Production of Cellulase and Xylanase from Oil Palm Trunk and Frond and Their Applications

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การผลิตเซลลูเลสและไซลาเนสจากลำต้นและทางใบปาล์มน้ำมันและการประยุกต์ใช้

ชื่อผู้แต่ง นายธนาวุฒิ หนูทองแก้ว

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บทคัดย่อ

ลำต้นปาล์มน้ำมันและทางใบปาล์มน้ำมันจัดเป็นชีวมวลหลักอย่างหนึ่งในภาคใต้ของประเทศไทย ลำต้นปาล์มน้ำมัน มีองค์ประกอบหลักเปลี่ยนแปลงตามความสูงของลำต้น และพบว่ามีการสะสมแป้งตามความสูงของลำต้นโดยมีปริมาณสูงสุดที่ส่วนยอด (ยาว 1 เมตร) คิดเป็น 3.79 เปอร์เซ็นต์ (น้ำหนัก/น้ำหนัก ของส่วนยอด) เมื่อสกัดน้ำตาล พบว่า น้ำคั้นจากส่วนล่างและส่วนกลางของลำต้นปาล์มน้ำมันมีปริมาณกลูโคส (20.13 และ 12.74 กรัมต่อลิตร ตามลำดับ) และฟรุกโตส (3.04 และ 6.02 กรัมต่อลิตร ตามลำดับ) สูงสุดตามลำดับ ส่วนน้ำคั้นทางใบปาล์มน้ำมันมีปริมาณกลูโคสสูงสุด (25.42 กรัมต่อลิตร) รองลงมาคือฟรุกโตส (3.66 กรัมต่อลิตร)

เมื่อหมักเส้นใยของลำต้นและทางใบปาล์มน้ำมันที่บดแล้วที่อุณหภูมิต่างๆเป็นเวลา 15 วัน พบว่า การหมักเส้นใยของลำต้นปาล์มน้ำมันที่อุณหภูมิห้องเป็นเวลา 15 วัน ให้ค่ากิจกรรมเอนไซม์ carboxymethyl cellulase (CMCase) สูงสุด (0.48 ยูนิตต่อกรัมสารตั้งต้นแห้ง) ในขณะที่ค่ากิจกรรมเอนไซม์ไซลาเนสสูงสุด (0.44 ยูนิตต่อกรัมสารตั้งต้นแห้ง) ได้จากการหมักทางใบปาล์มน้ำมัน ที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 3 วัน และไม่พบค่ากิจกรรมเอนไซม์จากการหมักชีวมวลทั้ง 2 ชนิด ที่อุณหภูมิ 50 องศาเซลเซียส เมื่อศึกษาโครงสร้างประชากรของจุลินทรีย์ด้วยเทคนิค PCR-DGGE จากตัวอย่างการหมักลำต้นและทางใบปาล์มน้ำมันดังกล่าว รวมทั้งตัวอย่างของแผ่นไม้ลำต้นปาล์มน้ำมันที่วางทิ้ง พบว่าตัวอย่างมีทั้งเชื้อแบคทีเรีย ยีสต์ และรา กลุ่มประชากรแบคทีเรียมีความหลากหลายสูง ส่วนกลุ่มยีสต์และกลุ่มรามีความหลากหลายน้อย สามารถแยกเชื้อราได้ 20 สายพันธุ์ และมีเพียง 8 สายพันธุ์ที่สามารถเจริญบนอาหารที่มีกากลำต้นและกากทางใบปาล์มน้ำมัน (หลังการบีบอัดน้ำตาลออกแล้ว) เป็นสารตั้งต้น ได้แก่ สายพันธุ์ TT1, TT2, TT3, TT4, TT5, TM1, TM2 และ TM3 เมื่อนำเชื้อรา 8 สายพันธุ์นี้มาทดสอบการผลิตเอนไซม์

เซลลูเลสและไซลาเนสในการหมักแบบอาหารแข็งและแบบอาหารเหลว พบว่าเชื้อราสายพันธุ์ TT1, TM3 และ TT2 ผลิตเอนไซม์ได้สูงสุด และจำแนกเชื้อรา ได้เป็น *Ceratocystis paradoxa*, *Trichoderma koningiopsis* และ *Hypocrea nigricans* ตามลำดับ ทำการศึกษาระยะเวลาในการผลิตเอนไซม์จากเชื้อราเหล่านี้ พบว่า กากลำต้นปาล์มน้ำมันเป็นสารตั้งต้นที่ดีกว่ากากทางใบปาล์มน้ำมัน และเชื้อ *C. paradoxa* TT1 ผลิตเอนไซม์ CMCase สูงสุด (18.16 ยูนิตต่อกรัมสารตั้งต้นแห้ง) ในการหมักแบบอาหารเหลว ขณะที่เชื้อ *T. koningiopsis* TM3 ผลิตเอนไซม์ไซลาเนสสูงสุด (56.46 ยูนิตต่อกรัมสารตั้งต้นแห้ง) และเอนไซม์ FPase สูงสุด (2.13 ยูนิตต่อกรัมสารตั้งต้นแห้ง) ในการหมักแบบอาหารแข็งที่ใช้กากลำต้นปาล์มน้ำมันเป็นแหล่งคาร์บอน

