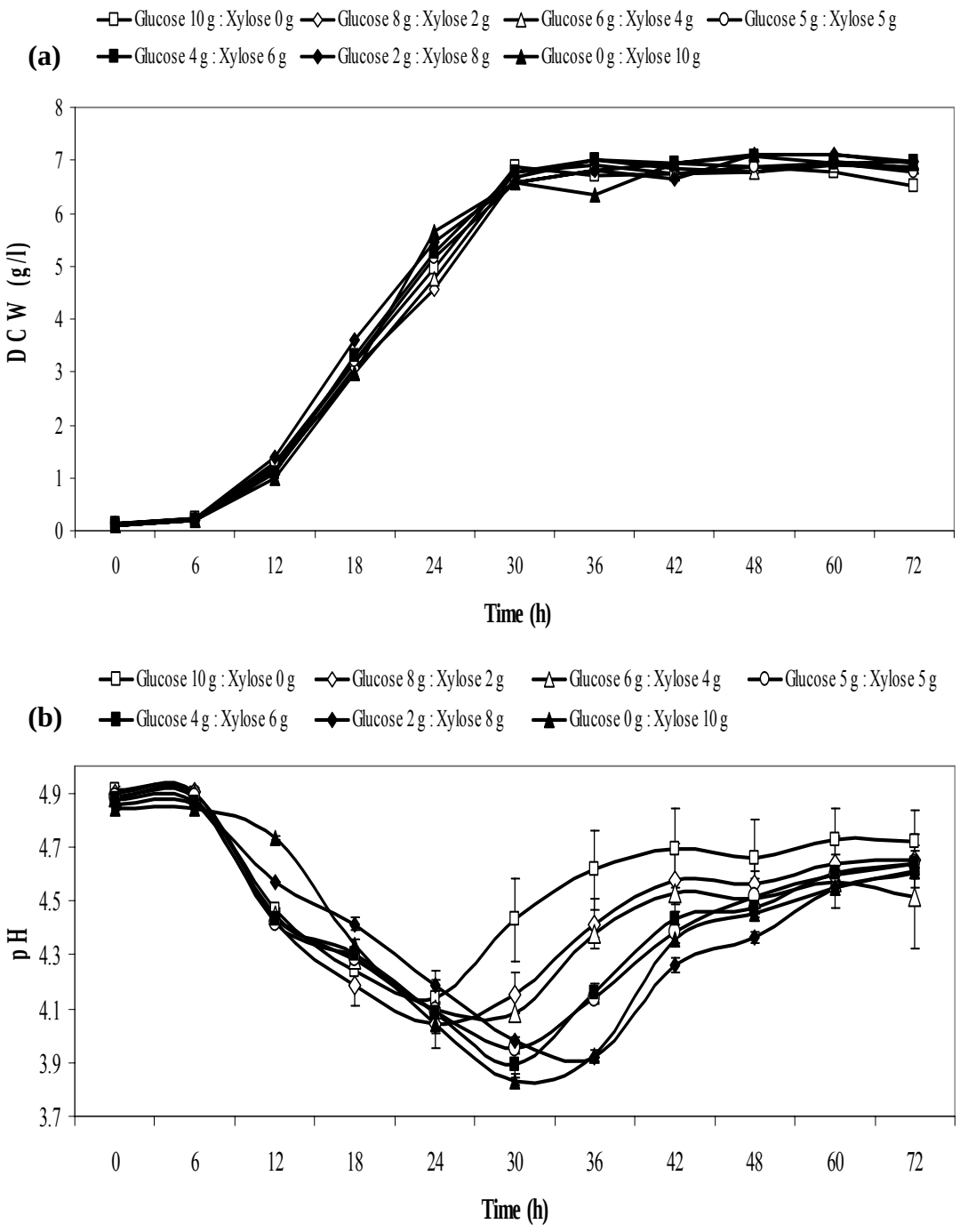


Figure 40. Time course of DCW (a) and pH changes (b) during ethanol production by *Candida shehatae* TISTR5843 in various glucose to xylose ratios at 180 rpm, room temperature (28-30°C).

3.8.4 Effect of initial pH

pH of the medium plays important role in cell growth and ethanol production. The several of initial pH of medium were ranged of 3.0-6.0. Fermentation was carried out in synthetic medium containing glucose to xylose ratios of 2:8 (w/w) which is the same sugars ratio presented in PPF hydrolysate (Section 3.10). The incubation conditions were 180 rpm at room temperature (30°C) for 72 h.

At initial pH of 3.0, 4.0, 4.5, 5.0 and 6.0, the ethanol yields and ethanol productivities were 0.35, 0.36, 0.44, 0.45 and 0.34 g ethanol/g sugar and 0.052, 0.089, 0.134, 0.136 and 0.081 g/l/h, respectively. Results indicated that initial pH of 4.5-5.0 was optimum for ethanol production and cells growth because it gave the highest ethanol concentration (3.22-3.27 g/l), the highest ethanol yield (0.44-0.45 g ethanol/g sugar), the highest ethanol productivity (0.134-0.136 g/l/h) as shown in Fig. 41a, and as well as the highest DCW (7.01-7.11 g/l) as shown in Fig. 42b. The fermentation of *C. shehatae* FPLY-049 in wood hydrolysate, the maximum ethanol production was found at pH 6.0 with completely fermented within 48 h and had low acetate content (Sreenath and Jeffries, 2000). All sugars in culture medium were consumed almost completely at 48 h cultivation (Fig. 41b).

The initial pH of 4.5 gave the highest and the fastest of cells growth observed from increase of DCW (Fig. 42b). The initial pH of 4.0, 4.5 and 5.0 slightly decreased within 24 h and then slightly increased to nearly pH 4.5 (Fig. 42c). There was no acetate formation whereas 0.16-0.17 g/l furfural was detected and it could be converted to the less toxic compounds such as furfuryl alcohol and furoic acid (described in section 3.8.2) within 30-36 h (Fig. 42a).

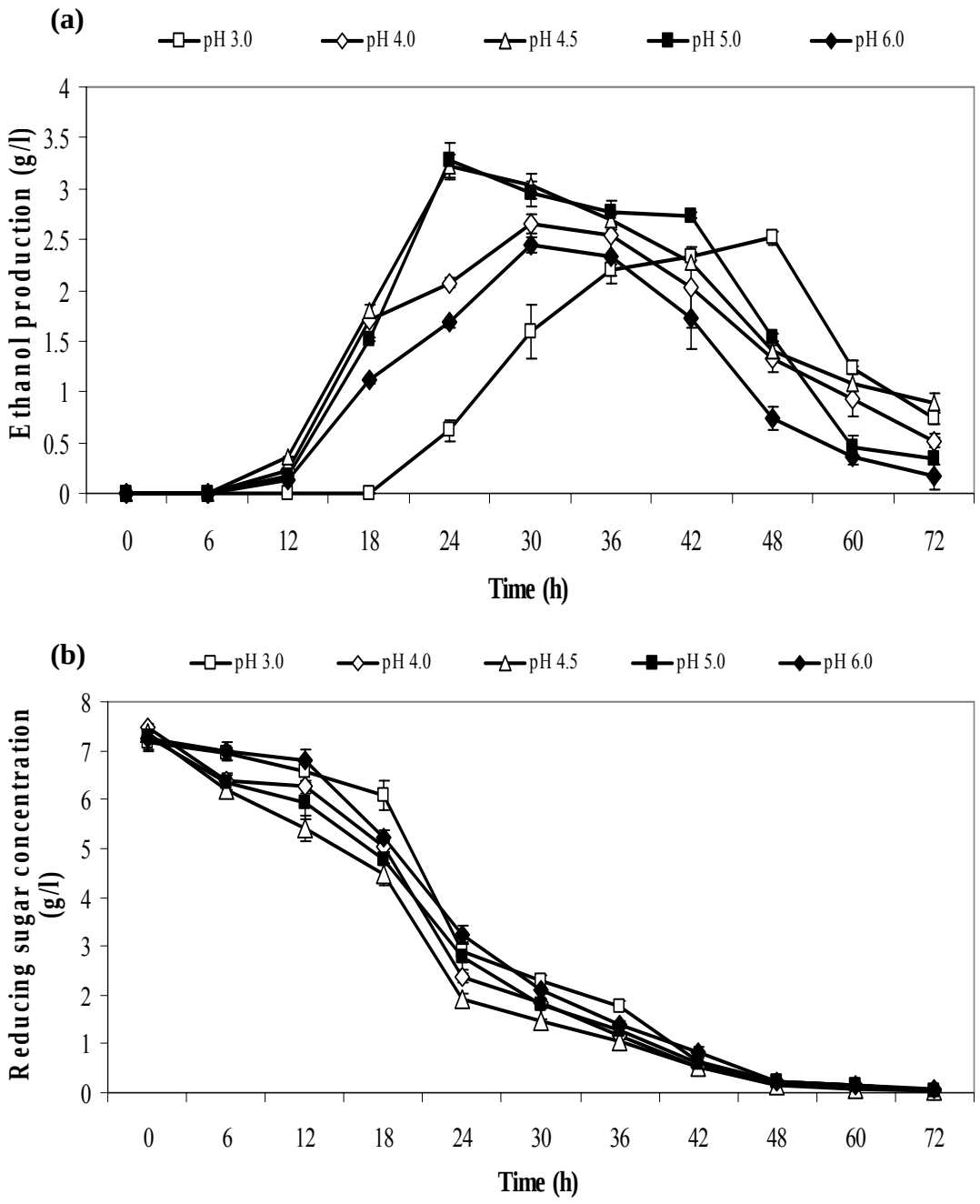


Figure 41. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in various initial pH (3.0-6.0) under 2:8 (w/w) of glucose to xylose ratios at 180 rpm, room temperature (30°C).

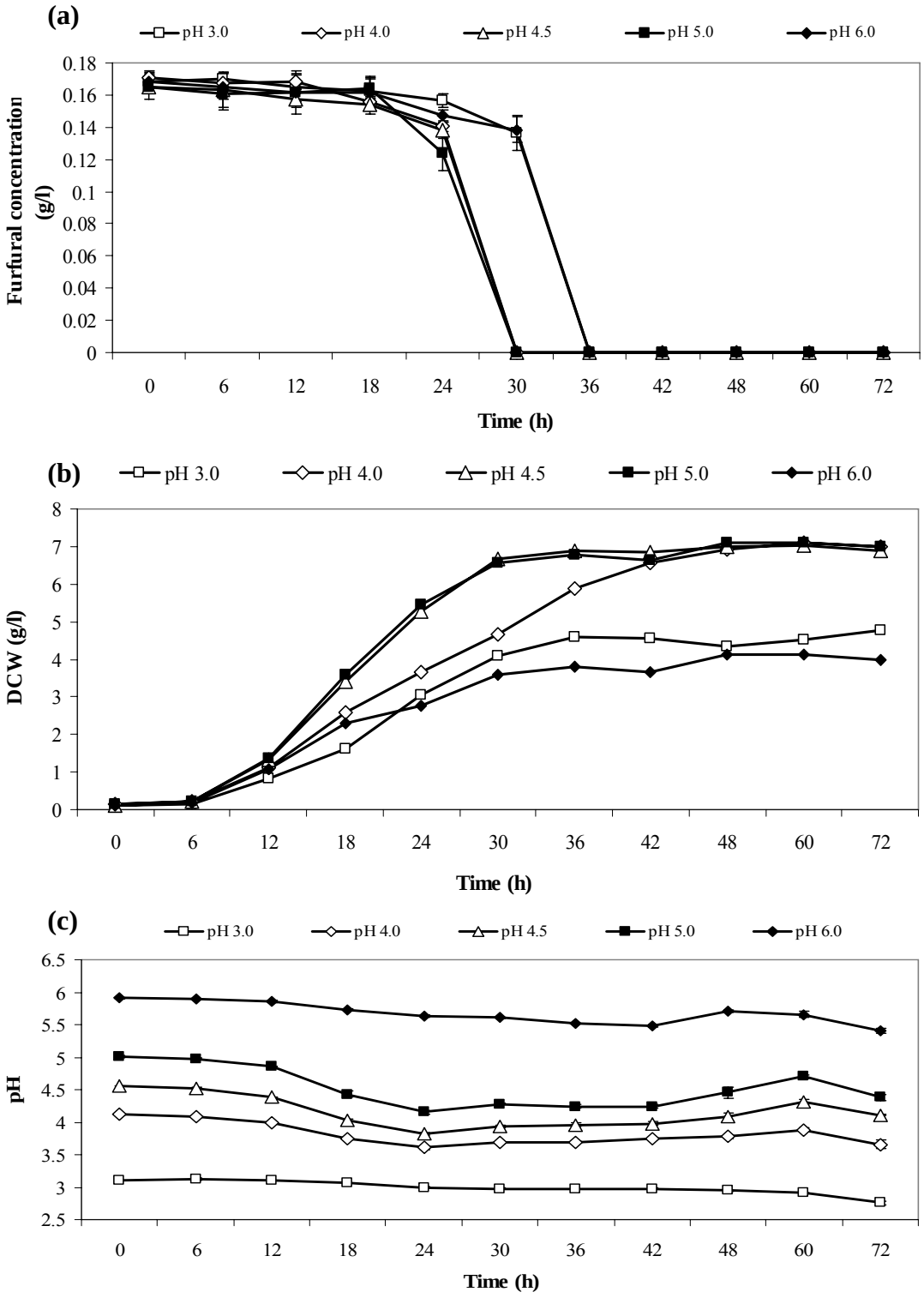


Figure 42. Time course of furfural concentrations (a), DCW (b) and pH changes (c) during ethanol production by *Candida shehatae* TISTR5843 in various initial pH (3.0-6.0) under 2:8 (w/w) of glucose to xylose ratios at 180 rpm, room temperature (30°C).

3.8.5 Effect of temperature

Temperature for cultivating *C. shehatae* TISTR5843 is an important factor concerning metabolism of cells and enzymes activities. At 35°C incubation, no cell growth (Fig. 43b) was detected with reveal to less sugar consumption (Fig. 44b). At room temperature (30±2°C), 3 g/l ethanol was produced at 30 h cultivation (Fig. 43a) giving ethanol yield of 0.42 g ethanol/g substrate and ethanol productivity of 0.103 g/l/h.

Acetate was consumed by *C. shehatae* TISTR5843 within 18 h cultivation (Fig. 44a) and furfural was transformed to lower toxic compound within 24 h (Fig. 44a) at room temperature. No consumptions of acetate and no transformation of furfural were also detected at 35°C (Fig. 44a).

The pH was changed between 4.0-4.5 at the room temperature (Fig. 44b) whereas no pH change was appeared under 35°C due to no growth. In additionally, temperature had no effect on any enzyme level over the range of 20-30°C (Alexander *et al*., 1988). However, when the temperature is higher than 30°C, enzyme activity reduced. The stationary phase of cells growth at room temperature was 42 h (Fig. 44b).

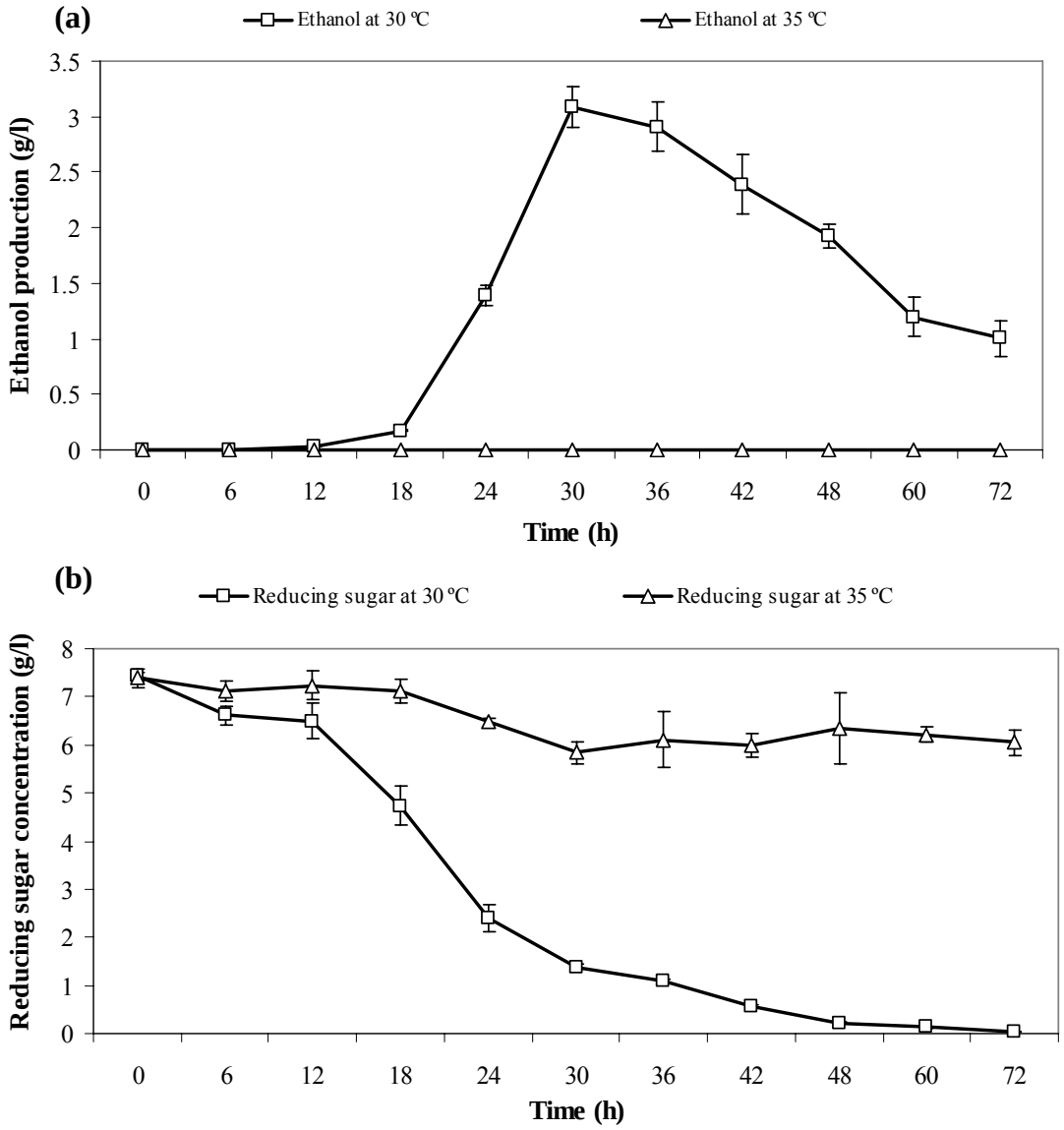


Figure 43. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in 2:8 (w/w) of C6 to C5 ratios at 180 rpm, pH 5.0 under 30°C and 35°C.

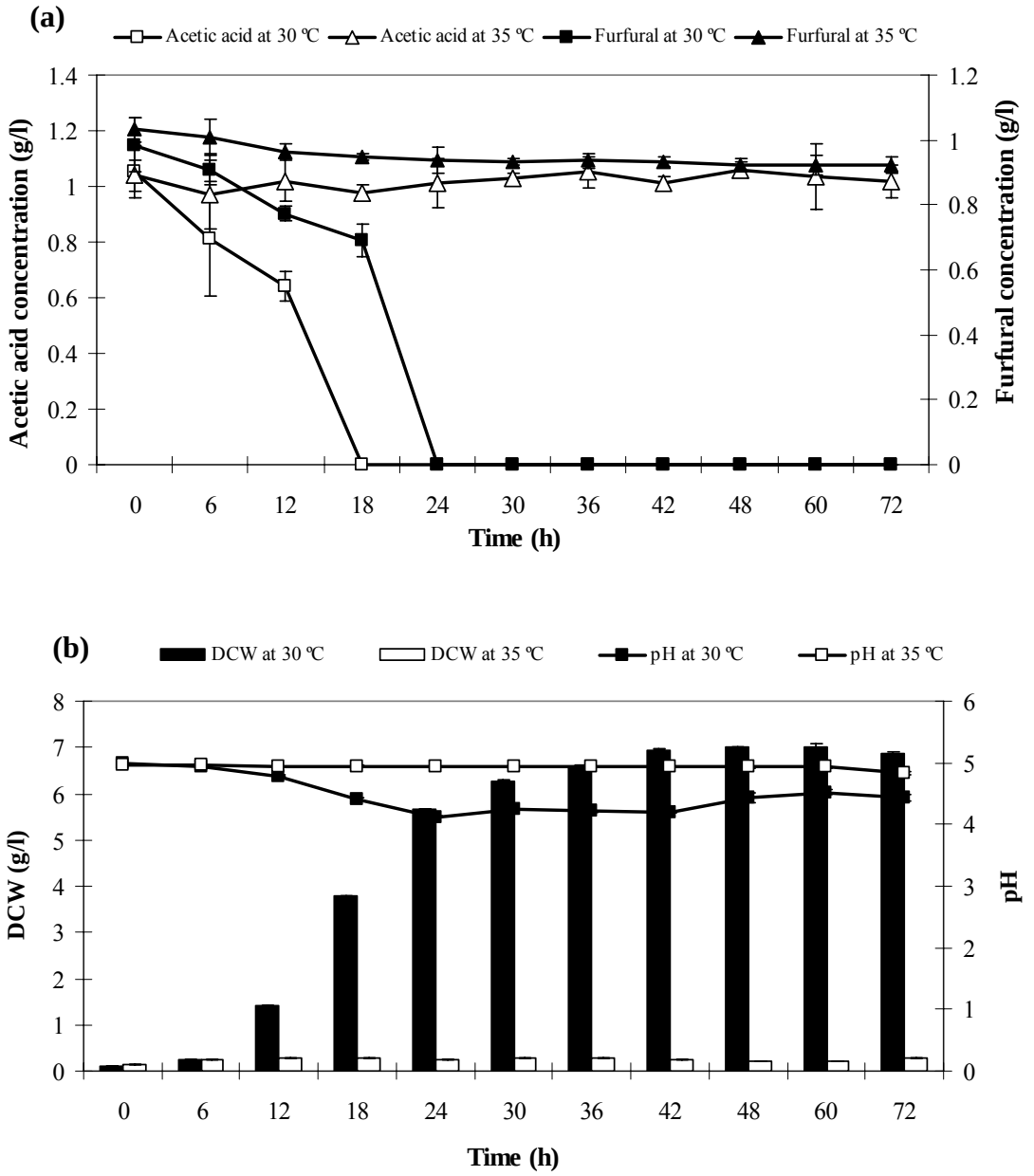


Figure 44. Time course of acetic acid and furfural concentrations (a) DCW and pH changes (b) during ethanol production by *Candida shehatae* TISTR5843 in 2:8 (w/w) of glucose to xylose ratios at 180 rpm, pH 5.0 under room temperature (30°C) and 35 °C.

3.8.6 Effect of shaking speed

Effect of shaking speed at 60, 120, 180 and 240 round per minute (rpm) was studied. Cultivation was conducted in the synthetic medium containing 2:8 (w/w) of glucose to xylose ratios with the initial pH of 5 at room temperature (30°C) for 72 h.

Ethanol yields and ethanol productivities of this factor were 0.35, 0.39, 0.43 and 0.40 g ethanol/g sugar, and 0.051, 0.091, 0.106 and 0.071 g/l/h, respectively. The results clearly indicated that at the optimum shaking speed was 180 rpm, giving the highest ethanol concentration of 3.17 g/l, the highest ethanol yield and productivity, as well as the fastest ethanol production (within 30 h cultivation) (Fig. 45a). Sugar consumption under various shaking speed of 120, 180 and 240 rpm were closely to zero within 48 h whereas the sugar consumption under shaking speed of 60 rpm slightly decreased during cultivation because *C. shehatae* TISTR5843 required more oxygen for cell growth (Fig. 45b) (Delgenes *et al*., 1996).

Furfural concentration of 0.16 g/l presented in the culture medium was produced by xylose degradation after sterilization at 110°C for 15 min. Furfural was converted to the less toxic compounds within 30 h under shaking speed of 120, 180 and 240 rpm while furfural was transformed at 60 h cultivation at shaking speed of 60 rpm (Fig. 46a). For pH investigation, pH slightly decreased within 30-36 h and then slightly increased to pH 3.9-4.2 thereafter in all shaking speed (Fig. 46c). However, at the shaking speed of 240 rpm gave the highest cells growth (Fig. 46b) and the lowest ethanol production (Fig. 45a) because of high oxygen resulted in high metabolism of cells growth (Jeffries and Alexander, 2000). These results are similar with the experiments of Prior *et al*. (1988). When the oxygen supply to an aerobic condition of *C. shehatae* was reduced to oxygen-limited and anoxic conditions, the accumulation of ethanol and the specific activity of alcohol dehydrogenase increased upto 4-folds (Prior *et al*., 1988). Additionally, Jeffries and Alexander (2000) suggested that at low level of aeration must be maintained to obtain good xylose fermentation and growth. However, too much oxygen supply is detrimental because xylose-fermenting yeast appears to both produce and consume ethanol at the same time.

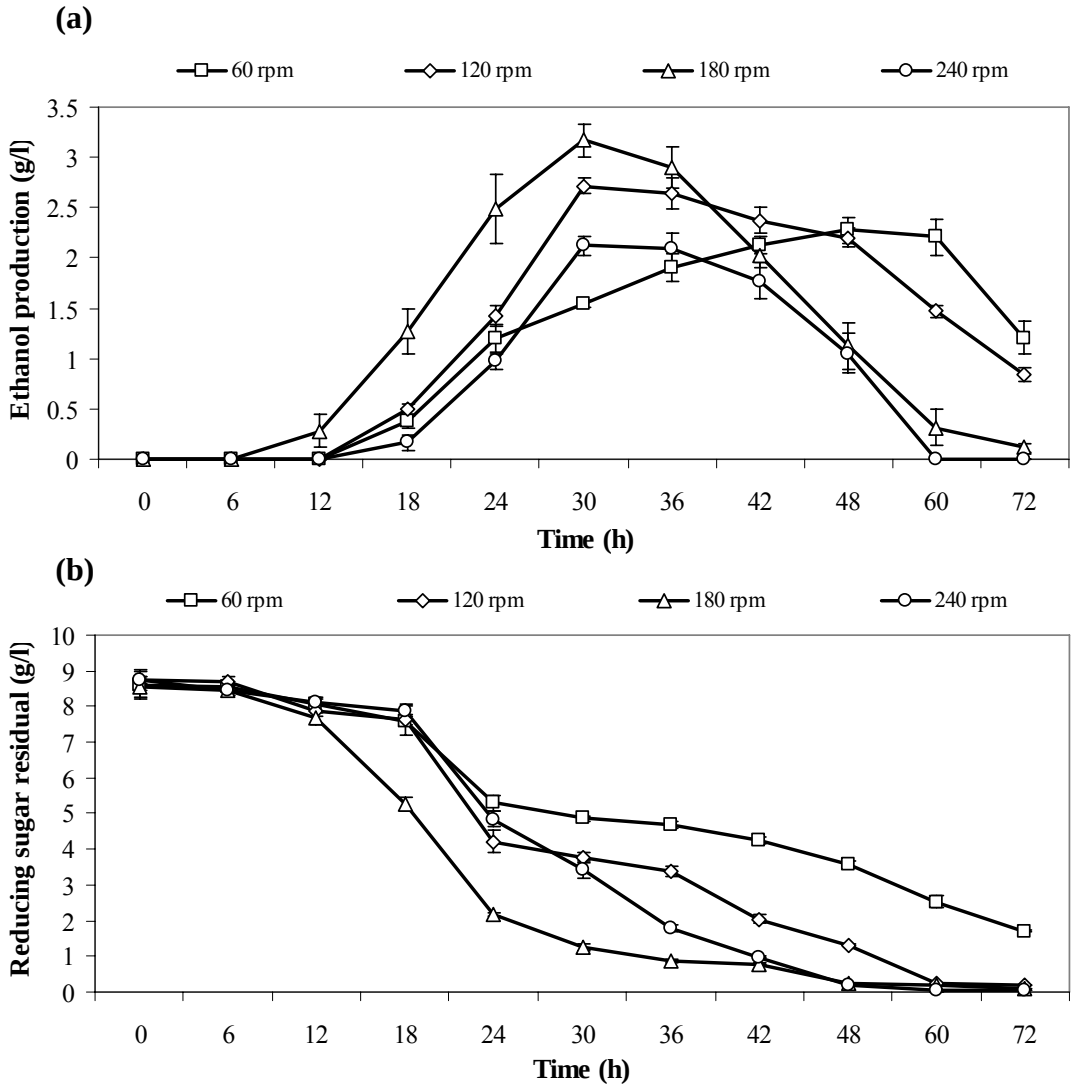


Figure 45. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in various shaking speed in synthetic medium containing 2:8 (w/w) of glucose to xylose ratios, pH 5 at room temperature (30°C).

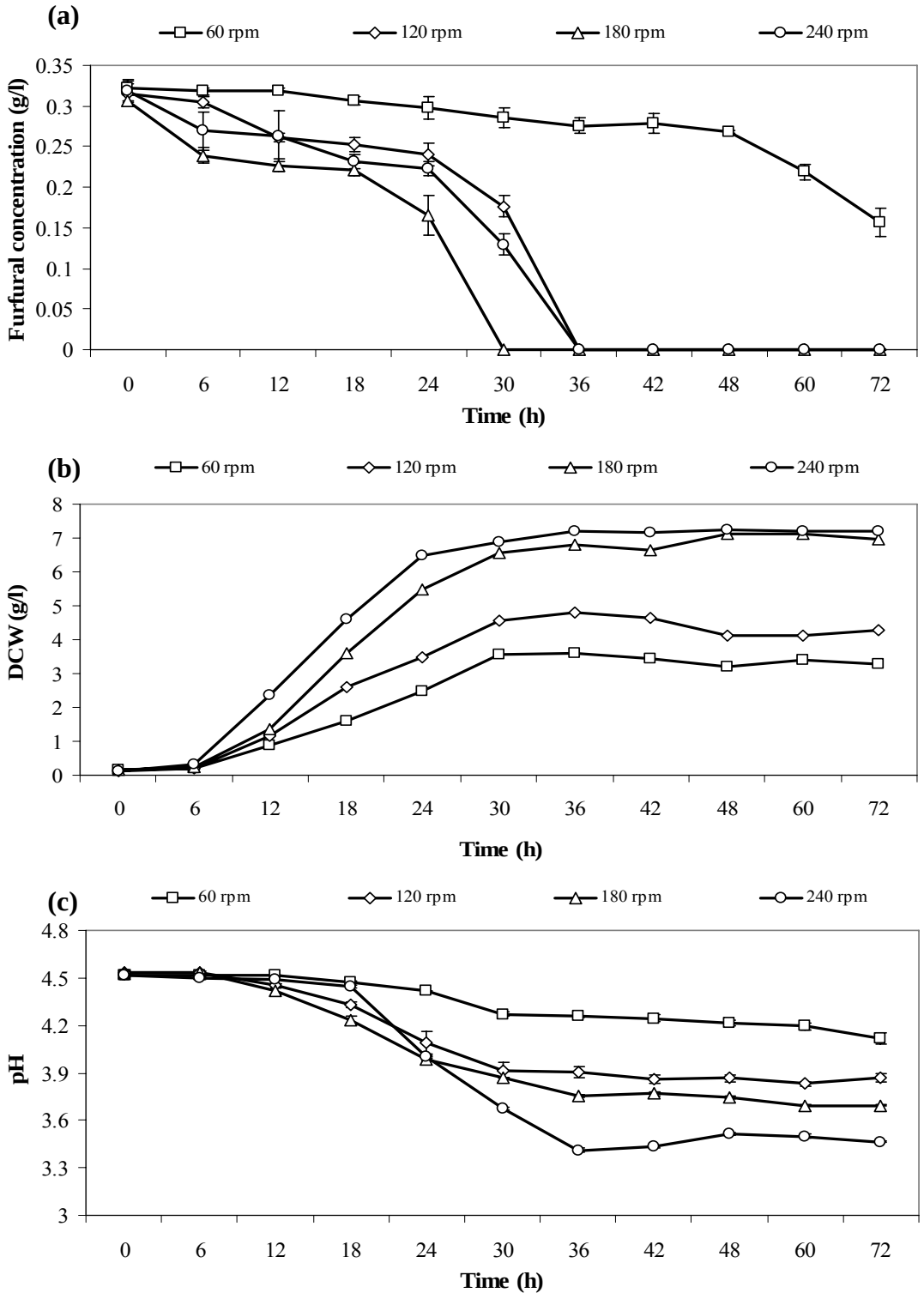


Figure 46. Time course of furfural concentrations (a), DCW (b) and pH changes (c) during ethanol production by *Candida shehatae* TISTR5843 in various shaking speed in synthetic medium containing 2:8 (w/w) of glucose to xylose ratios, pH 5 at room temperature (30°C).

The ethanol yields, ethanol productivities and ethanol concentrations at the optimum condition are summarized in Table 30. When cultured the yeast cell in the synthetic medium containing glucose as a sole carbon source, the optimum concentration was 24 g/l. However, when cultured the yeast cell in the synthetic medium containing xylose as a sole carbon source, the optimum concentration was 20 g/l. The co-substrate, glucose and xylose were studied. The optimum glucose to xylose ratio in the medium was 2:8 w/w with initial pH of 5. Incubation conditions were achieved at room temperature (30°C) with the shaking speed of 180 rpm. The highest ethanol yields, ethanol productivities and ethanol concentrations at optimum condition were 0.39-0.43 g ethanol/ g sugar, 0.103-0.330 g/l/h and 0.37-1.25%, respectively.

Table 30. Comparisons of ethanol yields, ethanol productivities and ethanol percentages by *Candida shehatae* TISTR5843 in synthetic medium at the optimum parameters affecting on ethanol production.

Factors		Ethanol yield (g ethanol/g sugar)	Ethanol productivity (g/l/h)	Ethanol concentration (g/l)
1. Glucose concentration	4 g/l	0.31	0.054	1.29
	7 g/l	0.37	0.106	2.55
	12 g/l	0.46	0.221	5.32
	24 g/l	0.45	0.343	10.29
	45 g/l	0.37	0.359	17.22
	75 g/l	0.24	0.290	17.40
2. Xylose concentration	4 g/l	0.33	0.058	1.39
	8 g/l	0.45	0.122	3.67
	20 g/l	0.42	0.194	8.14
	40 g/l	0.29	0.186	11.15
	60 g/l	0.22	0.138	13.29
	90 g/l	0.16	0.146	14.00
3. Glucose to xylose ratio	10:0 (w/w)	0.45	0.175	4.20
	8:2 (w/w)	0.43	0.178	4.28
	6:4 (w/w)	0.44	0.178	4.26
	5:5 (w/w)	0.44	0.174	4.18
	4:6 (w/w)	0.43	0.139	4.17
	2:8 (w/w)	0.43	0.111	3.99
	0:10 (w/w)	0.42	0.109	3.91
4. Temperature	30 °C	0.42	0.103	3.09
	35 °C	0	0	0
5. Initial pH	3.0	0.35	0.052	2.51
	4.0	0.36	0.089	2.66
	4.5	0.44	0.134	3.22
	5.0	0.45	0.136	3.27
	6.0	0.34	0.081	2.44
6. Shaking speed	60 rpm	0.35	0.051	2.27
	120 rpm	0.39	0.091	2.72
	180 rpm	0.43	0.106	3.17
	240 rpm	0.40	0.071	2.12

3.9 Ethanol production from enzymatic cellulosic hydrolysate

3.9.1 Selection of ethanolic producing yeasts and bacteria

In order to obtain the maximum ethanol production from cellulosic hydrolysate (almost glucose 7.9 g/l) obtained from enzymatic hydrolysis (Section 3.6), *C. shehatae* TISTR5843, *S. cerevisiae* TISTR5017, and *Z. mobilis* TISTR405 were studied for ethanol production from cellulosic hydrolysate at room temperature (30 ± 2°C) with shaking speed of 180 rpm for 72 h (Abbi *et al*., 1996). The maximum ethanol production were 2.25 ± 0.06 g/l, 2.82 ± 0.11 g/l and 2.46 ± 0.04 g/l achieved at 60, 24, and 30 h, respectively (Table 31). The results clearly indicated that *S. cerevisiae* TISTR5017 was the best ethanolic producing strain from using cellulosic hydrolysate containing glucose, giving not only the highest ethanol concentration but also ethanol yield (0.34 g/g sugar) and ethanol productivity (0.118 g/l/h).

Table 31. Comparison of ethanol production in cellulosic hydrolysate (glucose 7.9 g/l) by *C. shehatae* TISTR5843, *S. cerevisiae* TISTR5017, and *Z. mobilis* TISTR405.

Strains	Ethanol concentration (g/l)	Ethanol yield (g/g sugar)	Ethanol productivity (g/l/h)
C. shehatae TISTR5843	2.25 ± 0.06	0.27	0.047
S. cerevisiae TISTR5017	2.82 ± 0.11	0.34	0.118
Z. mobilis TISTR405	2.46 ± 0.04	0.30	0.068

3.9.2 Optimization of ethanol production in cellulosic hydrolysate by selected strain

RSM was used as a statistical tool to find the optimum condition for maximizing ethanol production in cellulosic hydrolysate by the selected strain, *S. cerevisiae* TISTR5017. Three major parameters, initial pH (X_{13}), shaking speed (X_{14}) and initial cell concentration (X_{15}), were studied. The responses of RSM model, ethanol concentration (Y_{12}), ethanol yields (Y_{13}), and ethanol productivity (Y_{14}), were

shown in Table 32. At the initial pH of 4.0 (run 1-5), no ethanol production was detected because of no cells growth. Low ethanol concentration (2.00-2.49 g/l), ethanol yield (0.24-0.30 g ethanol/g sugar) and ethanol productivity (0.083-0.104 g/l/h) were obtained at the condition of initial pH of 5.00 and 6.00 and shaking speed of 240 rpm (run 14, 19 and 20). High ethanol concentration (3.14-3.94 g/l), ethanol yield (0.38-0.48 g ethanol/g sugar) and ethanol productivity (0.131-0.164 g/l/h) were achieved at the initial pH of 5.00 and 6.00 and shaking speed of 120 and 180 rpm (run 6-13 and 15-18). To evaluate the results, the data in Table 32 were subjected to regression analysis, using the following quadratic equations 37-39:

$$Y_{12} = -55.59 + 20.21X_{13} + 0.06X_{14} + 1.30X_{15} - 0.0045X_{13}X_{14} - 0.22X_{13}X_{15} - 0.0005X_{14}X_{15} - 1.77X_{13}^2 - 0.0001X_{14}^2 - 0.12X_{15}^2 \quad (37)$$

$$Y_{13} = -6.86 + 2.49X_{13} + 0.007X_{14} + 0.16X_{15} - 0.0006X_{13}X_{14} - 0.028X_{13}X_{15} - 0.00004X_{14}X_{15} - 0.22X_{13}^2 - 0.00001X_{14}^2 - 0.016X_{15}^2 \quad (38)$$

$$Y_{14} = -2.33 + 0.85X_{13} + 0.002X_{14} + 0.05X_{15} - 0.0002X_{13}X_{14} - 0.009X_{13}X_{15} - 0.00002X_{14}X_{15} - 0.075X_{13}^2 - 0.000005X_{14}^2 - 0.004X_{15}^2 \quad (39)$$

These models presented the high determination coefficients (R^2 = 0.97, 0.97 and 0.97, respectively) (Table 33) explaining 0.97% of variability in the responses of ethanol concentration, ethanol yield and ethanol productivity. The adjusted determination coefficients (adjusted R^2 = 0.95) indicated the high significance of these models. The ANOVA quadratic regression demonstrated that among models were significant, as evidenced from high *F*-values (*F=41.54, 41.44* and *42.33*, respectively) with a very low probability (*P<0.0001*). Low variation coefficient value (C.V. = 14.01%, 14.07% and 13.89%, respectively) indicated a high precision and reliability of the experiments (O-Thong *et al*., 2008). The significance of each coefficient was determined by probability values. The variables with a significant effect on ethanol production were the initial pH (X_{13}) and shaking speed (X_{14}) (*P<0.05*). Linear terms of X_{13} and X_{14} and quadratic terms of X_{13}^2 and $X_{13}X_{14}$ were significant (*P<0.05*), demonstrating that maximizing ethanol production required a suitable value of initial pH and shaking speed.

Estimation of ethanol concentration, ethanol yield and ethanol productivity over X_{13}, X_{14} and X_{15} in terms of response surfaces are shown in Fig. 47. The effect of initial pH and shaking speed on ethanol production, fixed the initial cells concentration at 0.7 g/l, are shown in Fig. 47(a), 47(d) and 47(g). The maximum ethanol concentration (3.94 g/l), ethanol yield (0.48 g ethanol/g sugar) and ethanol productivity (0.164 g/l/h) were achieved at initial pH of 5.0 and shaking speed of 120 rpm. The results of ethanol production indicated that both initial pH and shaking speed had significant effect on ethanol production. The pH affects on cell membrane permeability and the solubility of some components of the medium: thus, a modification in the pH might also cause some micronutrient to precipitate and so become impossible to be assimilated (Sánchez *et al*., 1997). The dissolved oxygen tension (DOT) is also particularly critical in attaining maximal ethanol production with xylose-fermenting yeasts. *C. shehatae* require aeration for maximal ethanol production (Jeffries and Jin, 2000). In addition, rotary of the shaker should be effective enough to provide gentle mixing and surface aeration during the first period of the growth phase (Phisalaphong *et al*., 2006). However, much more oxygen supplied into fermentation system would be caused of reduction of ethanol production because the pathway of *C. shehatae* produced cell mass more than ethanol (Jeffries and Jin, 2000).

The response surface plots of the initial pH and initial cells concentration interaction were shown in Fig. 47(b), 47(e) and 47(h) when shaking speed was fixed at 180 rpm. The initial pH had a significant effect on ethanol production while the initial cells concentration had no effect. The response surface plots of shaking speed and initial cells concentration interaction are shown in Fig. 47(c), 47(f) and 47(i) when initial pH was fixed at 5.0. Shaking speed had a significant effect on ethanol production while the initial cells concentration had no effect.

Table 32. Central composite experimental design matrix defining initial pH (X_{13}), shaking speed (rpm) (X_{14}), and initial cells concentration (g/l) (X_{15}) and results on ethanol production in cellulosic hydrolysate after cultivation of *S. cerevisiae* TISTR5017 for 72 h at room temperature (30°).

Run	Parameter			Ethanol concentration (Y_{12}) (g/l)	Ethanol yield (Y_{13}) (g /g sugar)	Ethanol productivity (Y_{14}) (g/l/h)
	X_{13}	X_{14}	X_{15}			
1	4	120	0.40	0	0	0
2	4	120	1.00	0	0	0
3	4	180	0.70	0	0	0
4	4	240	1.00	0	0	0
5	4	240	0.40	0	0	0
6	5	180	0.40	3.18	0.39	0.133
7	5	180	1.00	3.54	0.43	0.148
8	5	180	0.70	3.66	0.45	0.153
9	5	180	0.70	3.66	0.45	0.153
10	5	180	0.70	3.66	0.45	0.153
11	5	180	0.70	3.66	0.45	0.153
12	5	180	0.70	3.66	0.45	0.153
13	5	180	0.70	3.66	0.45	0.153
14	5	240	0.70	2.05	0.25	0.086
15	5	120	0.70	3.94	0.48	0.164
16	6	120	0.40	3.52	0.43	0.147
17	6	120	1.00	3.14	0.38	0.131
18	6	180	0.70	3.23	0.39	0.134
19	6	240	0.40	2.49	0.30	0.104
20	6	240	1.00	2.00	0.24	0.083

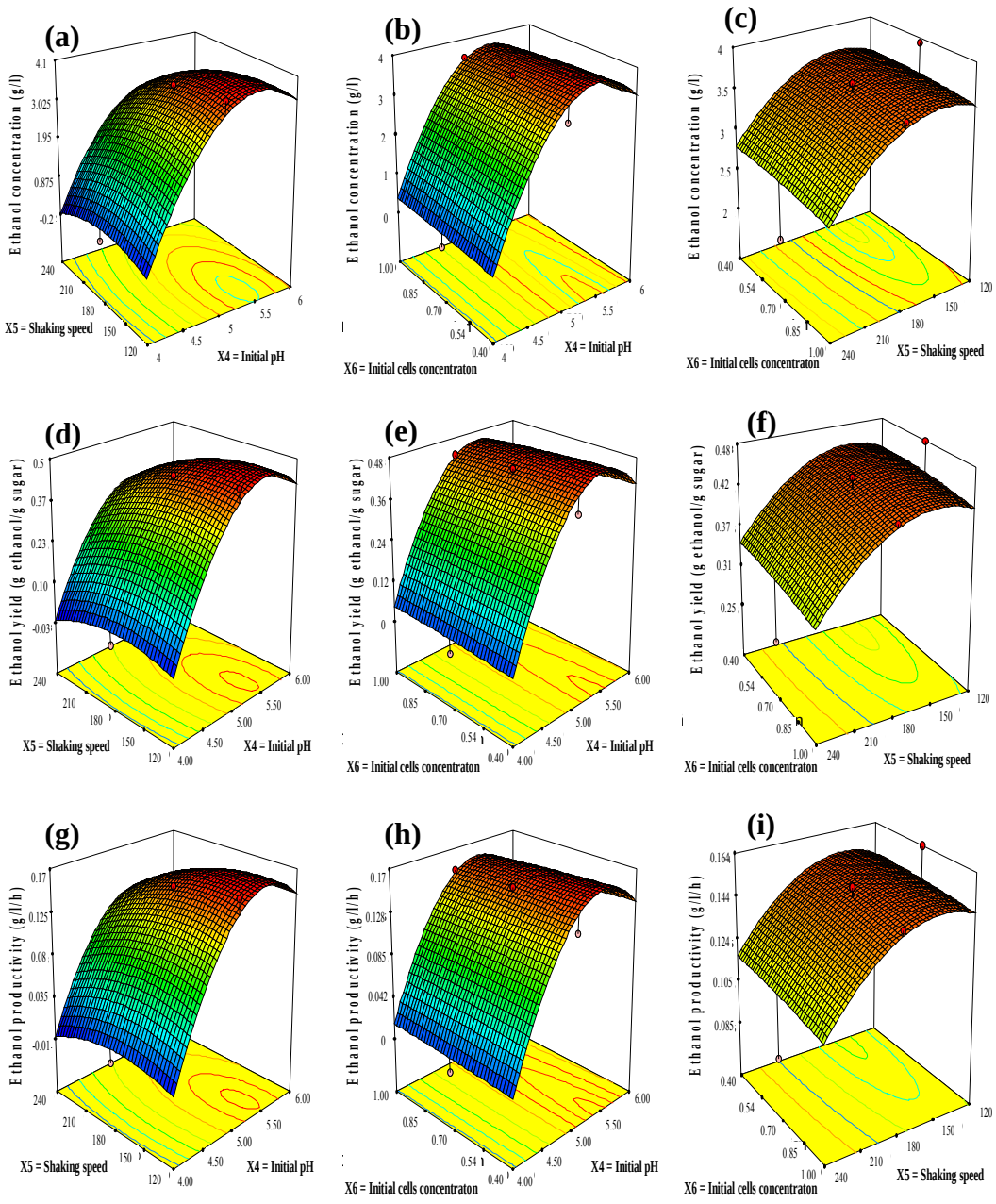


Figure 47. Three-dimensional graphs of the quadratic model of ethanol production in a cellulosic hydrolysate medium by *S. cerevisiae* TISTR5017. for ethanol concentration (a-c), ethanol yield (d-f) and ethanol productivity (g-i) within the central composite design (CCD): a, d and g; fixed initial cells concentration at centre point of 0.70 g/l, b, e and h; fixed shaking speed at centre point of 180 rpm, c, f and i; fixed initial pH at centre point of 5.0.

Table 33. Analysis of variance (ANOVA) for ethanol production in a cellulosic hydrolysate.

Responses (*Y*)	Source	Sum of square	Degree of freedom	Mean square	*F*-value	*P*-value
Ethanol concentration (Y_{12})	Model R^2 Adjusted R^2 C.V.	44.15 0.97 0.95 14.01	9	4.91	41.54	< 0.0001
Ethanol yield (Y_{13})	Model R^2 Adjusted R^2 C.V.	0.66 0.97 0.95 14.07	9	0.074	41.44	< 0.0001
Ethanol productivity (Y_{14})	Model R^2 Adjusted R^2 C.V.	0.077 0.97 0.95 13.89	9	0.009	42.33	<0.0001

3.9.3 Confirmation experiments and adequacy of the models of ethanol production in cellulosic hydrolysate

To confirm the validity of the statistical experimental strategies of ethanol production from cellulosic hydrolysate, three replicates of batch experiments were performed under the optimal condition conducted by RSM with the initial pH of 5.40, shaking speed of 137 rpm and initial cells concentration of 0.72 g/l. Results of confirmation experiments (Table 34) indicated that the experimental values of ethanol production (3.98 ± 0.42 g/l, 0.48 ± 0.02 g ethanol/g sugar, and 0.167 ± 0.04 g/l/h) were similar to the predicted values (4.06 g/l, 0.49 g ethanol/g sugar, and 0.170 g/l/h, respectively). There was no significant difference between the experimental values and the predicted values *(P<0.05)*. The ethanol production using the initial condition (control) (Table 34) and the optimum condition (Table 34) was 2.82 and 3.98 g/l, respectively. After optimization, ethanol production from this hydrolysate medium increased 1.41-fold (from 2.82 to 3.98 g/l).

Table 34. The confirmation experiments for ethanol production in cellulosic hydrolysate by *S. cerevisiae* TISTR5017 cultivated under the optimal condition.

Substrates	Trials	Condition	Initial pH	Shaking speed (rpm)	Initial cells concentration (g/l)	Ethanol concentration (g/l)	Ethanol yield (g/g sugar)	Ethanol productivity (g/l/h)
Cellulosic hydrolysate	-	Optimal[a]	5.40	137	0.72	3.98 ± 0.42	0.48 ± 0.02	0.167 ± 0.04
	9	Central	5.00	180	0.70	3.75	0.46	0.156
	-	Selected	5.00	180	0.4	2.82	0.34	0.118

[a] Based on ethanol production.

3.10 Ethanol production from PPF hydrolysate

3.10.1 Comparison of ethanol production between synthetic xylose medium and PPF hydrolysate by *C. shehatae* TISTR5843

Prior to produce ethanol from PPF hydrolysate, comparison of ethanol production from both synthetic xylose medium (28 g/l xylose) without supplementation of acetate (4.25 g/l) and furfural (0.67 g/l) and PPF hydrolysate (28 g/l xylose approximately) by *C. shehatae* TISTR5843 was investigated. *C. shehatae* TISTR5843 was selected due to its ability to consume xylose (Delgenes *et al*., 1996). Meanwhile, *S. cerevisiae* TISTR5517, selected and used to produce ethanol from cellulosic hydrolysate, could not use xylose as a substrate to produce ethanol. The optimum extrinsic parameters of ethanol production in synthetic xylose medium by *C. shehatae* TISTR5843 were set at initial pH of 5.0, cell concentration of 0.725 g/l (OD_{600}=0.5), shaking speed of 180 rpm, and incubated at room temperature (30°C) (Section 3.8: Table 30).

For synthetic xylose medium, ethanol production was started at 18 h (0.29 g/l) cultivation and increased rapidly until 48 h (8.14 g/l). In PPF hydrolysate medium, ethanol production was also begun at 18 h cultivation (0.17 g/l) and slightly increased until 96 h (3.30 g/l) (Fig. 48) because of generation of inhibitory compounds from dilute acid hydrolysis (Delgenes *et al*., 1996; Rahman *et al*., 2006) i.e. 4.25 g/l acetate and 0.67 g/l furfural were generated. The ethanol yields and ethanol productivities of both synthetic xylose and PPF hydrolysate medium were 0.43 and 0.16 g ethanol/g xylose, and 0.194 and 0.034 g/l/h, respectively. The results showed that the inhibitory played an important role in ethanol production by *C. shehatae* TISTR5843. Therefore, xylanase usage might be a good method to produce xylose without any inhibitors generation. However, xylose production by using xylanase have to study in case of the cost of enzyme was expensive. Therefore, the further studied of this research was an investigation of the effect of inhibitory compounds in PPF hydrolysate for the production of ethanol by *C. shehatae* TISTR5843. Consequently, the effect of nitrogen source and its concentration and carbon to nitrogen (C/N) ratio (concerning to cells growth) were studied in synthetic xylose medium.

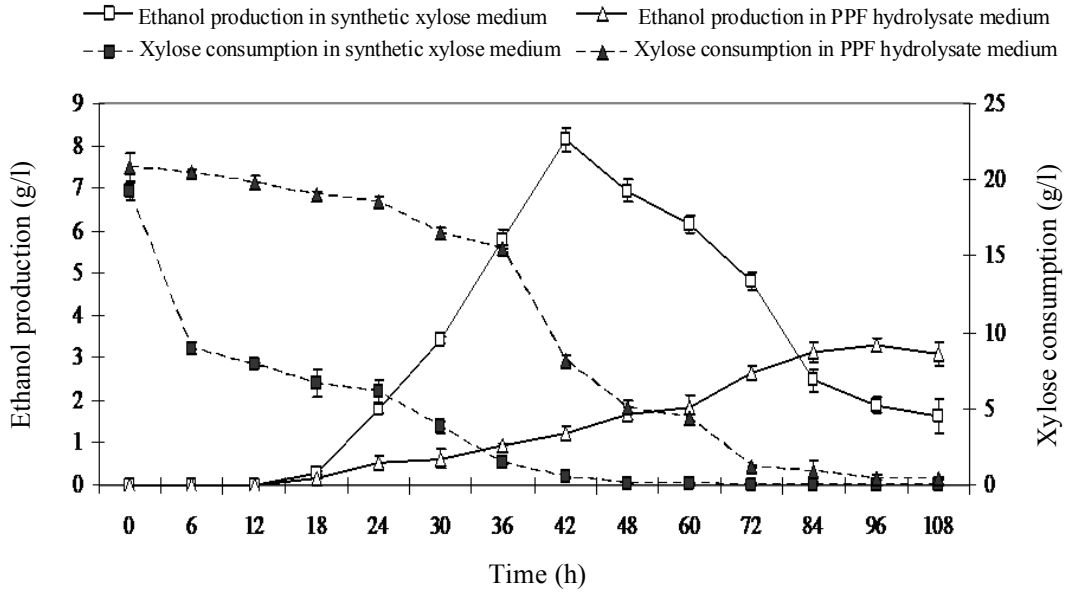


Figure 48. Time course comparison of ethanol production in synthetic xylose and PPF hydrolysate mediums by *Candida shehatae* TISTR5843 under 20 g/l xylose, initial pH of 5.0, shaking speed of 180 rpm at room temperature (30°C).

3.10.2 Effect of nitrogen source, nitrogen concentration and C/N ratio on ethanol production

3.10.2.1 Effect of nitrogen source

NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3PO_4$, urea, yeast extract, peptone and tryptone were used as nitrogen sources. Each of nitrogen sources was supplemented into the xylose medium in amount of 3 g/l based on nitrogen content in each compound. The synthetic xylose medium was 28 g/l xylose with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l), which is the similar xylose concentration and inhibitors concentration in PPF hydrolysate. The optimum extrinsic parameters of ethanol production in synthetic xylose medium by *C. shehatae* TISTR5843 were set at initial pH of 5.0, cell concentration of 0.725 g/l (OD_{600}=0.5), shaking speed of 180 rpm, and incubated at room temperature (30°C) (Section 3.8:

Table 30). In this case, the cultures were incubated for 24 h and 48 h. As seen in Fig. 49 peptone was the optimum nitrogen source giving the maximum ethanol production of 3.05 g/l. The reduction of ethanol production (8 to 3 g/l) resulted from furfural and acetate added into the synthetic xylose medium.

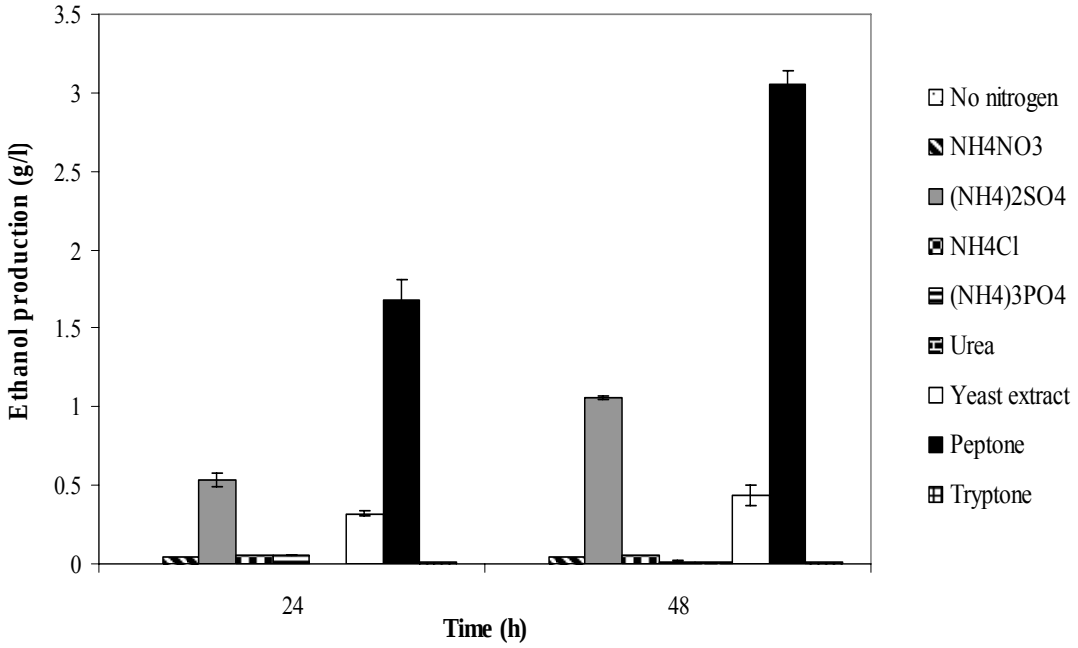


Figure 49. Effect of nitrogen sources on ethanol production by *Candida shehatae* TISTR5843 with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l) at optimum pH of 5.0 and initial cell concentration of 0.725 g/l incubated at 30°C on a rotary shaker (180 rpm) for 24 and 48 h.

3.10.2.2 Effect of peptone concentration

The highest ethanol production (4.75 g/l) was obtained at 3 g/l peptone with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l) corresponded to a C/N ratio of 9.3 (Fig. 50). Substantial reduction in ethanol production was observed at peptone concentration higher than 5 g/l. Ethanol production decreased with the increase of nitrogen content because of presented much more nitrogen (Abd-Aziz *et al*., 2001). In this study, therefore, peptone at 3 g/l was the optimal nitrogen concentration for producing ethanol by *C. shehatae* TISTR5843.

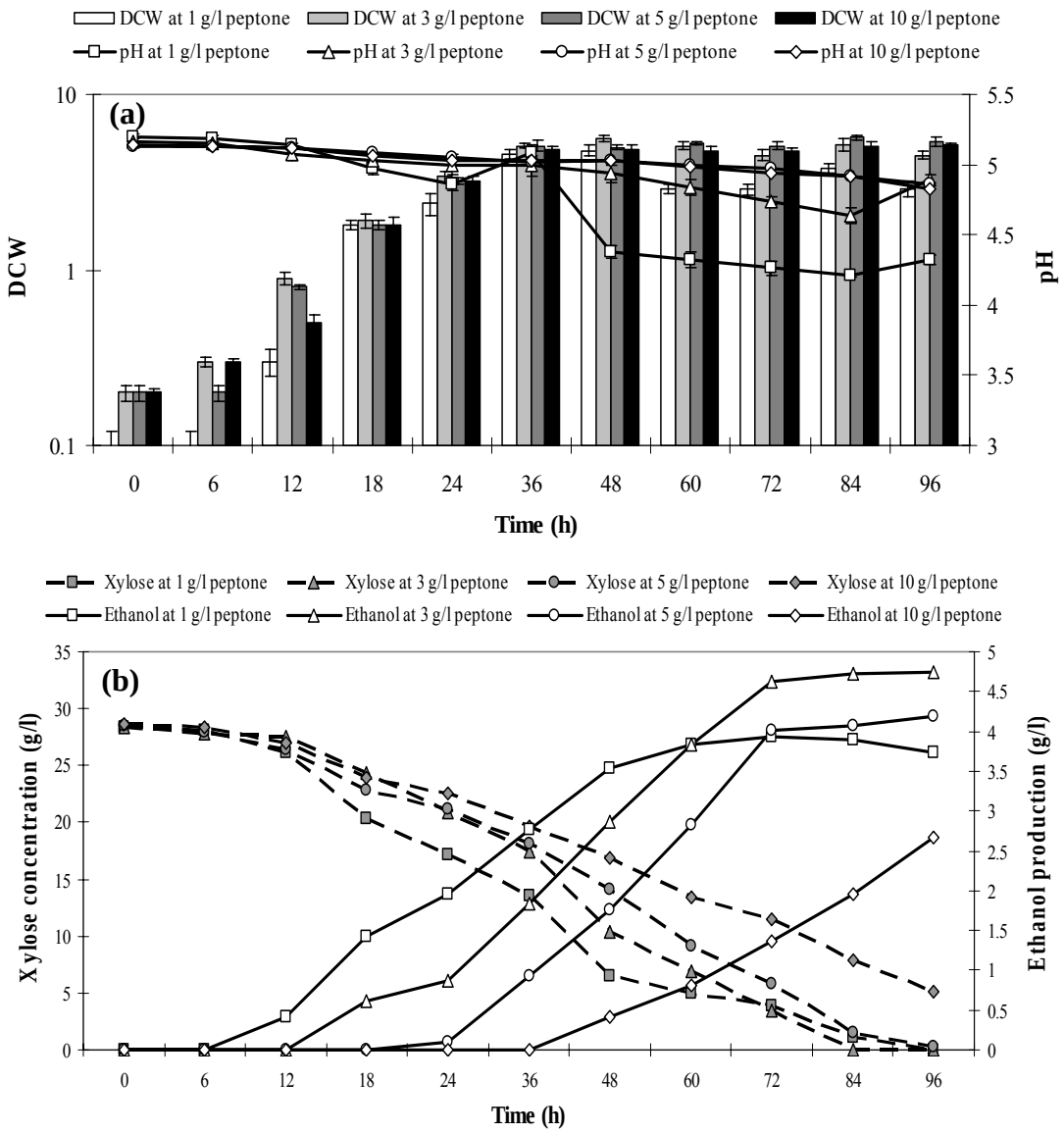


Figure 50. Time course of cell growth and pH change (a), and xylose consumption and ethanol production (b) by *Candida shehatae* TISTR5843 under various peptone concentrations of 1-10 g/l in synthetic xylose medium with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l), pH5 with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

To set the composition in synthetic medium as close as in PPF hydrolysate, the 28 g/l xylose medium was supplemented with 4.25 g/l acetate and 0.67 g/l furfural (as the same concentration in PPF hydrolysate). The performance of direct fermentation of xylose to ethanol by *C. shehatae* TISTR5843 at different C/N ratios is shown in Table 35 and described in the previous section. On the other hand, *C. shehatae* TISTR5843 grew faster and reached a maximum cell concentration (5.6 g/l) and produced a maximum ethanol production (4.75 g/l) at the C/N ratio of 9.3.

Table 35. The performance of ethanol production from xylose medium with supplementation of 4.25 g/l acetate and 0.67 g/l furfural at different C/N ratios by *Candida shehatae* TISTR5843.

Fermentation performance	C/N ratio			
	2.8	5.6	9.3	28
Maximum cell concentration (g/l)	5.2	5.3	5.6	4.8
Maximum ethanol concentration (g/l)	2.68	4.20	4.75	3.93
Ethanol yield (g of ethanol per g of xylose)	0.12	0.17	0.19	0.16
Cell yield (g of cell per g of xylose)	0.221	0.279	0.311	0.218
Fermentation time (h)	48	48	48	48
Overall productivity (g/h)	0.056	0.087	0.099	0.081

28 g/l xylose was used in all fermentations

3.10.3 Effect of inhibitory compounds in PPF hydrolysate on ethanol production

Normally, after diluted acid hydrolysis of biomass, several inhibitor compounds were generated such as acetate, furfural, vanillin, cinnamaldehyde, ρ-hydroxybenzaldehyde and syringaldehyde (Olsson and Hahn-Hägerdal, 1996; Delgenes *et al*., 1996). In this study, acetate, vanillin and furfural were studied because they are the major inhibitor compounds in PPF hydrolysate.

Compared to the control culture (without any inhibitor supplementations), all supplemented cultures of *C. shehatae* TISTR5843 showed a

decrease in cells growth and ethanol production (Table 36). Vanillin, derivative of lignin, showed the highest inhibitory effect (Fig. 51). Both growth and ethanol production processes were almost totally inhibited at an initial vanillin concentration of 1.0 g/l (Table 36). The high toxicity of vanillin on activities by xylose-fermenting microorganisms was reported by Tran and Chambers (1985), Nishikawa *et al* (1988) and Delgenes *et al* (1996). The ethanol produced by *P. stipitis* CBS 5776 in the xylose fermentation with 0.09 g/l of vanillin was 2.2 times lower of that in the control (Tran and Chambers, 1985). *K. pneumoniae* ATCC 8724 grown in the presence of 0.5 g/l of vanillin showed that growth and 2,3-butanediol production from xylose were lower 80 and 56.1% , respectively compared to the control (Nishikawa *et al*., 1988). An initial vanillin concentration of 1.0 g/l, both growth and ethanol production processes by *C. shehatae* ATCC22984, *P. stipitis* NRRL Y 7124 and *S. cerevisiae* CBS 1200 were almost totally inhibited (Delgenes *et al*., 1996).

In contrast to vanillin, allowing for concentration effects, acetate was the less toxic compound for *C. shehatae* TISTR5843. At the highest acetate concentration of 10 g/l, growth and ethanol production decreased 26.78% and 43.01%, respectively, compared to the control culture (Table 36 and Fig. 52). The sensitivity of the fermentation activities toward acetate appeared to be strain dependent as illustrated by Tran and Chambers (1985), Watson *et al* (1984) and Delgenes *et al* (1996). With an acetate concentration of 11.9 g/l, the ethanol production by *P. stipitis* CBS 5776 decreased 24% (Tran and Chambers, 1985), whereas 13 g/l of acetic acid almost completely inhibited ethanol production from xylose by *P. tunnophilus* (Watson *et al*., 1984). At an initial acetate concentration of 15 g/l, ethanol production by *C. shehatae* and *P. stipitis* decreased 64% and 25%, respectively (Delgenes *et al*., 1996). The undissociated acetic acid permeates the cell membrane and then dissociates in the cytoplasm where the pH is almost neutral. The cell uses energy to pump out surplus H^+ ions in order to maintain its intracellular pH. This eventually leads to cell death at high acetic acid concentrations (Palmqvist *et al*., 1996; Nigam, 2001).

Table 36. Growth and ethanol production by *C. shehatae* TISTR5843 in synthetic xylose medium in the presence of inhibitory compounds.

Compounds	Concentration (g/l)	DCW (g/l)	Ethanol concentration (g/l)	DCW (% of control)	Ethanol (% of control)
Control	Acetate = 1.6 Furfural = 0.2	12.96	12.88 (0.46)	100	100
Acetate	2.5	4.92	8.41 (0.30)	37.96	65.30
	5	4.25	7.58 (0.27)	32.79	58.85
	10	3.47	5.54 (0.20)	26.78	43.01
Furfural or furaldehyde	0.5	4.97	7.51 (0.27)	39.35	58.31
	1	1.32	5.94 (0.21)	11.19	46.12
	2	0	0	0	0
Vanillin	0.5	4.62	3.95 (0.14)	35.65	30.67
	1	0.24	0.04 (0)	1.85	0.31
	2	0	0	0	0
Acetate and furfural	4.25 and 0.67[a]	4.18	4.56 (0.16)	32.25	35.40

[a]The same amount of inhibitor concentration presented in the PPF hydrolysate
Control: amount of malt extract was 3 g/l

For furfural effect showed that the furfural concentration of 1 g/l gave cell growth and ethanol production of 11.19% and 46.12 %, respectively, of the control whereas the completely inhibited effect of cell growth and ethanol production was obtained from the initial furfural concentration of 2 g/l (Table 36). In general, furfural is inhibitory to yeast metabolism at a level of 1.0 g/l and greater (Nigam, 2001). Furfural affects the growth and metabolism of microorganisms. It delays the onset of fermentation while it is assimilated and degraded (Palmqvist *et al*., 1996). However, a suitable amount of furfural present in lignocellulosic hydrolysate can be beneficial for ethanolic fermentation of xylose because furfural uses NADH to generate NAD^+, which is used for transformation of xylitol to xylulose by xylitol dehydrogenase (XDR) in xylose phosphate pathway (Wahlbom and Hahn-Hägerdal, 2002). Furfural can be converted to furfuryl alcohol by *C. shehatae* TISTR5843

(Martín *et al*., 2007) with the maximal conversion rate of 0.025 g/l/h (Fig. 53c and 54), which is 3-folds lower than that of *S. cerevisiae* (Martín *et al*., 2007). However, the limited substrate concentration and the present of furfuryl alcohol decreased the growth rate as well as the specific rate of ethanol production (Fig. 53c and 54) because furfuryl alcohol inhibited alcohol dehydrogenase (ADH) and xylose reductase, which are enzymes for furfural reduction (Nigam, 2001). Consisting of individual (furfural, acetic acid and others), combination and all together in medium had the different inhibitory effected on fermentability. Nigam (2001) reported that the effect of all together > combination > individual are proved.

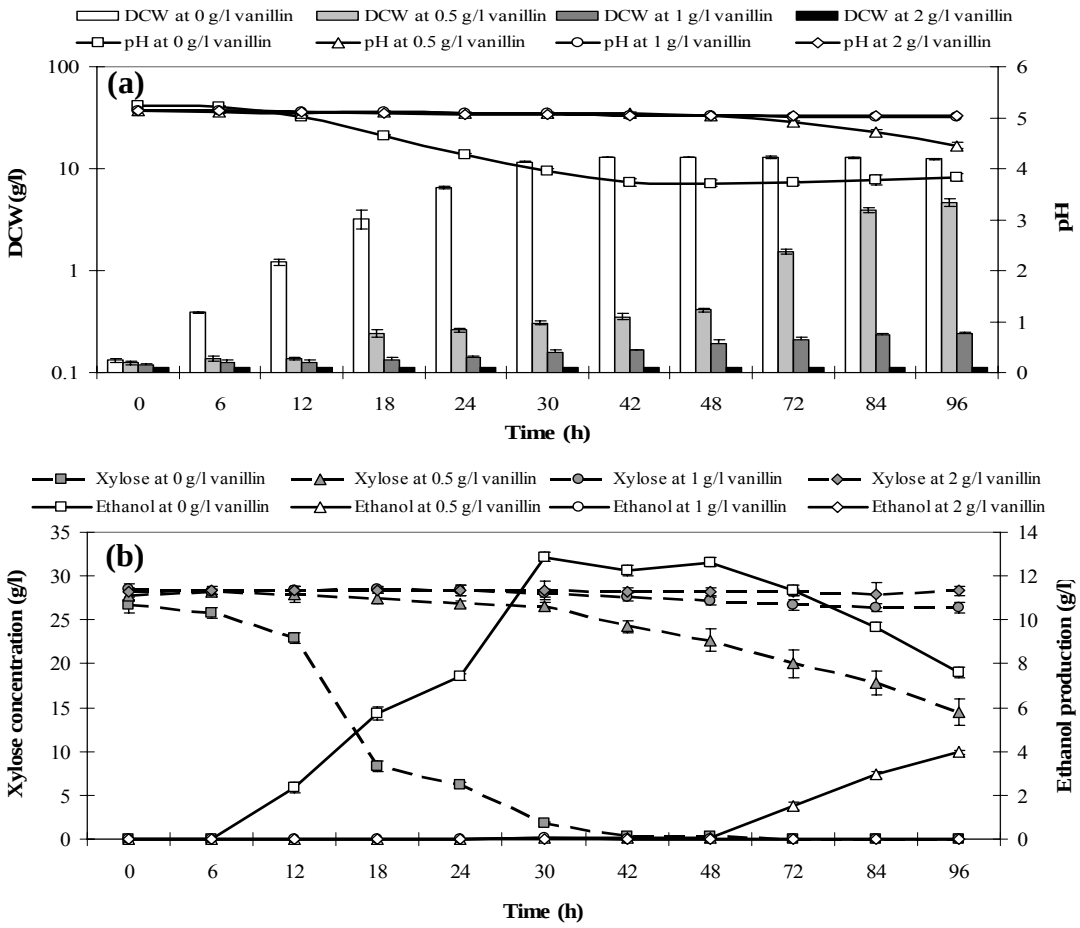


Figure 51. Time course of growth and pH change (a), and xylose consumption and ethanol production (b) by *Candida shehatae* TISTR5843 under various vanillin supplementations of 0-2 g/l in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

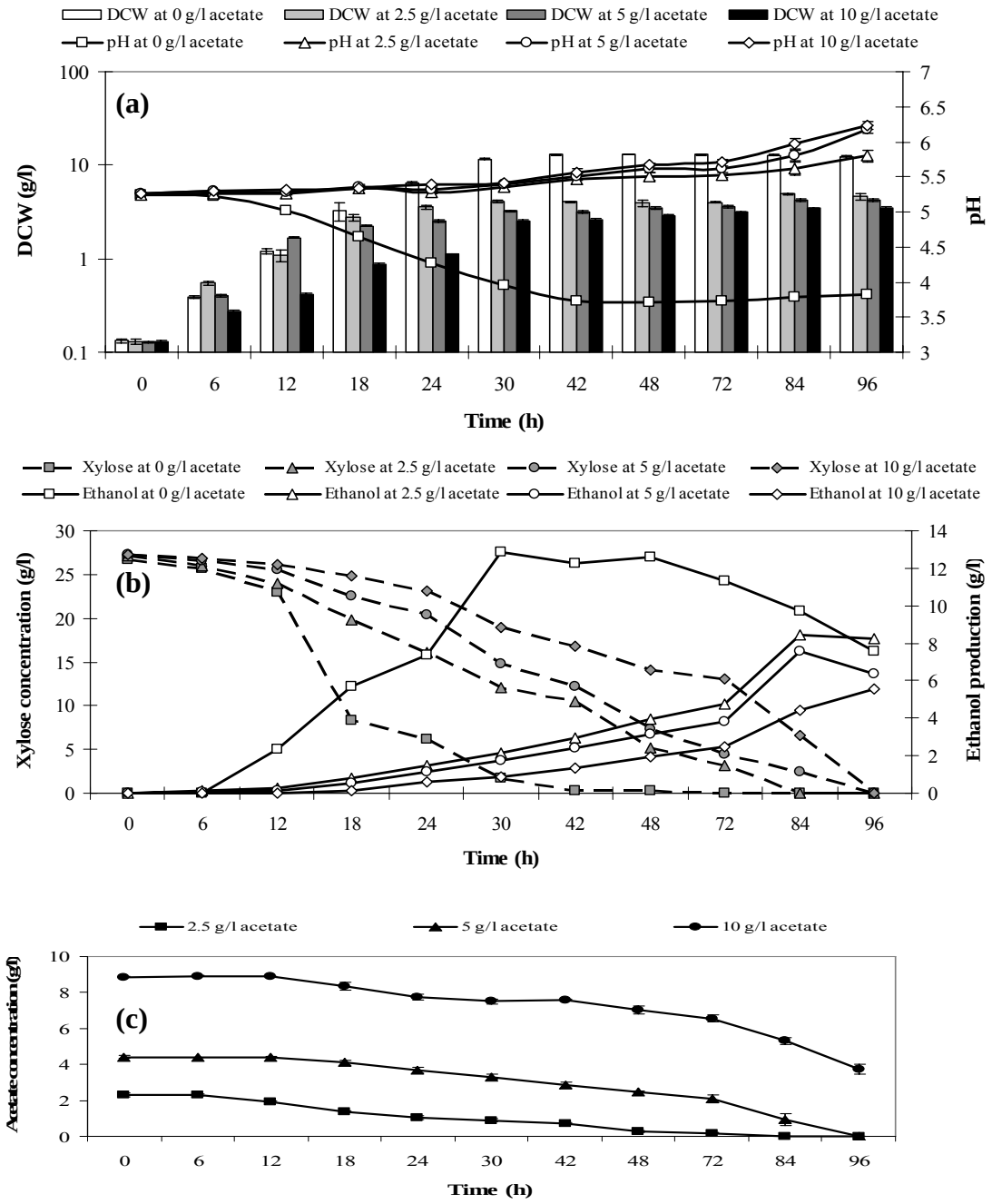


Figure 52. Time course of growth and pH change (a), xylose consumption and ethanol production (b), and acetate reduction (c) by *Candida shehatae* TISTR5843 under various acetate supplementations of 0-10 g/l in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm) in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l.

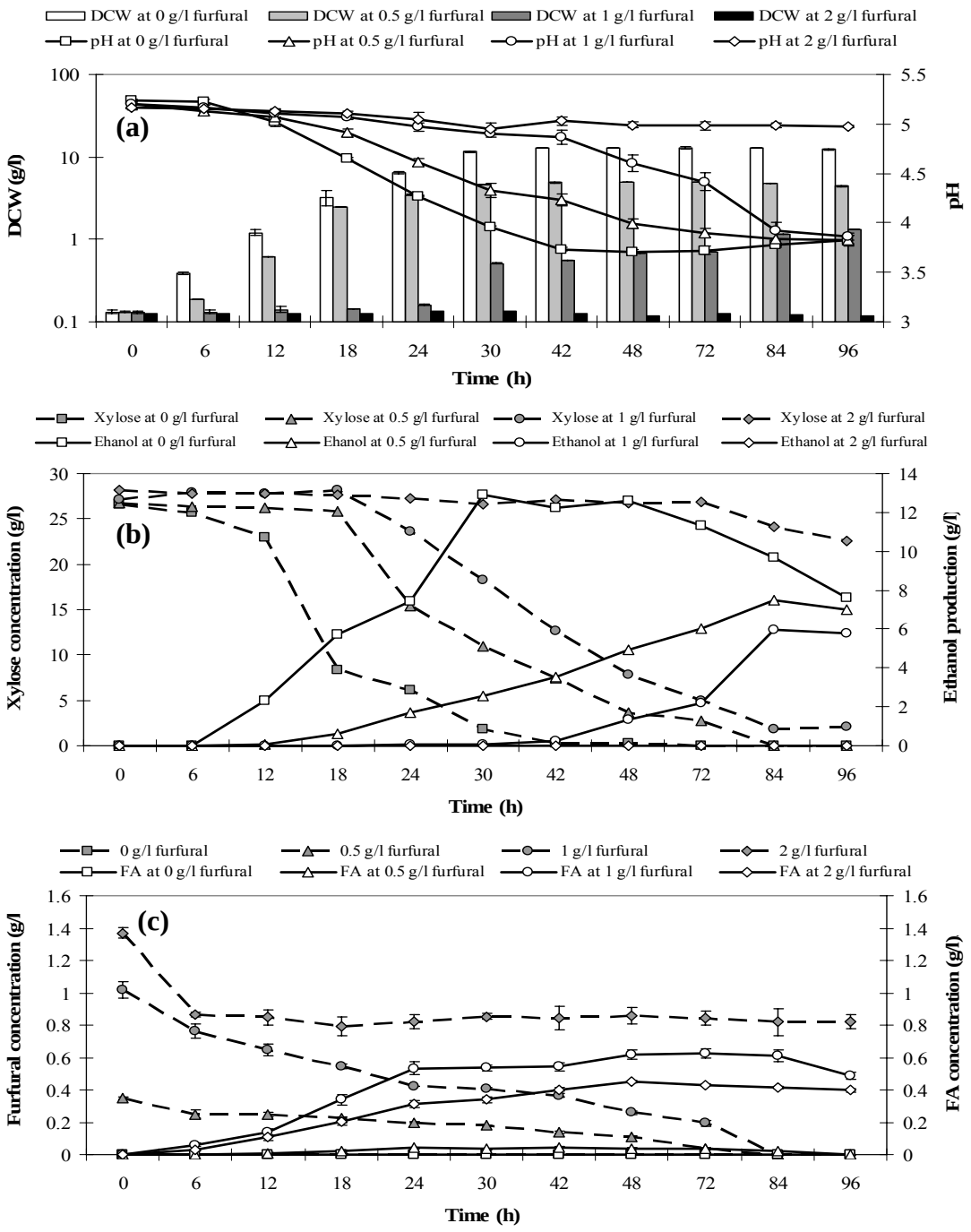


Figure 53. Time course of growth and pH change (a), xylose consumption and ethanol production (b), and furfural reduction with furfuryl alcohol (FA) generation (c) by *Candida shehatae* TISTR5843 under various furfural supplementations of 0-2 g/l in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

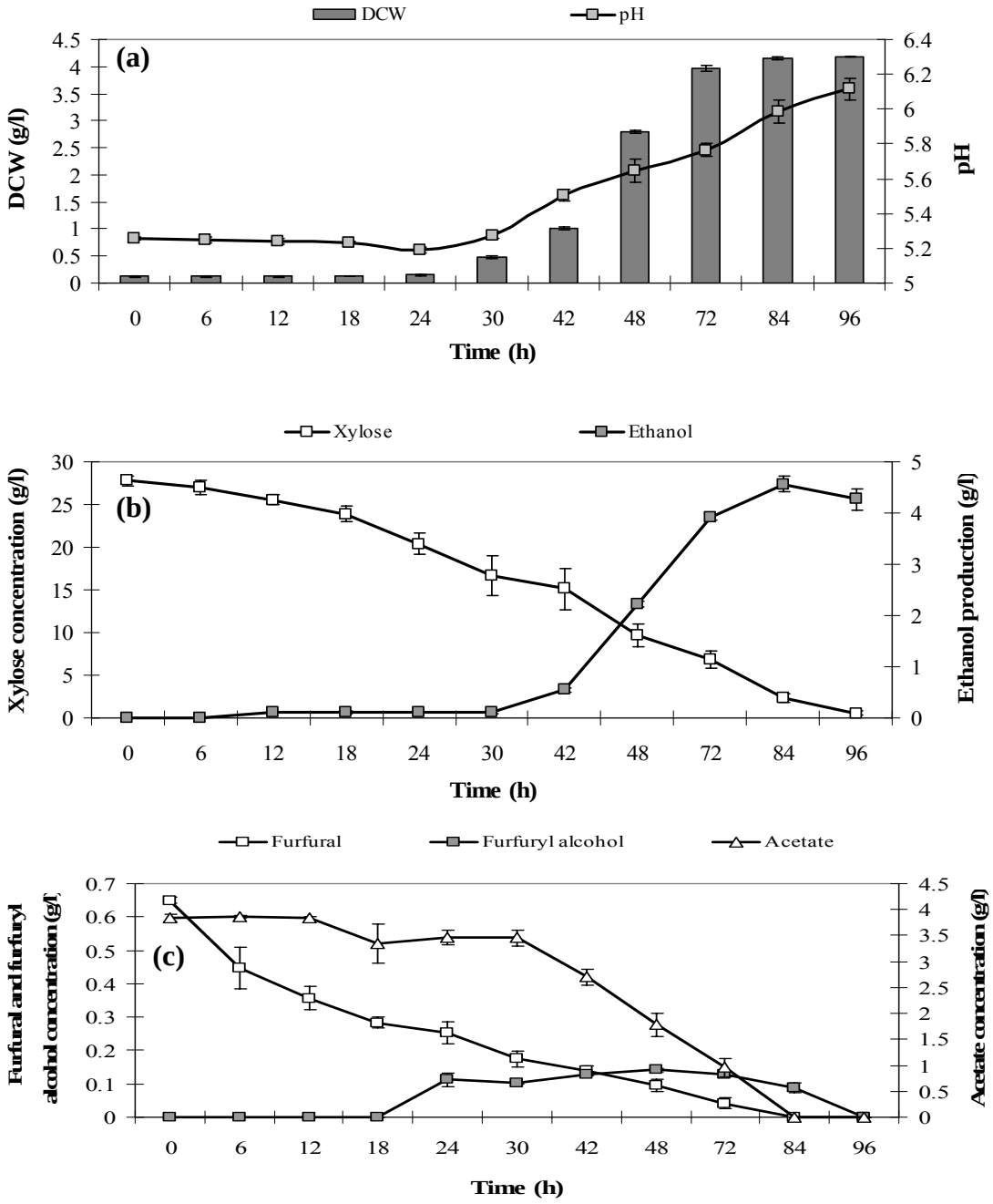


Figure 54. Time course of growth and pH change (a), xylose consumption and ethanol production (b), furfural and acetate reduction with furfuryl alcohol (FA) generation (c) by *Candida shehatae* TISTR5843 under mixture of 4.25 g/l acetate and 0.67 g/l furfural in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l at 30°C on a rotary shaker (180 rpm).

3.10.4 Effect of dilution factor of PPF hydrolysate on ethanol production

A large amount of inhibitory compounds in PPF hydrolysate resulted in the inhibition of cell growth and ethanol production, using diluted PPF hydrolysate may be a suitable method in order to reduce inhibition in the fermentation process. Inhibitors can be abated prior to fermentation through this process (Martín *et al*., 2007; Nichols *et al*., 2010). Detoxification improves the fermentability of hydrolysates, it is for economical reasons desirable to limit the requirements for detoxification to a minimum when compared to chemical and physical detoxifications (Martín *et al*., 2007). In this study, no dilution, 1/2 dilution, 1/3 dilution and 1/5 dilution were studied.

Cells growth at 1/5 dilution of PPF hydrolysate was found to be the fastest of growth rate within 36 h because of the lowest content of inhibitory compounds presented in hydrolysate (Fig. 55a and 55b). However, cells growth of no dilution, 1/2 dilution and 1/3 dilution of PPF hydrolysate increased rapidly after all inhibitors were consumed and transformed to less toxic compound at 60 h cultivation time (Fig. 55a and 56).

Xylose concentration of no dilution, 1/2 dilution, 1/3 dilution and 1/5 dilution of PPF hydrolysate were consumed closely to zero by *C. shehatae* TISTR5843 within 84, 36, 36 and 24 h (Fig. 55b), respectively, giving maximum of ethanol production of 5.21, 4.51, 3.02 and 1.56 g/l, respectively (Fig. 55b). Ethanol yields and productivities of these dilutions were 0.19, 0.32, 0.30, 0.27 g/g, and 0.062, 0.125, 0.084, 0.065 g/l/h, respectively. Thus, the 1/2 dilution was a suitable dilution for production of ethanol because of giving the maximum ethanol yield and ethanol productivity. The 1/2 dilution was used in the further experiment of optimization of ethanol production in PPF hydrolysate.

Acetate was consumed by *C. shehatae* TISTR5843 within 48-84 h cultivation time (Fig. 56a) for 1/2-1/5 dilutions, while acetate consumption of no dilution was consumed from 6.00 to 0.92 g/l (84.67 % reduction). Furfural concentrations could be transformed totally to furfuryl alcohol within 60 h (Fig. 56b).

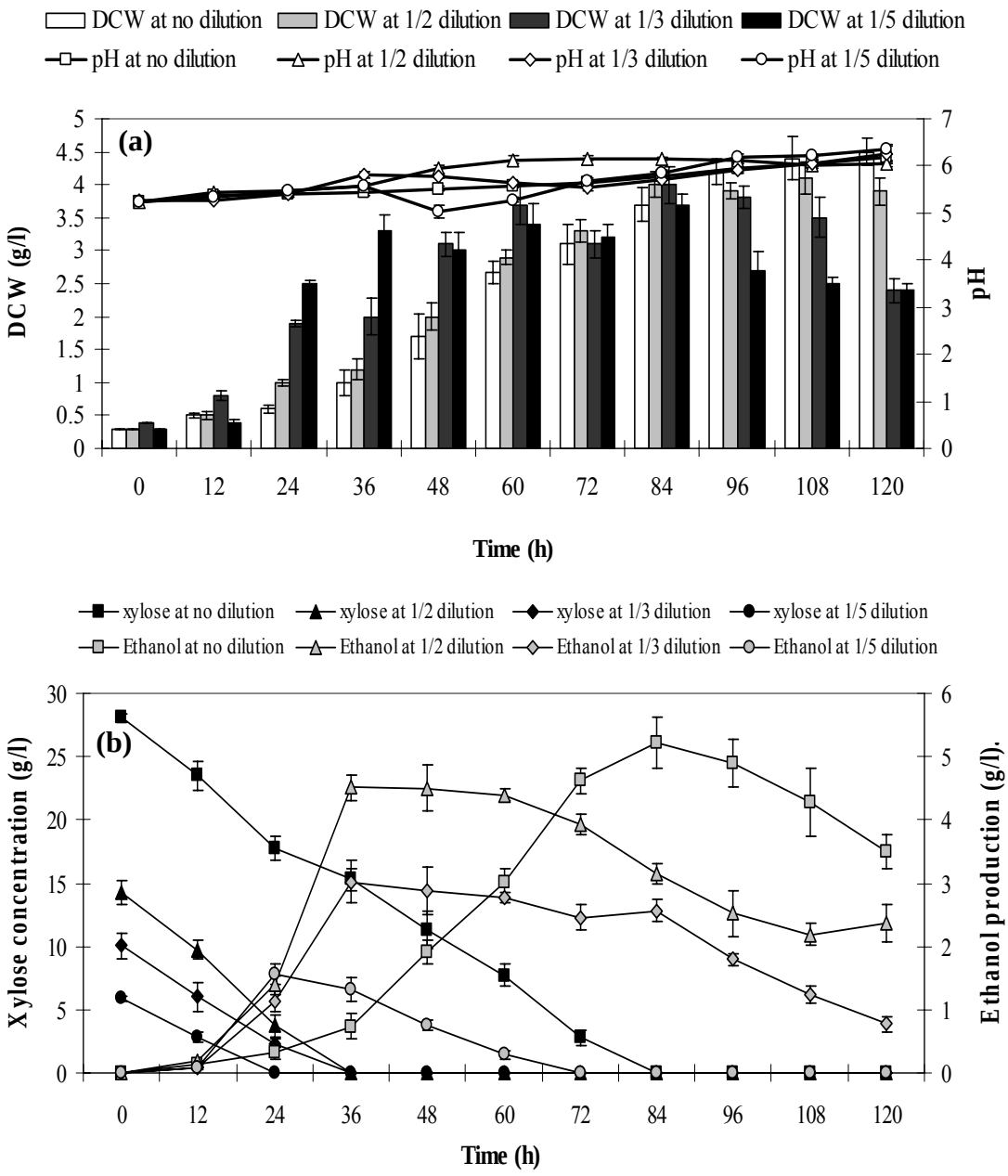


Figure 55. Time course of growth and pH change (a) and xylose consumption and ethanol production (b) by *Candida shehatae* TISTR5843 in various PPF hydrolysate dilutions (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

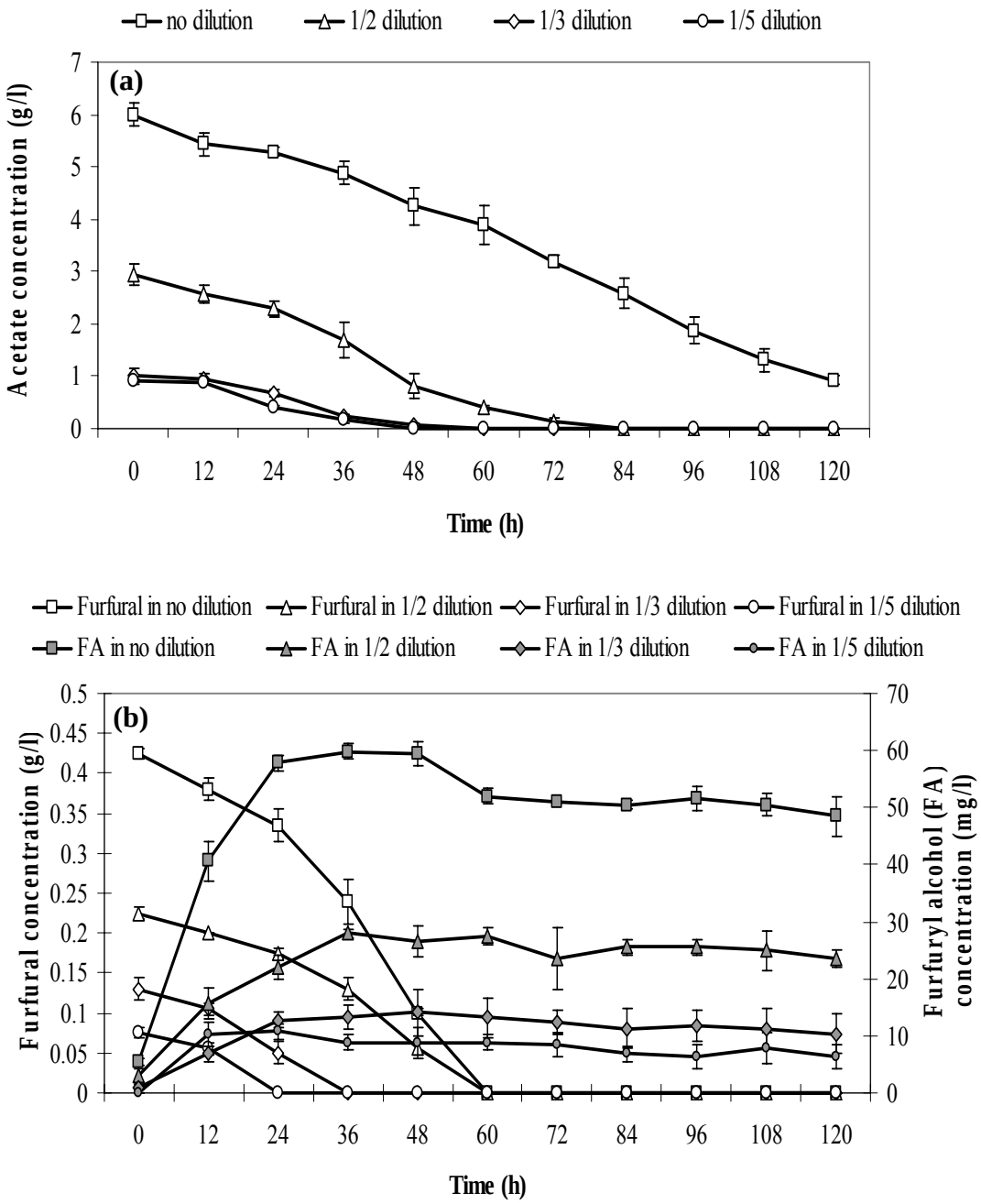


Figure 56. Time course of acetate consumption (a) and furfural reduction with furfuryl alcohol (FA) generation (b) by *Candida shehatae* TISTR5843 in various PPF hydrolysate dilutions (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

3.10.5 Optimization of ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate

3.10.5.1 Effect of initial pH, cells inoculum and shaking speed on ethanol production from PPF hydrolysate using RSM

Effect of initial pH, cells inoculum (OD_{600}) and shaking speed had been done in synthetic xylose medium. However, these parameters have to be optimized again due to the different ingredients in both synthetic xylose medium and PPF hydrolysate medium. Initial pH was varied in the range of 4-6, shaking speed was set in the range of 60-180 rpm and cells inoculum was controlled in the range of 0.9-1.5 g/l (Table 37). These experiments were operated at dilution of PPF hydrolysate medium of 1/2 dilution giving the initial xylose concentration of 13 g/l. The RSM was used a technique to find the optimal condition for maximizing ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate.

The ethanol concentration ranged from 0 to 5.15 g/l, the ethanol yield ranged from 0 to 0.40 g ethanol/g sugar and ethanol productivity ranged from 0 to 0.143 g/l/h were obtained in this study (Table 37). Low ethanol concentration (0-2.18 g/l), ethanol yield (0-0.17 g ethanol/g sugar) and ethanol productivity (0-0.061 g/l/h) were obtained using the initial pH of 4.00 and 6.00 (Table 37 at run 1-5 and run 16-17) and shaking speed of 60 rpm (run 16-17). Moderate ethanol concentration (2.93-4.65 g/l), ethanol yield (0.24-0.36 g ethanol/g sugar) and ethanol productivity (0.081-0.129 g/l/h) were obtained using the initial pH of 5.00 and 6.00 (run 6, 15 and run 18-20) and shaking speed of 60, 120 and 180 rpm (run 6, 15 and run 18-20). High ethanol concentration (5.04-5.15 g/l), ethanol yield (0.39-0.40 g ethanol/g sugar) and ethanol productivity (0.140-0.143 g/l/h) were achieved by using the moderate initial pH of 5.00 (run 7-14) and the moderate shaking speed of 120 rpm (run 7-14). However, no significance ($P<0.05$) of ethanol concentration (5.04-5.08 g/l), ethanol yield (0.39 g ethanol/g sugar) and ethanol productivity (0.140-0.141 g/l/h) were obtained using the initial pH of 5.00, shaking speed of 120 rpm and the initial cell concentration of 0.9 and 1.5 g/l (run 8 and 7). Therefore, there was no effect of the initial cell concentration in the range of 0.9-1.5 g/l on ethanol production from hemicellulosic hydrolysate by *C. shehatae* TISTR5843.

Table 37. Central composite experimental design matrix defining initial pH (X_{16}), shaking speed (rpm) (X_{17}), and initial cells concentration (g/l) (X_{18}) and results on ethanol production from hemicellulosic hydrolysate after cultivation of *Candida shehatae* TISTR5843 under 1/2 dilution factor of PPF hydrolysate medium (13 g/l initial xylose content) for 72 h at room temperature (30°).

Run	Parameter			Response (Y_{15}) Ethanol concentration (g/l)	Response (Y_{16}) Ethanol yield (g ethanol/g sugar)	Response (Y_{17}) Ethanol productivity (g/l/h)
	X_{16}	X_{17}	X_{18}			
1	4	120	1.2	0	0	0
2	4	60	0.9	0	0	0
3	4	180	0.9	0	0	0
4	4	180	1.5	0	0	0
5	4	60	1.5	0	0	0
6	5	180	1.2	4.65	0.36	0.129
7	5	120	1.5	5.08	0.39	0.141
8	5	120	0.9	5.04	0.39	0.140
9	5	120	1.2	5.13	0.39	0.142
10	5	120	1.2	5.13	0.39	0.142
11	5	120	1.2	5.15	0.40	0.143
12	5	120	1.2	5.10	0.39	0.142
13	5	120	1.2	5.15	0.40	0.143
14	5	120	1.2	5.14	0.40	0.143
15	5	60	1.2	3.89	0.31	0.108
16	6	60	0.9	2.10	0.16	0.058
17	6	60	1.5	2.18	0.17	0.061
18	6	180	0.9	2.93	0.24	0.081
19	6	120	1.2	4.42	0.35	0.123
20	6	180	1.5	3.00	0.24	0.083

To evaluate the results, the data in Table 37 were subjected to regression analysis, using the following quadratic equations (40)-(42):

$$Y_{15} = -77.97 + 30.20X_{16} + 0.04X_{17} + 2.02X_{18} + 0.003X_{16}X_{17} + 0.08X_{16}X_{18} - 0.0001X_{17}X_{18} - 2.92X_{16}^2 - 0.0002X_{17}^2 - 1.16X_{18}^2 \quad (40)$$

$$Y_{16} = -5.98 + 2.31X_{16} + 0.003X_{17} + 0.23X_{18} + 0.0003X_{16}X_{17} + 0.01X_{16}X_{18} - 0.22X_{16}^2 - 0.00002X_{17}^2 - 0.14X_{18}^2 \quad (41)$$

$$Y_{17} = -2.16 + 0.84X_{16} + 0.001X_{17} + 0.056X_{18} + 0.0001X_{16}X_{17} + 0.0025X_{16}X_{18} - 0.000008X_{17}X_{18} - 0.08X_{16}^2 - 0.00001X_{17}^2 - 0.03X_{18}^2 \quad (42)$$

The models presented the high determination coefficients (R^2 = 0.98, 0.98 and 0.98, respectively) (Table 38) explaining 98% of variability in all responses of ethanol concentration, ethanol yield and ethanol productivity. The adjusted determination coefficients (adjusted R^2 = 0.97, 0.97 and 0.97, respectively) indicated the high significance of these models (O-Thong *et al*., 2008). In addition, the ANOVA quadratic regression demonstrated that among models were significant, as evidenced from high *F*-values (F=65.77, 60.90 and 64.72, respectively) with a very low probability ($P<0.0001$). Low variation coefficient value (C.V.=11.86%, 12.27% and 11.96%, respectively) indicated a high precision and reliability of the experiments (Table 38) (O-Thong *et al*., 2008). The significance of each coefficient was determined by probability values. The variables with a significant effect on ethanol production were the initial pH (X_{16}) and shaking speed (X_{17}) ($P<0.05$). Linear term of X_{16} and quadratic terms of X_{16}^2 and X_{17}^2 were significant ($P<0.05$), demonstrating that maximizing ethanol production required a suitable value of initial pH and shaking speed.

Estimation of ethanol concentration (Y_{15}), ethanol yield (Y_{16}) and ethanol productivity (Y_{17}) over X_{16}, X_{17} and X_{18} in terms of response surfaces are shown in Fig. 57. The effect of initial pH and shaking speed was studied on ethanol production when initial cells concentration was fixed at 1.2 g/l (Fig. 57a, 57d and

57g). The maximum ethanol concentration (5.15 g/l), ethanol yield (0.40 g ethanol/g sugar) and ethanol productivity (0.143 g/l/h) were achieved at initial pH of 5.20 and shaking speed of 120 rpm whereas the minimum value (0-3 g/l, 0-0.24 g ethanol/g sugar and 0-0.081 g/l/h, respectively) was obtained at initial pH of 4.0 and 6.0, and at shaking speed of 60 and 180 rpm. These results indicated that both initial pH and shaking speed had significant effect on ethanol production as they affected directly on fermentation concerning on alcohol dehydrogenase (ADH) activity (Prior *et al*., 1988). The optimal initial pH of 5.20 gave the highest ADH activities (Nie *et al*., 2007). For the effect of shaking speed (optimum at 120 rpm), oxygen-limitation condition was reported to increase the specific activity of ADH up to 4-folds with the occurrence of ethanol accumulation in *C. shehatae* (Prior *et al*., 1988). Excess oxygen is detrimental because xylose-fermenting yeasts appear to produce as well as consume ethanol at the same time (Alexander *et al*., 1988).

The response surface plots of initial pH and initial cells concentration interaction were given in Fig. 57b, 57e and 57h when shaking speed was fixed at 120 rpm. These figures indicated that only the initial pH had a significant effect on ethanol production while the initial cells concentration had no effect. The effect of initial pH on ethanol production has been studied in *Saccharomyces cerevisiae* and *Zymomonas mobilis*. The optimum initial pH of both strains was 4.18-6.39 (Wang *et al*., 2008; Yu *et al*., 2009) and 4.93 (Bandaru *et al*., 2006), respectively, giving the highest ethanol production. The response surface plots of shaking speed and initial cells concentration interaction were given in Fig. 57c, 57f and 57i when the initial pH was fixed at 5.0. These figures illustrate that the shaking speed had a significant effect on ethanol production while the initial cells concentration had no effect. These results are similar with the report of Sreekumar et al (1999). The inoculum concentration (6 and 10 log CFU) had no effect on ethanol production by *Zymomonas mobilis* (6.57 and 5.71% w/v). However, the effect of inhibition is reduced when higher cells inocula were used (Palmqvist *et al*., 1996). The substances give rise to a lag phase proportional to their concentration. The ethanol productivity decreased, but the maximum ethanol yield was constant (Palmqvist *et al*., 1996).

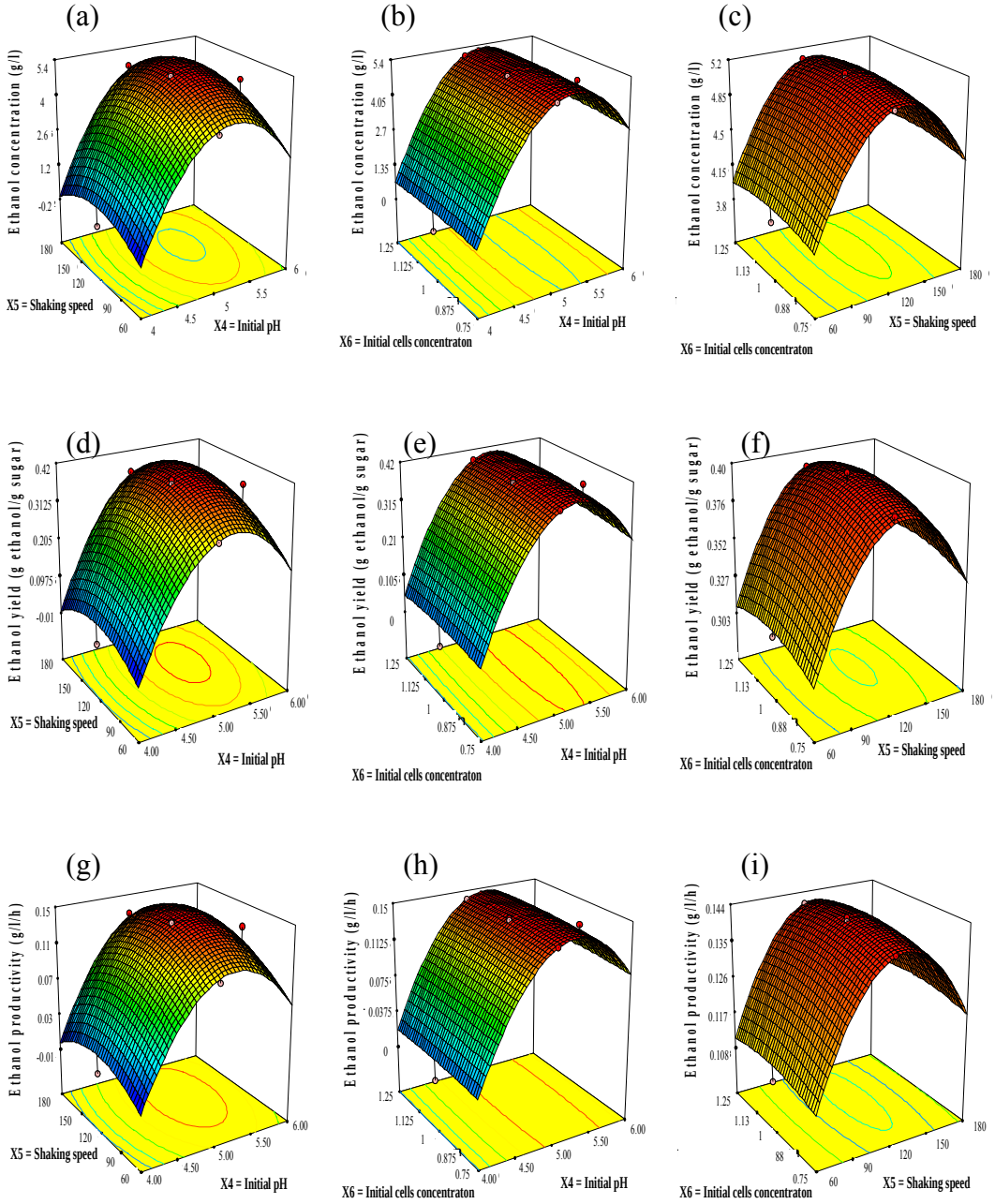


Figure 57. Three-dimensional graphs of the quadratic model of ethanol production in PPF hydrolysate by *Candida shehatae* TISTR5843: ethanol concentration (a-c), ethanol yield (d-f) and ethanol productivity (g-i) within the central composite design (CCD): a, d and g; fixed initial cells concentration at centre point of 1.2 g/l, b, e and h; fixed shaking speed at centre point of 120 rpm, c, f and i; fixed initial pH at centre point of 5.0.

Table 38. Analysis of variance (ANOVA) for ethanol production in PPF hydrolysate medium.

Responses (Y)	Source	Sum of square	Degree of freedom	Mean square	F-value	P-value
Ethanol concentration (Y_{15})	Model	85.53	9	9.50	65.77	< 0.0001
	R^2	0.98				
	Adjusted R^2	0.97				
	C.V.	11.86				
Ethanol yield (Y_{16})	Model	0.51	9	0.057	60.90	< 0.0001
	R^2	0.98				
	Adjusted R^2	0.97				
	C.V.	12.27				
Ethanol productivity (Y_{17})	Model	0.066	9	0.001	64.72	< 0.0001
	R^2	0.98				
	Adjusted R^2	0.97				
	C.V.	11.96				

3.10.5.2 Confirmation experiments and adequacy of the models of ethanol production from PPF hydrolysate

To confirm the validity of the statistical experimental strategies of ethanol production from PPF hydrolysate, three replicates of batch experiments were performed under the optimal condition calculated by RSM, containing initial pH of 5.25, shaking speed of 135 rpm and initial cells concentration of 1.08 g/l (OD_{600}=0.9). The results from confirmation experiments indicate that the experimental values of ethanol production (5.25 ± 0.72 g/l, 0.40 ± 0.02 g ethanol/g sugar, and 0.146 ± 0.011 g/l/h) were agreed with the predicted values (5.32 g/l, 0.41 g ethanol/g sugar, and 0.148 g/l/h). There was no significant difference between the experimental values and the predicted values *(P>0.05)*. The ethanol production using the initial condition (control) (Table 39) and the adjusted condition (Table 39, trial 10) were 4.12 and 5.25 g/l, respectively. After optimizing the condition of ethanol production from this hydrolysate medium, ethanol concentration, ethanol yield and ethanol productivity increased 1.27, 1.21 and 2.56 folds, respectively.

3.10.6 Effect of sterilized and non-sterilized PPF hydrolysate mediums on ethanol production by *C. shehatae* TISTR5843

Due to inhibitors contained in PPF hydrolysate medium, sterilization and non-sterilization of this medium for ethanol production have to compare in order to prevent inhibitors generation and saving of energy. This study has been controlled under optimum condition of initial pH of 5.25, shaking speed of 135 rpm, cells inoculums of 1.08 g/l (OD_{600}=0.9) (Section 3.10.5) and room temperature for 108 h. Xylose concentration of sterilized and non-sterilized PPF hydrolysate medium were consumed closely to zero by *C. shehatae* TISTR5843 within 36 h (Fig. 58a) giving maximum of ethanol production of 4.71 and 4.47 g/l, respectively (Fig. 58a). Ethanol yields and productivities of both mediums were 0.38, 0.37 g/g, and 0.131, 0.124 g/l/h, respectively. After statistical determination, there were not significant differences in both ethanol yield and ethanol productivity between sterilized and non-sterilized PPF hydrolysate medium (*P>0.1*). Thus, non-sterilized PPF hydrolysate medium was used for production of ethanol in the scale up experiment. The pH change, acetate and furfural reduction of both mediums had the same trend (Fig. 58b and 58c).

Table 39. The confirmation experiments for ethanol production in PPF hydrolysate medium (13 g/l xylose) by *C. shehatae* TISTR5843 cultivated under the optimal condition.

Substrates	Trials	Condition	Initial pH	Shaking speed (rpm)	Initial cells concentration (g/l)	Ethanol concentration (g/l)	Ethanol yield (g/g sugar)	Ethanol productivity (g/l/h)
PPF	-	Optimal[a]	5.25	135	1.08	5.25 ± 0.72	0.40 ± 0.02	0.146 ± 0.011
hydrolysate	13	Central	5.00	120	1.20	5.13 (36 h)	0.39	0.142
medium	-	Selected	5.00	180	0.5	4.12 (72 h)	0.33	0.057

[a] Based on ethanol production.

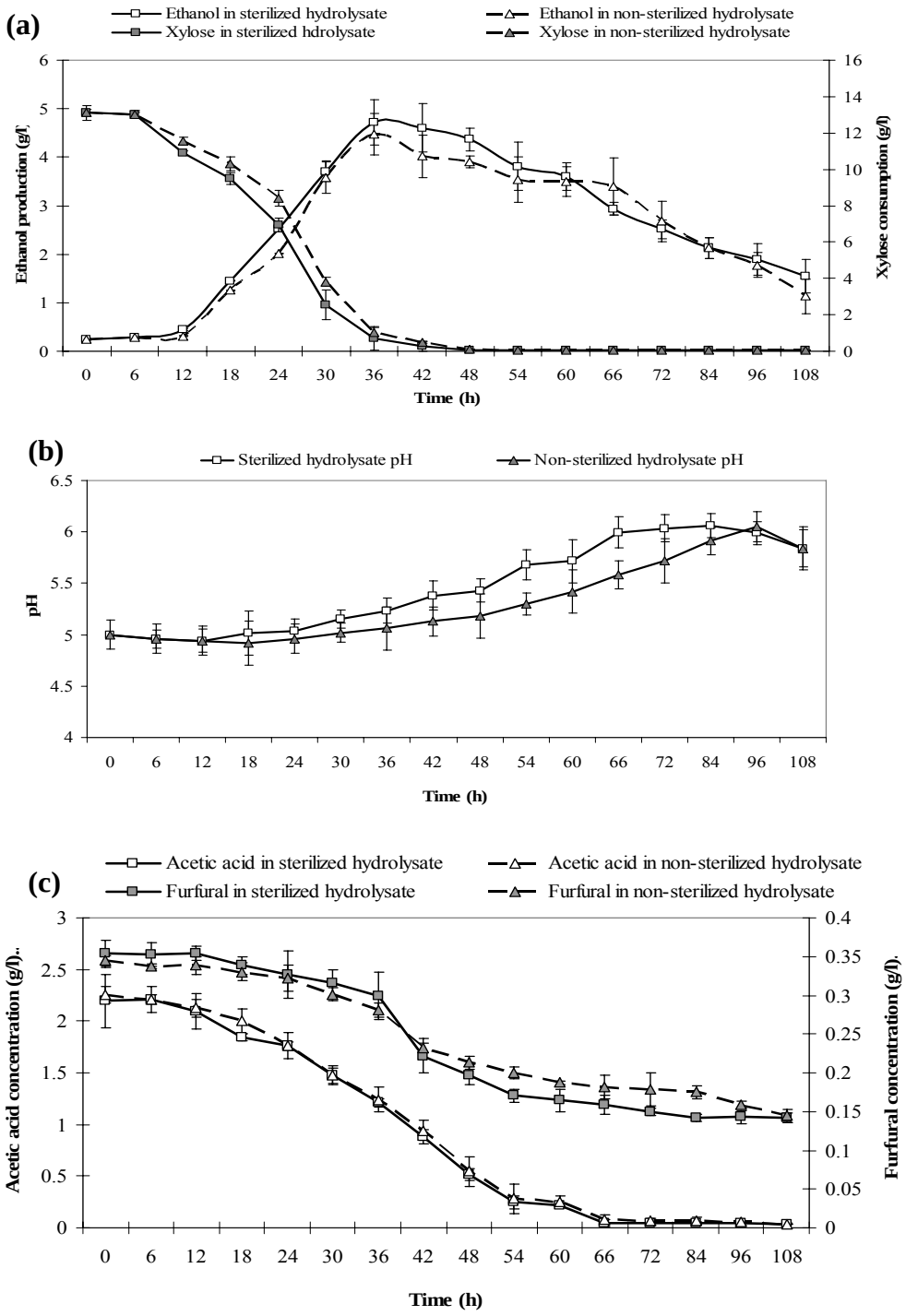


Figure 58. Time course of xylose consumption and ethanol production (a), pH change (b), and acetate and furfural reduction (c) by *Candida shehatae* TISTR5843 under sterilized and non-sterilized PPF hydrolysate medium (pH5.25) with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm).

3.10.7 Ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate medium in 3 L reactor

3.10.7.1 Comparison of ethanol production from Erlenmeyer flask and 3-L reactor using batch process

Prior to study the scale up of ethanol production in PPF hydrolysate medium by *C. shehatae* TISTR5843, comparison on ethanol production in Erlenmeyer flask (250 ml) and reactor (3 liters) have to been done in order to obtain some available data, i.e. cultivation for maximum ethanol production, cultivation for inhibitors reduction, and comparison of ethanol production between flask and reactor due to the difference of container, which is perhaps affected. In this experiment, PPF hydrolysate medium was prepared and operated under non-sterilized with 1/2 dilution with initial pH of 5.25, and agitation speed of 135 rpm. The initial cells inoculum was 1.08 g/l. The routine experiment was carried out at room temperature (30°C) on an agitation speed (135 rpm) for 120 h.

Xylose concentration of non-sterilized PPF hydrolysate medium in both flask and reactor were consumed closely to zero by *C. shehatae* TISTR5843 within 48 h giving maximum of ethanol production of 4.47 and 4.07 g/l, respectively (Fig. 59a). Ethanol yields and productivities of both systems were 0.40, 0.39 g/g, and 0.093, 0.084 g/l/h, respectively. However, the ethanol productivity was decreased from 0.124 (see section 3.10.6) to 0.084 g/l/h due to expansion of cultivation for maximum ethanol production from 36 h (see section 3.10.6) to 48 h. A longer lag phase might be from the inhibitory compounds in hydrolysate. The furfural content in this study was approximately 360 mg/l in both flask and reactor (Fig. 59d) which was higher than that furfural content in the section 3.10.6 (330-340 mg/l) resulting in longer cultivation time. Meanwhile the acetic acid content (~2200 mg/l) was constant (Fig. 59c). Moreover, there was no significant difference between flask and reactor in both pH change and DCW (P>0.05) (Fig. 59b).

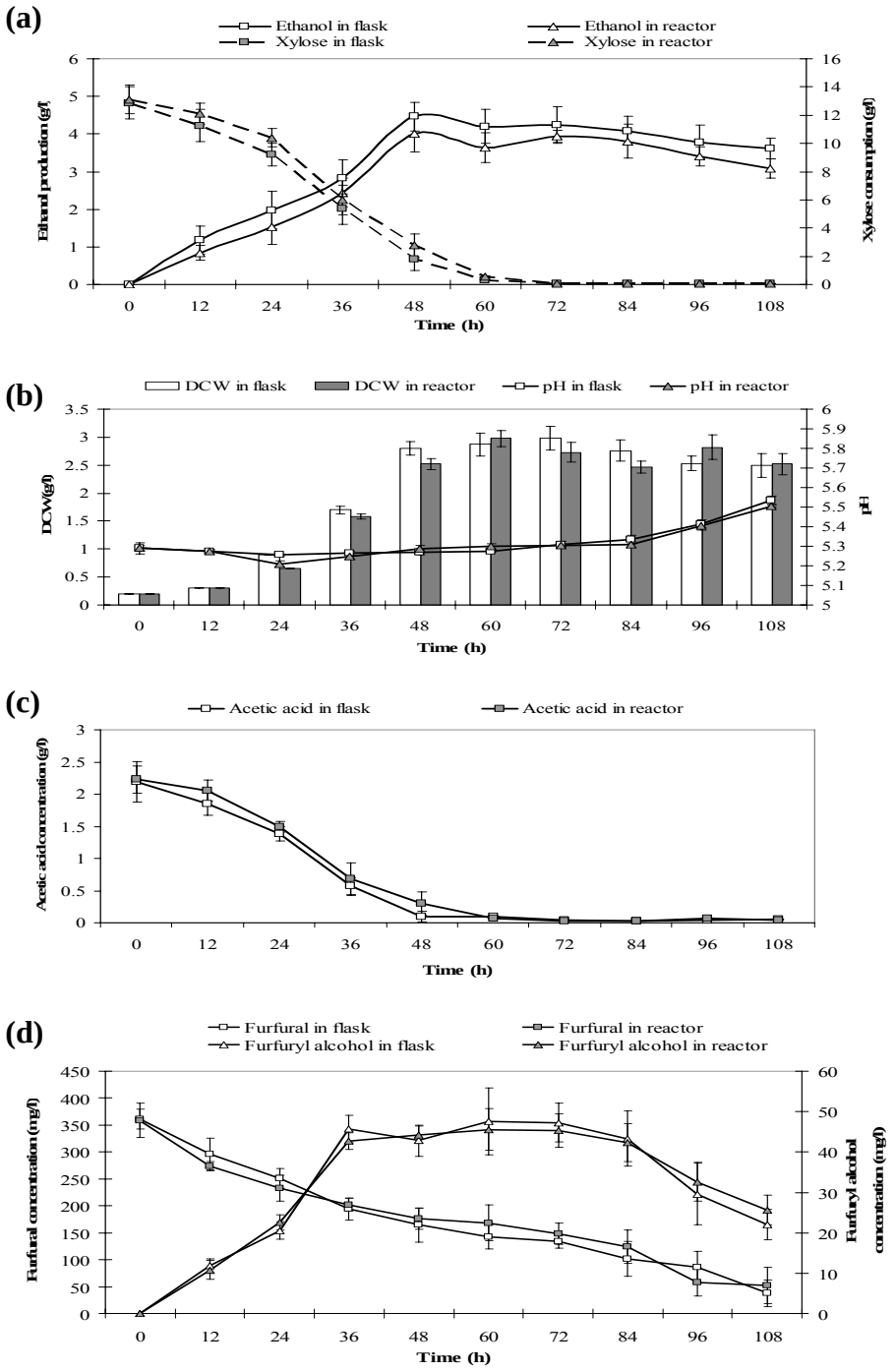


Figure 59. Time course of xylose consumption and ethanol production (a), pH change and DCW (b), acetic acid reduction (c), furfural reduction and furfuryl alcohol production (d) by *Candida shehatae* TISTR5843 under non-sterilized with 1/2 dilution of PPF hydrolysate medium (pH 5.25) in both flask and reactor with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm).

3.10.7.2 Ethanol production in PPF hydrolysate medium under fed-batch fermentation

To increase the ethanol production from PPF hydrolysate medium by *C. shehatae* TISTR5843, a fed-batch process was studied. Fed-batch culture is a batch culture feeding continuously or sequentially with substrate without the removal of fermentation broth. It is widely used for the production of microbial biomass, ethanol, organic acids, antibiotics, vitamins, enzymes and other compounds (Roukas, 1996). Fed-batch culture compared to the conventional batch culture has several advantages including very low concentration of residual sugars, higher dissolved oxygen in the medium (from added fresh medium), decreased fermentation time, higher productivity and reduced toxic effects of the medium components which are present at high concentrations (Roukas, 1996) as well as eliminating substrate inhibition (Ozmichi and Kargi, 2007). This work was carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium. The fresh medium added into the reactor in the second and next cycle has been no diluted in order to control the system was 1/2 dilution. The data from the batch process (section 3.10.7.1) were used for operating the fed-batch system including maximum ethanol production time (48 h, Fig. 59a), xylose residue and inhibitory compounds residues. The experimental kinetic values of ethanol production, cells growth and inhibitors reduction in the bioreactor during 6 days (3 cycles) of fed-batch fermentation of PPF hydrolysate medium (13 g/l of xylose) are presented in Table 40. The ethanol concentration in broth from bioreactor gave the maximal value of 3.92 g/l in the first cycle and slightly decreased in the further cycles (from 3.92 to 3.61 g/l and finally to 2.99 g/l at the last cycle (Table 40)). The cause of this phenomenon is from inhibitory compounds (furfural and acetate) presented in PPF hydrolysate medium (Fig. 60c and 60d) (Delgenes *et al*., 1996). Fig. 60c and 60d indicated that *C. shehatae* TISTR5843 was not able to completely reduce furfural and acetate within 48 h (the first cycle) resulted in the accumulation of these toxic compounds to the next cycles (Fig. 61). Therefore, substrate uptake rate and ethanol production were decreased in the subsequent cycles (Fig. 61).

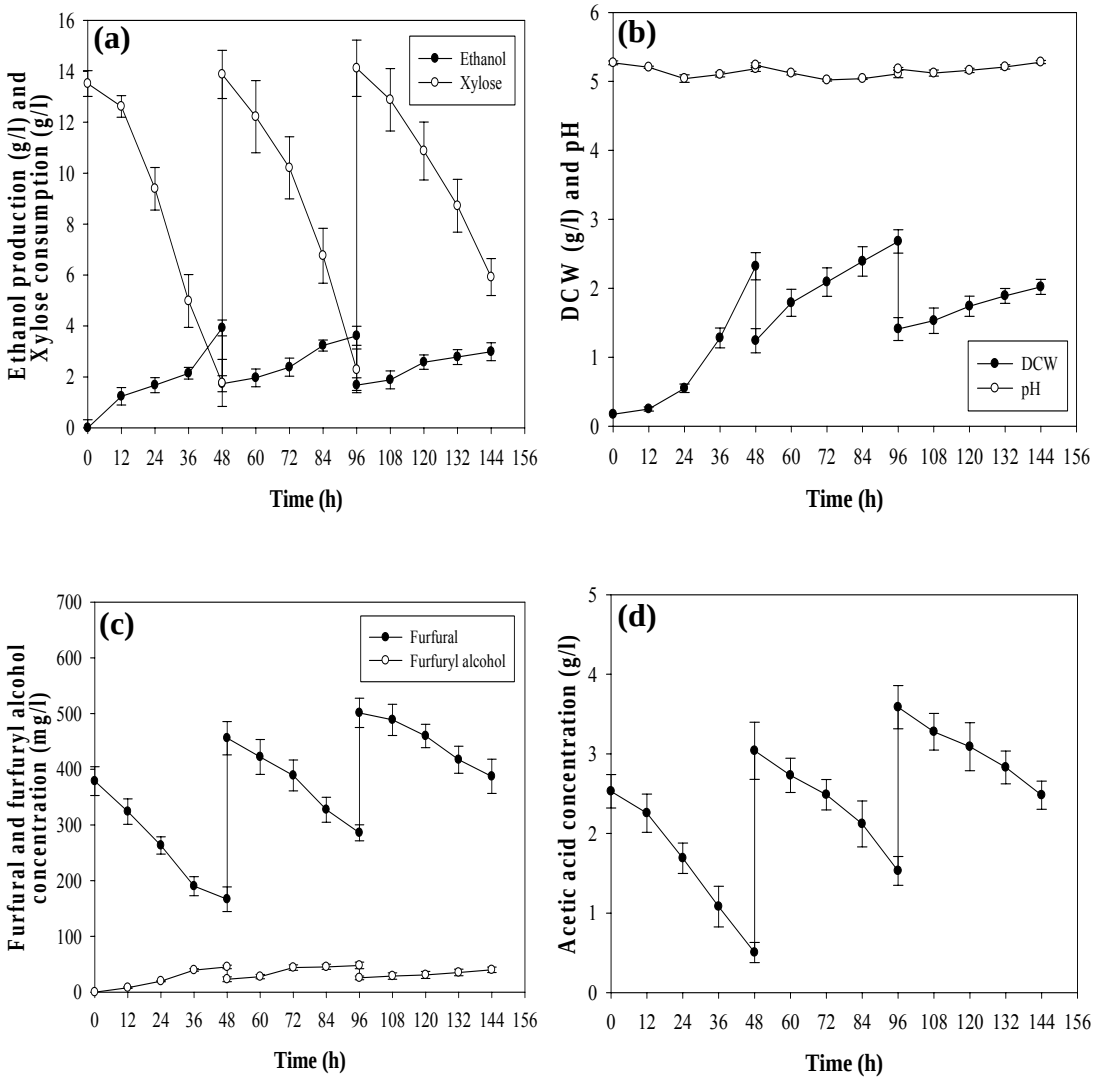


Figure 60. Time course of xylose consumption and ethanol production (a), pH change and DCW (b), furfural reduction and furfuryl alcohol generation (c), acetic acid reduction (d) by *Candida shehatae* TISTR5843 under non-sterilized fed-batch process with 1/2 dilution (pH5.25) in 3 liters reactor with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm) for 144 h.

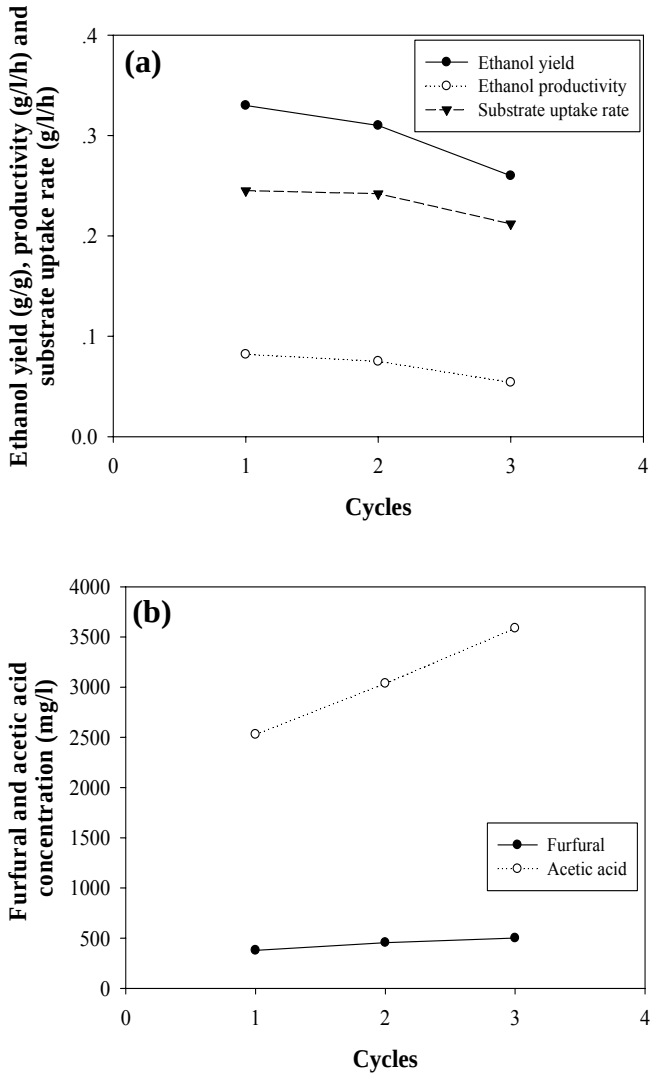


Figure 61. Reduction of ethanol production and substrate uptake rate (a) and accumulation of furfural and acetate (b) in all cycles of fed-batch fermentation by *Candida shehatae* TISTR5843 in 3 liters non-sterilized PPF hydrolysate.

Table 40. Fermentative kinetics of fed-batch and semi-continuous processes in PPF hydrolysate medium by *Candida shehatae* TISTR5843.

	Fed-Batch			Semi-continuous		
	1st	2nd	3rd	1st	2nd	3rd
Maximum ethanol (*P*, g/l)	3.92	3.61	2.99	4.02	3.71	2.79
Ethanol yield (*Yp/s*, g/g)	0.33	0.31	0.26	0.36	0.34	0.20
Substrate uptake rate (*Qs*, g/l.h)	0.245	0.242	0.212	0.232	0.229	0.181
Ethanol productivity (*Qp*, g/l.h)	0.082	0.075	0.054	0.084	0.077	0.058
Conversion of substrate to ethanol (%)	86.98	83.57	72.24	85.06	79.82	63.88

3.10.7.3 Ethanol production in PPF hydrolysate medium under semi-continuous fermentation

When a portion of the fermentation broth is withdrawn at intervals and the residual part of the culture is used as an inoculum for the next batch culture, the system is operated as a repeated fed-batch culture or semi-continuous culture. In addition to increased productivity, semi-continuous culture has the advantages which are (i) it doesn't require new inocula for each consecutive fed-batch and (ii) the contamination of the medium is lower than in the continuous culture. Thus semi-continuous culture is considered one of the most useful systems for economical ethanol production (Roukas, 1996). This work was also carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium.

The experimental kinetic values of ethanol production in the bioreactor during 6 days (3 cycles) of semi-continuous fermentation of PPF hydrolysate medium (13 g/l of xylose) are presented in Table 40. The ethanol concentration in broth drained from bioreactor gave the maximal value of 4.02 g/l in the first cycle and slightly decreased in the further cycles (from 4.02 to 3.71 g/l and finally to 2.79 g/l at the last cycle (Table 40)). The causes of this phenomenon were described in the previous section 3.10.7.2 and shown in Fig. 62 and 63.

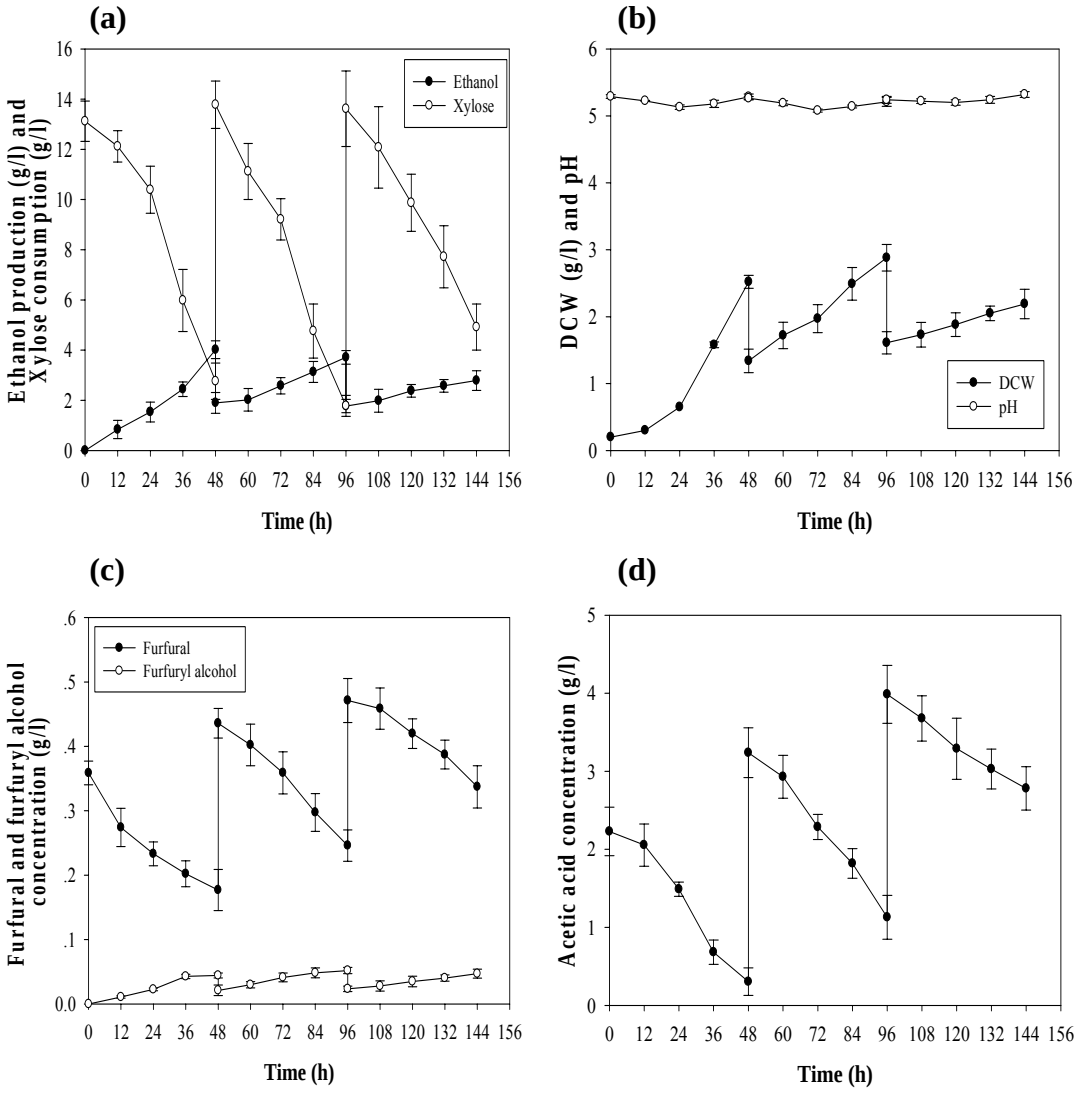


Figure 62. Time course of xylose consumption and ethanol production (a), pH change and DCW (b), furfural reduction and furfuryl alcohol generation (c), acetic acid reduction (d) by *Candida shehatae* TISTR5843 under non-sterilized semi-continuous process with 1/2 dilution of PPF hydrolysate medium (pH5.25) in 3 liters reactor with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm) for 144 h.

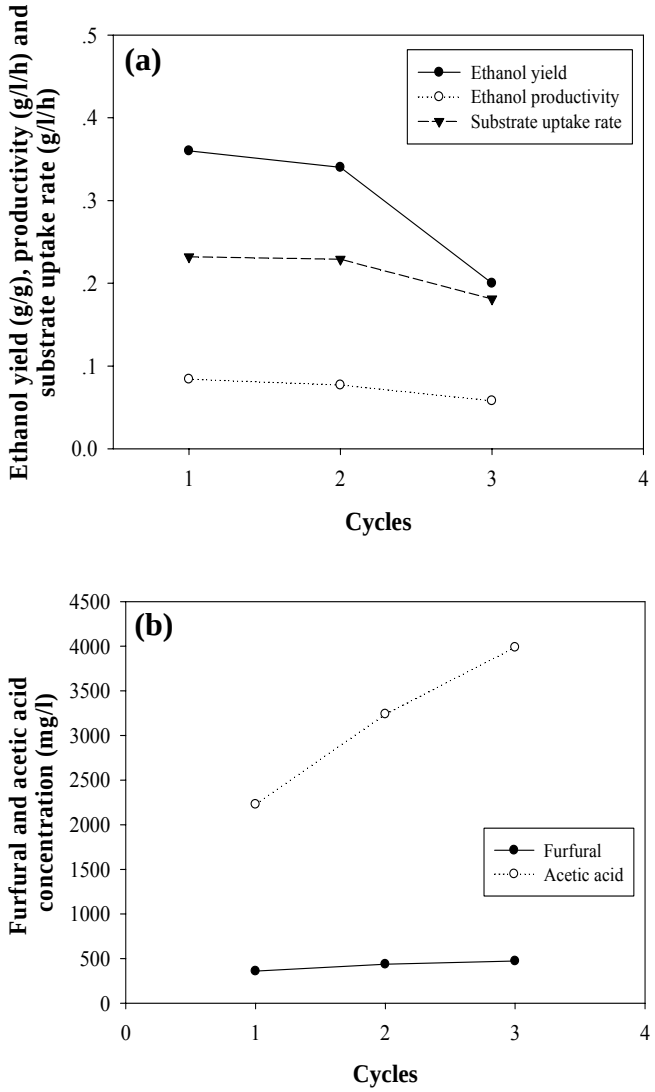


Figure 63. Reduction of ethanol production and substrate uptake rate (a) and accumulation of furfural and acetate (b) in all cycles of semi-continuous fermentation by *Candida shehatae* TISTR5843 in a 3 liters non-sterilized PPF hydrolysate.

3.11 Potential of ethanol production by immobilized yeast cells on PPF as a natural support

3.11.1 Immobilization of yeast cells on PPF and delignified PPF (dPPF)

A limitation of the operational stability, which is a cell desorption in immobilization system has been an issue (Yu *et al*., 2007). Scanning electron microscopic technique was used as a tool to study the yeast cells adsorption in sPPF, lPPF and sDPPF (Fig. 64). The lPPF is fibrous and non porosity form (Fig. 64a). After milling process, the surfaces area and porosity increased in sPPF as well as in sDPPF (Fig. 64b and 64c). The yeast cells were observed on the outer surface of lPPF only (Fig. 64d); however, a population of yeast cells was observed on all surfaces of sPPF and sDPPF (Fig. 64e and 64f). The porous structure of sPPF and sDPPF increased mass transportation which resulted in high biomass concentration in the range of 3.71- 4.20 g DCW/l (Fig. 64d-64f).

3.11.2 Kinetic analysis of immobilization for ethanol production in the batch fermentation

The kinetic values were investigated with respect to the use of PPF as a natural support for ethanol production by *C. shehatae* TISTR5843 (Table 41). The maximum ethanol production (P_{max}) was observed at 36 h cultivation by immobilized yeast cells on lPPF, sPPF and sDPPF with the values of 10.7, 11.3 and 11.5 g l^{-1}, respectively (Fig. 65 and Table 41). The P_{max} values of immobilized cells on sPPF and sDPPF increased 4.2 and 6.2%, respectively; however, this value of immobilized cells on lPPF was not significantly different from the value of thoes free cells ($P<0.05$). The results are similar to the study of Behera et al (2010) which reported the ethanol productions by yeast cells entrapped in Ca-alginate was higher than free cells. The substrate uptake rate (*Qs*) of immobilized cells on sPPF and sDPPF were higher than that of free cells with the values of 5.25 and 5.46%, respectively. Similarly, the ethanol yield (*Yp/s*) obtained from immobilized cells was higher than that of free cells with the values of 4.55 and 6.82%, respectively. Correspondingly, the ethanol productivity (*Qp*) and sugar consumption (%) of immobilized cells were slightly more than to that of free cells. All cell concentrations were rapidly increased within 30 h cultivation and slightly increased thereafter due to the limitation of carbon source

(Fig. 65c). Generally, the performance of ethanol production by immobilized cells is much better than free cells because the immobilization system protect cell from ethanol inhibition (Abbi *et al*., 1996).

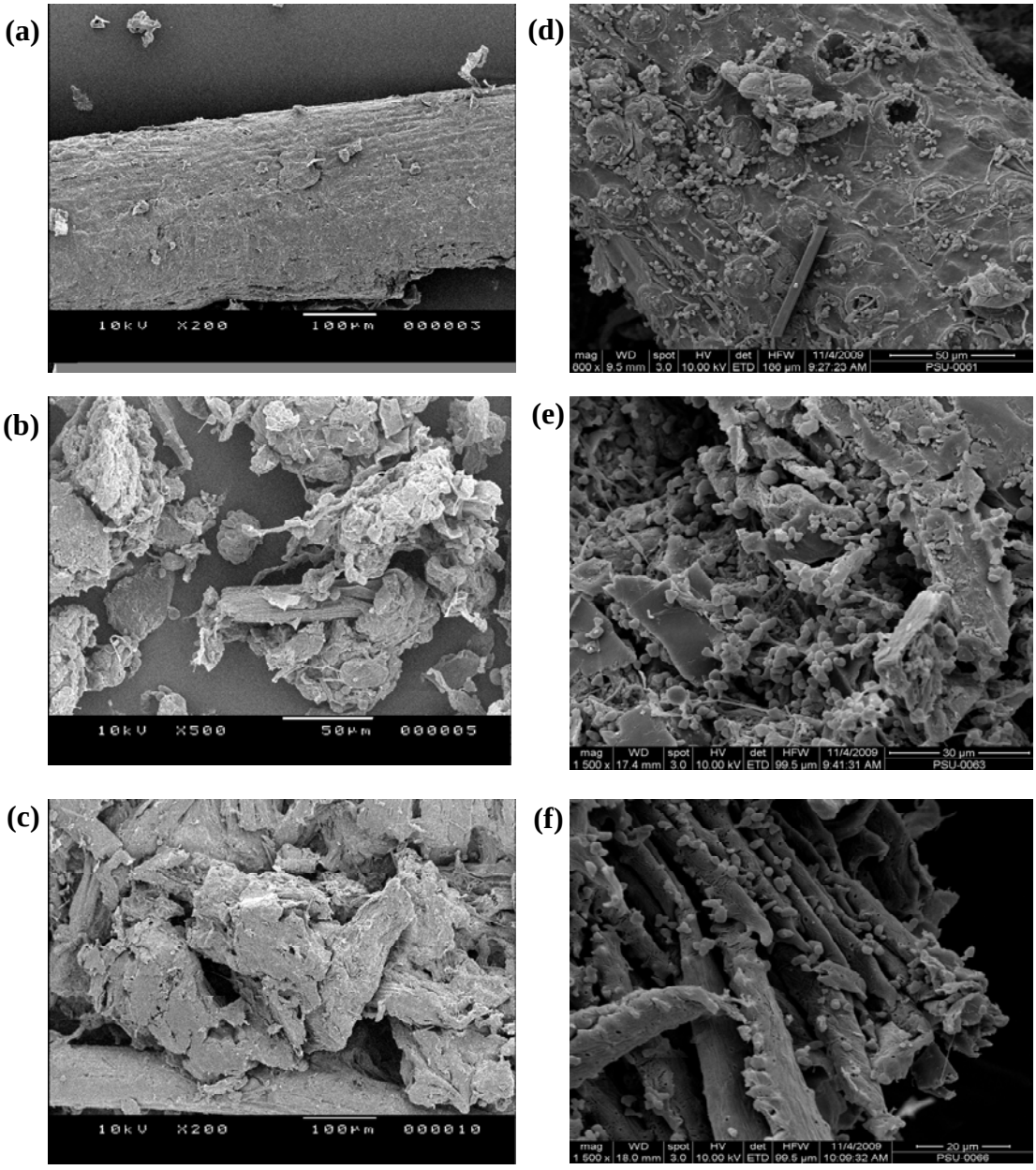


Figure 64. Scanning electron micrograph of (a) lPPF, (b) sPPF and (c) sDPPF; and immobilized cells adhere to the surface of (d) lPPF, (e) sPPF and (f) sDPPF.

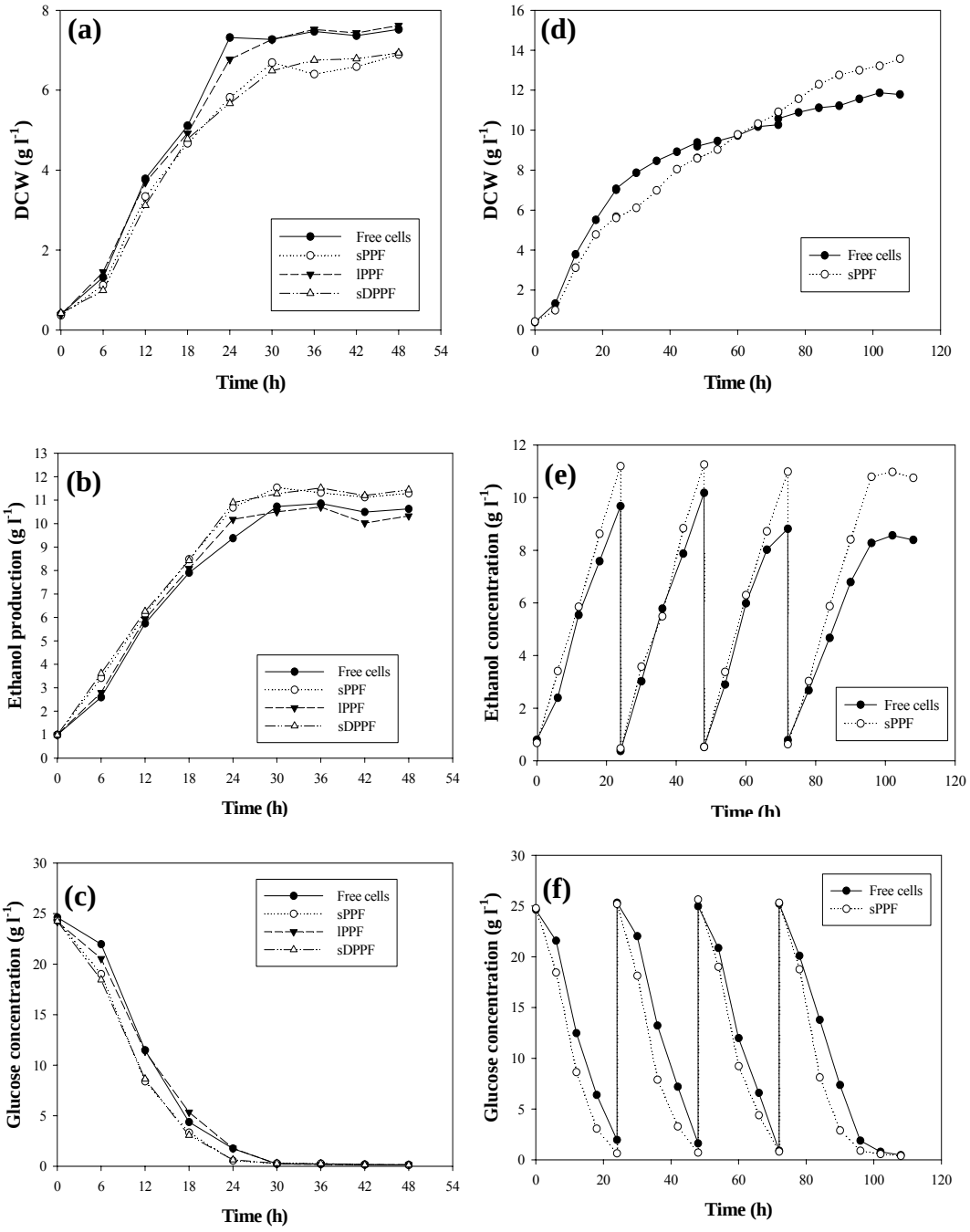


Figure 65. Kinetics of ethanol production by free and immobilized cells of *Candida shehatae* TISTR5843 in batch fermentation, in panel (a) cell growth, (b) ethanol production and (c) glucose consumption; and in repeated batches fermentation, in panel (d) cell growth, (e) ethanol production and (f) glucose consumption at room temperature (30°C) with shaking speed 150 rpm.

The ethanol yields by free and immobilized cells obtained from this study consist with theoretical value (Table 41). The sPPF was chosen as a support in further study of repeated batches fermentation because the ethanol yield was not significantly different compared to ethanol production by immobilized on sDPPF ($p<0.05$). Also, sDPPF is concerned in the sector of environment and economic because PPF need to be treated by chemical (delignification).

Table 41. Growth and fermentation kinetics of free and immobilized cells by *Candida shehatae* TISTR5843 adhered on various supports in batch fermentation.

	Free cells	Immobilized cells on		
		lPPF	sPPF	sDPPF
Maximum ethanol production (P_{max}, g ethanol/l)	10.8[a]	10.7[a]	11.3[b]	11.5[b]
Maximum cells concentration (X_{max}, g DCW/l)	7.52	7.62	6.89	6.93
Specific growth rate (μ, h^{-1})	0.02	0.02	0.02	0.02
Cell yield (*Yx/s*, g DCW/g glucose)	0.31	0.31	0.28	0.29
Ethanol yield (*Yp/s*, g ethanol/g glucose)[c]	0.44 [a]	0.44 [a]	0.46 [b]	0.47 [b]
Substrate uptake rate (*Qs*, g glucose/l/h)	0.95	0.93	1.00	1.00
Ethanol productivity (*Qp*, g ethanol /l/h)	0.30	0.30	0.31	0.32
Glucose consumption (%)[d]	98.2	98.0	99.1	99.0

All cases were performed in triplicate; values varied less that 10%.

[a] and [b] are significant difference at $p<0.05$.

[c] $$Ethanol\ yield\ (g\ ethanol\ g\ glucose^{-1}) = \frac{Ethanol\ production\ (g\ l^{-1})}{Glucose\ consumption\ (g\ l^{-1})}$$

[d] $$Glucose\ consumption\ (\%) = \frac{Glucose\ consumption\ (g\ l^{-1})}{Glucose\ content\ in\ medium\ (g\ l^{-1})} \times 100$$

3.11.3 Kinetic analysis of immobilization for ethanol production in the repeated batch fermentation

To increase ethanol productivity and retain the ethanol yield by recycling the cells, the repeated batches fermentation was studied. The free and immobilized cells were recycled into a fresh medium every 24 h. The cultivation conditions were pH 5.0 at room temperature (30°C) with an agitation speed of 150 rpm. A comparison of ethanol production by free and immobilized cells in four repeated batch experiments was shown in Fig. 65. The ethanol concentrations, ethanol yields and ethanol productivities of free and immobilized cells increased in repeated batches fermentation in the range of 10.78-30.12%, 9.52-22.22% and 11.90-32.35%, respectively (Table 42).The ethanol yield of immobilized cells decreased 9.52 % after the second cycle and 6.38% at the forth cycle (Table 42). It has been reported that ethanol production by immobilized cells on spent grain and delignified spent grain in four repeated batches process decreased 22.81% and 15.08%, respectively, (Kopsahelis *et al*., 2007). Ethanol production by immobilized *C. shehatae* NCL-3501 was constant for 3 cycles and decreased thereafter (Abbi *et al*., 1996).

Both ethanol productivity and ethanol yield of immobilized cells were greater than free cells. There is an evidence supported that the significantly higher percentage of saturated fatty acids in immobilized cells leads to the grater ethanol tolerance, greater yeast cells survival and greater ethanol productivity compared to free cells (Krisch and Szajáni, 1997). In addition, immobilized cells can retain enzyme activities for a long time because of the physiological changes in cells which affect the cell compositions such as proteins, lipids, RNA, DNA, and inorganic substances (Yu *et al*., 2007).

In conclusion, the pretreatment of PPF, milling and delignification increase surface area of natural support, which enhances cell adsorption and ethanol production. The immobilized yeast demonstrates the operational stability without decrease its activity. Therefore, PPF has a potential as a natural supporting material in the immobilization system.

Table 42. Fermentative kinetics of free and immobilized cells by *Candida shehatae* TISTR5843 adhered on sPPF in four repeated batches fermentation.

	Free cells				Immobilized cells on sPPF			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Maximum ethanol production (P_{max}, g ethanol l^{-1})	9.7	10.2	8.8	8.3	11.2	11.3	11.0	10.8
Ethanol yield (*Yp/s*, g ethanol g glucose^{-1})	0.42	0.42	0.38	0.36	0.46	0.47	0.44	0.44
Substrate uptake rate (*Qs*, g glucose $l^{-1}h^{-1}$)	0.94	0.99	1.00	0.97	1.00	1.02	1.03	1.02
Ethanol productivity (*Qp*, g ethanol $l^{-1}h^{-1}$)	0.40	0.42	0.37	0.34	0.47	0.47	0.46	0.45
Glucose consumption (%)	92.0	93.6	96.4	92.5	97.4	97.1	96.8	96.5

All cases were performed in triplicate; values varied less that 10%.

CHAPTER 4

CONCLUSION

4.1 Conclusion

1. Palm pressed fiber (PPF) compositions are 32.06% cellulose, 25.83% hemicelluloses and 17.28% lignin. The delignified palm pressed fiber (dPPF) contained 37.70% cellulose, 34.67% hemicelluloses and 7.31% lignin (based on 1 g PPF).
2. Extraction of hemicellulose from dPPF by alkaline method was optimized by the response surface methodology (RSM). The optimum condition was 28.8% (w/v) KOH concentration, the PPF to KOH ratio of 1:20 (w/v), and reaction time of 20 min, giving the highest hemicellulose yield of 38.67 ± 1.21% with 99.25% extraction. Under these conditions, the hemicellulose was composed of 80.8% xylose, 15.7% glucose, 3.2% acetic acid and <0.38% furfural.
3. The optimum condition of one-stage process for furfural production from extracted hemicelluloses was 150°C, sulfuric acid to hemicellulose ratio (liquid to solid, L/S ratio) of 8 ml/g, sulfuric acid concentration of 5% v/v for 90 min reaction time. The maximum furfural yield was 0.86 g/l or 3.44 wt%.

   Two-stage process for furfural production was consisted of hydrolysis followed by dehydration process. In the acid hydrolysis step, the optimum conditions were 120°C, 5.7% sulfuric acid, L/S ratio of 8.5 ml/g for 31 min (R^2 = 0.90). The maximum yield of xylose was 12.32 ± 2.42 g/l. In the dehydration process, the optimum reaction temperature was 135°C and reaction time of 90 min (R^2 = 0.93). The maximum furfural production was 8.67 ± 0.62 g/l.
4. After hemicelluloses extraction, the cellulose was used for glucose production by cellulase (*Trichoderma reesei*). The 7.9 g/l of maximum reducing sugar mainly glucose (60% saccarification) was achieved when incubated 12 g/l of

the extracted cellulose with cellulase of 4,166 U/g substrate under the optimum condition at pH 4.8 incubated for 50 °C for 900 min.

5. The glucose production from the extracted cellulose by acid hydrolysis was conducted by two-stage process, firstly it was treated by 72% sulfuric acid and followed by 4% sulfuric acid hydrolysis using solid/liquid ratio (SLR) of 1:16 (w/v) at 120 °C for 86 min. The 0.54 g/l of glucose was produced under those conditions.
6. Xylose production by dilute acid hydrolysis was achieved by RSM. The optimum condition was the 2% (v/v) H_2SO_4 at 120 ºC for 30 min, giving the highest xylose and glucose yields of 27.23 and 2.3 g/l. In addition, the concentrations of acetic acid and furfural, which are inhibitors in the fermentation, were 5.99 g/l and 0.42 g/l, respectively.

   The ethanol production in synthetic medium by *Candida shehatae* TISTR5843 was studied. When cultured the yeast cell in the synthetic medium containing glucose as a sole carbon source, the optimum concentration of glucose and xylose were found to be 24 g/l and 20 g/l, respectively. The optimum glucose to xylose ratio in the medium was 2:8 w/w with the initial pH of 5. Incubation conditions were achieved at room temperature (30°C) with the shaking speed of 180 rpm. The highest ethanol yields and ethanol productivities at optimum condition were 0.42-0.45 g ethanol/ g sugar, and 0.103-0.343 g/l/h, respectively.
7. *Saccharomyces cerevisiae* TISTR5017 was found to be the best strain for ethanol production in cellulosic hydrolysate. The highest ethanol yield and ethanol productivity were 0.34 g/g sugar, and 0.118 g/l/h, respectively.
8. The optimum condition for ethanol production in cellulosic hydrolysate by *S. cerevisiae* TISTR5017 was the initial pH of 5.40, shaking speed at 137 rpm and initial cells concentration of 0.56 g/l. The ethanol production was 3.98 g/l with the ethanol yield of 0.48 g ethanol/g sugar, and the productivity of 0.167 g/l/h.
9. The optimum nitrogen source and concentration was 3 g/l peptone, and C/N ratio of 9.3. The inhibitory compounds in PPF hydrolysate for ethanol

production by *Candida shehatae* TISTR5843 were acetate, furfural, and vanillin, should be less than 2.5, 0.5, and 0.5 g/l, respectively. The highest ethanol production was 4.75 g/l. The optimum dilution factor of PPF hydrolysate in ethanol production by *C. shehatae* TISTR5843 was 1/2 dilution giving the maximum ethanol yield of 0.32 g ethanol/g sugar and ethanol productivity of 0.125 g/l/h.

4. The optimum conditions for ethanol production in PPF hydrolysate by *C. shehatae* TISTR5843 was the initial pH of 5.25, shaking speed of 135 rpm and initial cells concentration of 1.08 g/l. The ethanol production was 5.25 g/l with the ethanol yield of 0.40 g ethanol/g sugar, and the productivity of 0.146 g/l/h.

จัดรูปแบบ: สัญลักษณ์แสดงหัวข้อย่อยและลำดับเลข

5. The maximum ethanol production of fed batch and semi-continuous process in 1/2 dilution PPF hydrolysate conducted in 3 cycles by *C. shehatae* TISTR5843 were 3.92 and 4.02 g/l, respectively, which were similar ethanol production from batch process (4.07 g/l).

6. The pretreatment of PPF, milling and delignification increased surface area of natural support, which enhances cell adsorption and ethanol production. The ethanol concentrations, ethanol yields and ethanol productivities of free and immobilized cells increased in repeated batch fermentation in the range of 10.78-30.12%, 9.52-22.22% and 11.90-32.35%, respectively. The immobilized cells on sPPF can be reused 4 times with retaining the activity of 93%. Therefore, PPF has a potential as a natural support in the immobilization system.

4.2 Suggestion

1. Study the lignin removal by steam explosion because of (i) chemicals cost reduction, (ii) pretreatment cost reduction after lignin removal (pH adjustment), and (iii) environmental friendly.
2. Study the lignin removal by white rot fungi.
3. Study how to reuse the acid in the process of furfural production.
4. Study the immobilized cells in PPF hydrolysate.
5. Produce ethanol from cellulose by fungi.
6. Study the down stream process of ethanol production by the process of evaporation (evaporation and permeation) which used for obtaining the ethanol when it's produced.
7. Apply the knowledge from this study to produce value added products from lignocellulosic materials.
8. Study the xylose production by using xylanase in order to no any inhibitory compounds generation.
9. Study how to reduce the cost of xylanase in xylose production.
10. Study the investment cost for each process, enzymatic hydrolysis and acid hydrolysis.

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APPENDICES

Appendix A
Chemical Reagents

All chemicals and their source in this study were given in Table A1.

Table A. List of some chemicals.

Lists	Sources
Acids;	
Acetic acid (CH_3COOH; F.W. 60.05),	Labscan
2-furoic acid ($C_5H_4O_3$; F.W. 112.08), 98%	Aldrich
Phosphoric acid	
Sulfuric acid (H_2SO_4; F.W. 98.08), 98%	Labscan
Tri-fluoro acetic acid (TFA, CF_3COOH; F.W. 114.02), ≥98%	Aldrich
Alkalis;	
Potassium hydroxide (KOH; F.W. 56.11), 85%	Labscan
Sodium hydroxide (NaOH; F.W. 40), 97%	Labscan
Solvents;	
Acetone (CH_3COCH_3; F.W. 58.08), ≥99%	Labscan
Acetonitrile (CH_3CN; F.W. 41.05), HPLC grade ≥99%	Labscan
Ethanol (CH_3CH_2OH; F.W. 46.07), HPLC grade ≥99.5%	J.T.Baker
Furfural, GC grade 98%	Wako
Furfuryl alcohol	Fluka
Hydroxy-methyl-furfural ($C_6H_6O_3$; F.W. 126.11), GC grade ≥95%	Fluka
Water for HPLC grade	Labscan

Table A. List of some chemicals (cont.)

Lists	Sources
Sugars;	
L-(+)-Arabinose ($C_5H_{10}O_5$; F.W. 150.13), ≥99%	Himedia
D-(-)-Fructose ($C_6H_{12}O_6$; F.W. 180.16), ≥98%	Fluka
D-(+)-Galactose ($C_6H_{12}O_6$; F.W. 180.16), ≥99%	Sigma
D-(+)-Glucose ($C_6H_{12}O_6$; F.W. 180.16), ≥99%	Merck
D-(+)-Mannose ($C_6H_{12}O_6$; F.W. 180.16), ≥99%	Fluka
α-L-Rhamnose ($C_6H_{12}O_5$·H2O; F.W. 182.17), ≥99%	Fluka
D-(+)-Arabinose ($C_5H_{10}O_5$; F.W. 150.13), ≥99%	Sigma
Salts:	
Ammonium sulphate ($(NH_4)_2SO_4$; M.W. 132.14), ≥99.5%	Labscan
Copper sulphate ($CuSO_4$; M.W. 159.68), 98.5%	Ajax Finechem
Potassium dihydrogen orthophosphate (KH_2PO_4, M.W. 136.09), ≥99%	Ajax Finechem
Sodium chloride (NaCl; M.W. 58.43), ≥99.5%	Sigma-Aldrich
Sodium acetate (CH3COONa.3H2O; M.W. 136.08), ≥99%	Ajax Finechem
Substances;	
Bacteriopeptone	Himedia
Bovine serum albumin (BSA)	Sigma
Carboxy-methyl-cellulose (CMC)	Fluka
Cellulase from Aspergillus niger	Fluka
Malt extract	Himedia
Sodium chlorite ($NaClO_2$)	Sigma-Aldrich
Vanillin	Sigma-Aldrich
Xylan	Sigma-Aldrich
Yeast extract	Labscan

Appendix B

Sugar analysis and standard curve

1. Colorimetric method

1.1 Somogji-Nelson reagents

Reaction

(1) $2Cu^{2+}$ + reducing sugars $\longrightarrow$ Cu_2O

(2) $Cu_2O + H_2SO_4 \longrightarrow 2Cu^{+}$

(3) $2Cu^{+} + MoO_4^{2-} + SO_4^{2-} \longrightarrow 2Cu^{2+}$ + molybdenum blue

1.2 Chemical reagents

1.2.1 Low-alkalinity reagent of Somogyi was prepared by following below;

Reagent A: 12 g of Nak tartrate and 24 g anhydrous Na_2CO_3 were dissolved in 250 ml boiled distilled water.

Reagent B: 4.0 g $Cu_2SO_4.5H_2O$ was dissolved in 50 ml distilled water.

Reagent C: 180 g anhydrous Na_2SO_4 was dissolved in 500 ml distilled water and then heated for releasing air. After that, it was cooled.

"Adding reagent B in to reagent A together with mixing and then 16 g $NaHCO_3$ was added slowly. After that, reagent C was added and finally 1000 ml was a final volume and adjusted by boiled distilled water. Keep this solution at 37ºC for a week."

1.2.2 Arsenomolybdate reagent of Nelson was prepared by following below;

Reagent D: 25 g ammonium molybdate was dissolved by 450 ml distilled water and then added by 21 ml of 96% sulfuric acid.

Reagent E: 3.0 g Na2HAsO4.7H2O was dissolved by 25 ml distilled water.

"Reagent E was added to reagent D and adjusted to 1000 ml by distilled water and then incubated at 37ºC for 24 h. Finally, Nelson reagent was stored by dark bottle."

1.3 Standard glucose

0.02 g glucose was dissolved by distilled water and adjusted to 100 ml. Lastly, 200 µg/ml will be obtained. It was diluted to various concentration of 50, 100, 150 µg/ml.

Std. glucose or sample 100 µl

⇓

Add 100 µl Somogyi reagent and mixed together

⇓

Heat at 100ºC for 10 min

⇓

Cool by ice

⇓

Add 100 µl Nelson reagent and mixed together until Cu_2O was dissolved completely

⇓

Adjust to the final volume of 2.5 ml by distilled water and wait for 15 min

⇓

Measure at OD 500 nm

Blank is water

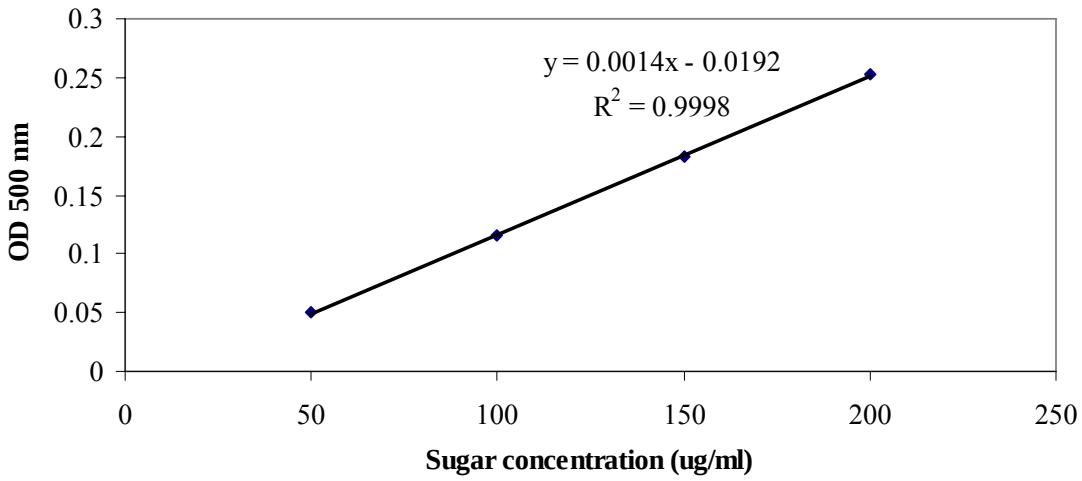

Figure B1. Standard curve of total sugar content (as glucose) at OD_{500} nm determined by Somogji-Nelson assay.

2. HPLC assay of reducing sugars

Condition: Mobile phase: acetonitrile (ACN) and HPLC water (v/v) (75:25 v/v)

Injection: 20 µl

Column: Zorbax NH_2 column (4.6×250 mm, 5-Micron, Agilent, USA) with RI detector

Operation temperature and flow rate: 25°C, flow rate of 0.7 ml min^{-1}

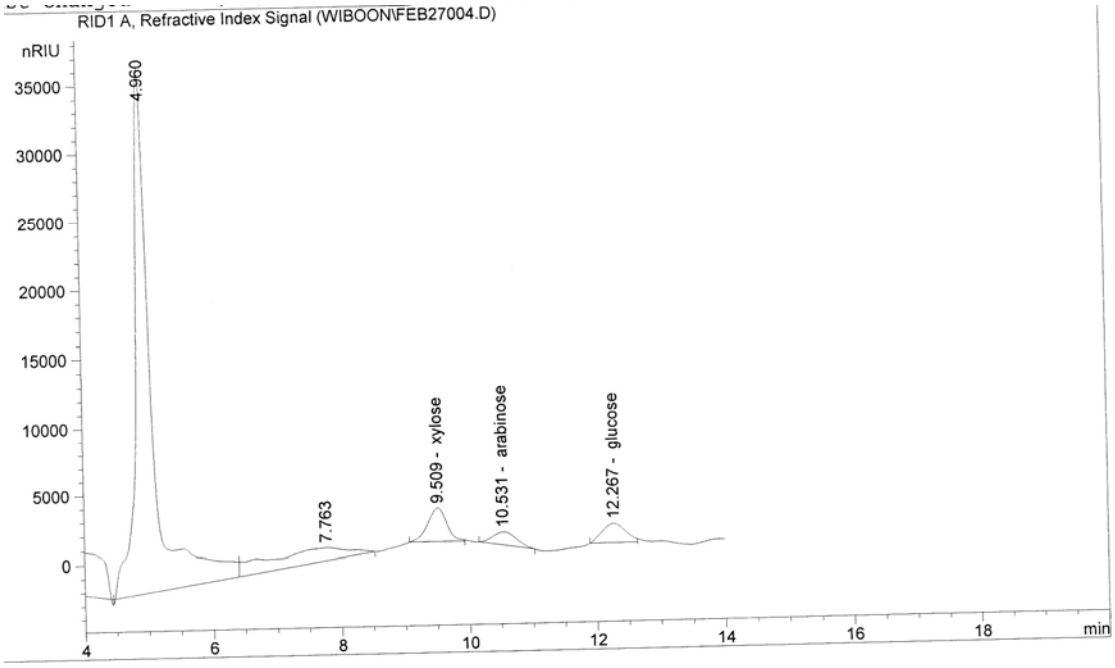

Figure B2. Standard curve of xylose, arabinose, and glucose determined by HPLC.

Appendix C

Protein analysis and standard curve

1. Chemical reagents

1) Reagent A: 2% (w/v) Na_2CO_3 in 0.1 N NaOH.

2) Reagent B: 0.5% (w/v) $CuSO_4.5H_2O$ in 1% sodium potassium tartrate.

3) Reagent C: 30 ml reagent A is mixed by 2 ml reagent B.

4) Folin-ciocatrus phenol reagent is diluted by distilled water in the ratio of 1:2.

5) Bovine Serum Albumen (BSA) 1 mg/ml

2. Method

0.5 ml BSA (0, 20, 40, 60, 80 and 100 µg/ml) and samples were added in each test tube

⇓

Add 0.5 ml reagent A and boiled for 5 min and cooled by ice

⇓

Add 2.5 ml reagent C and mixed well and incubated at room temperature for 10 min

⇓

Add Folin reagent 0.5 ml and mixed well and incubated at room temperature for 30 min

⇓

Measure OD at 750 nm

3. Curve of standard protein

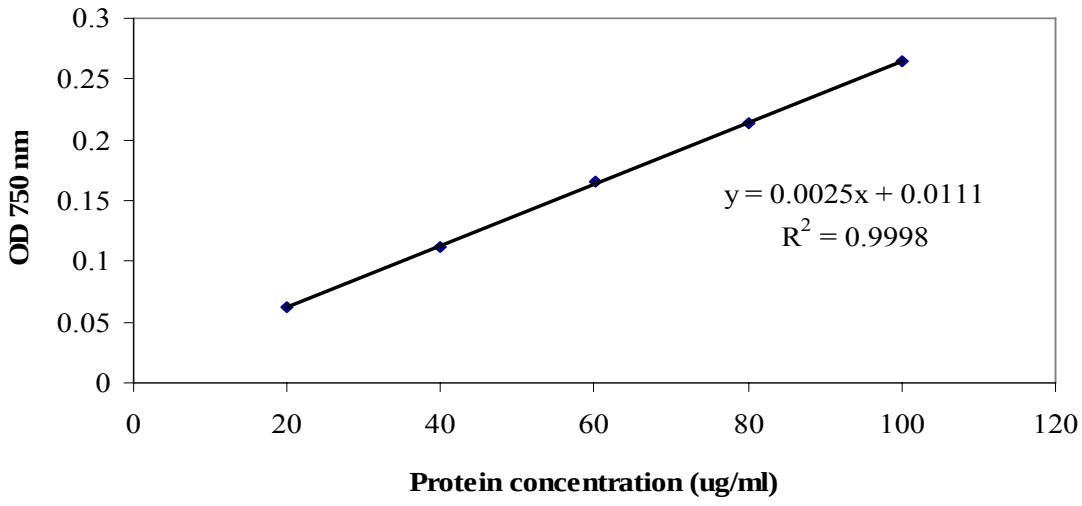

Figure C. Standard curve of protein as BSA at OD_{750} nm.

Appendix D

Furfural and acetate analysis and standard curves

Furfural and acetate were determined by Gas Chromatography (GC) assay.

Condition: Injection: 1 µl

Column: Stabilwax®-DA, Restek GC columns

Operation temperature at injection and detector port: 230 and 250°C

Oven: hold at 70°C for 3 min, (ii) ramp for 5 min at 20°C min^{-1} to a final temperature of 235°C, (iii) hold at 235°C for 3 min.

Carrier gas: Helium at flow rate 1.2 ml min^{-1}.

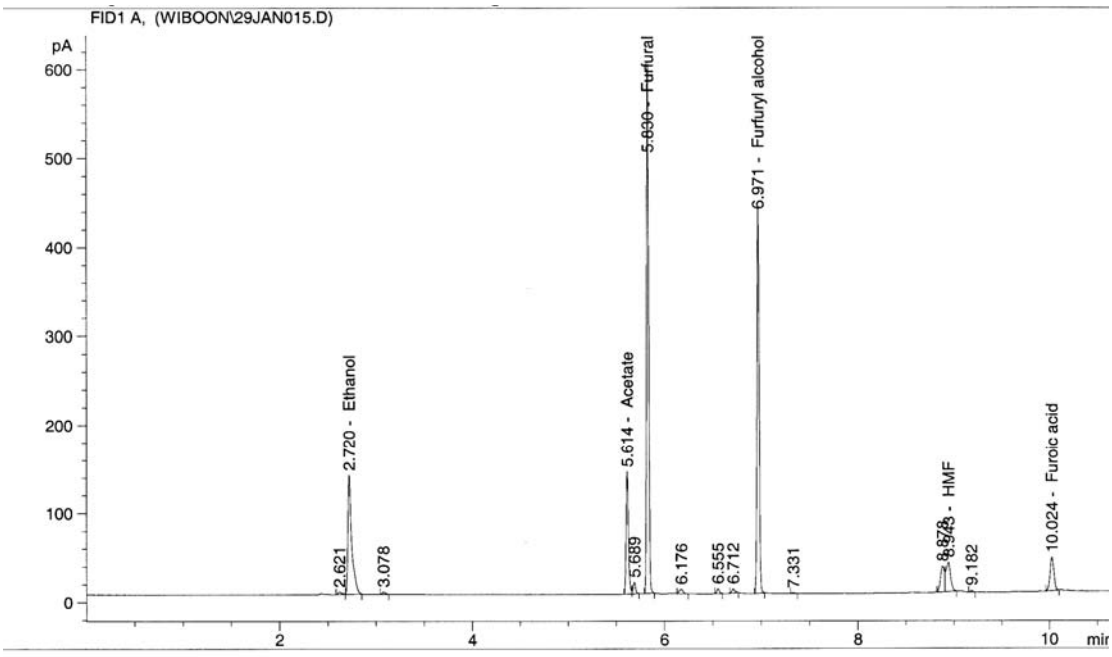

Figure D. Standard curve of ethanol, acetate, furfural, and furfuryl alcohol determined by GC.

Appendix E

Dry cell weight

1. The culture of *C. shehatae* TISTR5843, cultured under at 30°C for 24 h, was adjusted the turbidometrically at the wavelength of 600 nm (OD_{600}) to be 0.2-1.0.
2. Cell pellet was harvested and twice washed with distilled water by centrifuge (8,000 *xg* for 10 min).
3. Cell pellet remaining in tubes were baked at 105°C for 12 h. and weighted the dry cell until their constantly weight.

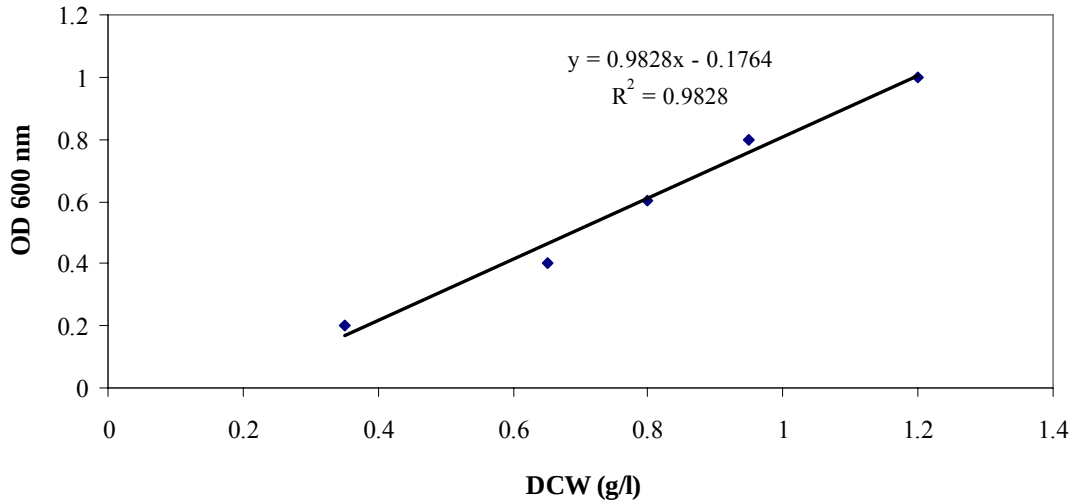

Figure E. Standard curve of dry cell weight of *C. shehatae* TISTR5843.

Appendix E

1. Calculation of glucose production

The ratio of dPPF to sulfuric acid $= \frac{1 \text{ g dPPF}}{10 \text{ ml}}$

When, glucose production = 1.32 g/l
Thus, solution 1000 ml contain glucose 1.32 g
If solution 10 ml contain glucose $= \frac{1.32 \times 10}{1000}$

= 0.0132 g glucose /10 ml

From Table xx,
1 g dPPF contain cellulose 0.42 g calculated to 100 %
If glucose 0.0132 g calculated to % $= \frac{0.0132 \times 100}{0.42}$

= 3.14 %

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List of Publications and Proceedings

Publications

Riansa-ngawong, W. and Prasertsan, P. 2010. Statistical approach for optimization of hemicellulose production from delignified palm pressed fiber and used as a bio-material for one-stage production of furfural. Int. J. Biotechnol. Biochem. 6(7): 1101-1116.

Riansa-ngawong, W. and Prasertsan, P. 2011. Optimization of furfural production from hemicellulose extracted from delignified palm pressed fiber using two-stage process. Carbohydr. Res. 346(1): 103-110.

Presentations

Riansa-ngawong, W., Prasertsan, P. and Iiyama, K. 2008. Statistical studies on hemicellulose production from delignified palm pressed fiber. Proceeding of International Biotechnology Symposium (IBS) on October 12-17, 2008, Dalian, China (Poster Presentation).

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