การเตรียมหัวเชื้อของเชื้อรา *C. paradoxa* TT1 และ *T. koningiopsis* TM3 ในรูปแบบแห้ง พบว่า มีปริมาณเชื้อ 1.2×10^9 และ 1.6×10^8 CFU ต่อกรัมน้ำหนักแห้ง ตามลำดับ เมื่อเก็บหัวเชื้อที่อุณหภูมิห้อง (30±2 องศาเซลเซียส) และที่ 4 องศาเซลเซียส เป็นเวลา 6 เดือน พบว่าหัวเชื้อ *T. koningiopsis* TM3 ยังคงมีปริมาณเชื้ออยู่เท่าเดิม (ประมาณ 10^8 CFU ต่อกรัมน้ำหนักแห้ง) ในขณะที่ปริมาณของหัวเชื้อ *C. paradoxa* TT1 ลดลงอย่างมาก (30-45 เปอร์เซ็นต์ ของค่าเดิม) เมื่อเปรียบเทียบการผลิตเอนไซม์แบบอาหารแข็งและอาหารเหลว จากหัวเชื้อในรูปแบบแห้ง สารละลายสปอร์ของเชื้อ *C. paradoxa* TT1 และ *T. koningiopsis* TM3 (แบบเชื้อเดี่ยวและแบบเชื้อผสม) กับหัวเชื้อของกรมพัฒนาที่ดิน (ซุปเปอร์ พด. 1) พบว่า หัวเชื้อ TM3 และ TT1 สามารถผลิตเอนไซม์ได้สูงกว่าหัวเชื้อซุปเปอร์ พด.1 และให้ค่าใกล้เคียงกับค่าที่ได้จากการใช้สารละลายสปอร์ของเชื้อ หัวเชื้อ TM3 มีศักยภาพในการผลิตเอนไซม์ได้สูงกว่าหัวเชื้อ TT1 จากการเปรียบเทียบการผลิตเอนไซม์จากหัวเชื้อ *T. koningiopsis* TM3 โดยการหมักแบบอาหารแข็งในกากลำต้นปาล์มน้ำมัน กากทางใบปาล์มน้ำมัน ทะลายปาล์มเปล่า กากตะกอนดีแคนเตอร์ และเส้นใยปาล์ม และการหมักแบบอาหารเหลวในน้ำทิ้งโรงงานสกัดน้ำมันปาล์ม พบว่า ค่ากิจกรรมของเอนไซม์ CMCase และไซลาเนสสูงสุด (4.44 และ 63.17 ยูนิตต่อกรัมสารตั้งต้นแห้ง ตามลำดับ) เมื่อใช้กากลำต้นปาล์มน้ำมันเป็นแหล่งคาร์บอน

จากการศึกษาลักษณะของเอนไซม์ พบว่า ค่าที่เหมาะสมต่อกิจกรรมของเอนไซม์ CMCase และไซลาเนส คืออุณหภูมิ 50 องศาเซลเซียส ในช่วงพีเอช 4.4-4.8 และ 4.8-5.6 ตามลำดับ ส่วนการทนต่อความร้อนของเอนไซม์ พบว่า CMCase ยังคงค่ากิจกรรมเอนไซม์มากกว่า 75

เปอร์เซ็นต์หลังการบ่มที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 5 ชั่วโมง และมีค่ากิจกรรมเอนไซม์น้อยกว่า 50 เปอร์เซ็นต์ ในการบ่มที่อุณหภูมิห้อง 50 และ 60 องศาเซลเซียส ส่วนกิจกรรมของเอนไซม์ไซลาเนส สามารถทนต่อความร้อนได้ต่ำกว่า โดยให้ค่ากิจกรรมเอนไซม์ 75 เปอร์เซ็นต์ที่อุณหภูมิต่ำกว่า 40 องศาเซลเซียส หลังการบ่ม 2 ชั่วโมง และสูญเสียกิจกรรมของเอนไซม์ 73 และ 79 เปอร์เซ็นต์ หลังจากบ่มเป็นเวลา 1 ชั่วโมง ที่อุณหภูมิ 50 และ 60 องศาเซลเซียส ตามลำดับ ขณะที่กิจกรรมเอนไซม์ CMCase ลดลงเพียง 21 และ 31 เปอร์เซ็นต์ ภายใต้สภาวะเดียวกัน ทำการผลิตเอนไซม์จากหัวเชื้อ *T. koningiopsis* TM3 ในอาหาร MMS โดยใช้กากลำต้นปาล์มน้ำมันเป็นแหล่งคาร์บอน ที่อุณหภูมิห้อง เป็นระยะเวลา 4 วัน เก็บเกี่ยวเอนไซม์โดยการตกตะกอนด้วยอะซิโตน พบว่า เอนไซม์เข้มข้นที่ได้ให้ค่ากิจกรรมเอนไซม์ CMCase และไซลาเนสเพิ่มขึ้น 6 เท่า และ 6.8 เท่า ตามลำดับ (3.22 และ 54.14 ยูนิตต่อมิลลิลิตร ตามลำดับ) ผลผลิตเอนไซม์ประมาณ 60 และ 68 เปอร์เซ็นต์ ตามลำดับ

เมื่อใช้เอนไซม์เข้มข้นที่ผลิตได้ไปย่อยกากลำต้นปาล์มน้ำมัน โดยใช้ความเข้มข้นของเอนไซม์ในช่วง 0-40 ยูนิตต่อกรัมกากลำต้นปาล์มน้ำมัน บ่มที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง พบว่าค่าที่เหมาะสม คือ ความเข้มข้นของเอนไซม์ 25 ยูนิตต่อกรัมกากลำต้นปาล์มน้ำมัน ระยะเวลาการย่อย 15 ชั่วโมง ได้ไฮโดรไลเสทที่มีปริมาณน้ำตาลรีดิวซ์สูงสุด 11.92 กรัมต่อลิตร คิดเป็น 0.48 กรัมต่อกรัมกากลำต้นปาล์ม เมื่อนำไฮโดรไลเสทจากกากลำต้นปาล์มน้ำมัน (ไม่มีการเติมสารอาหาร) ไปผลิตเอทานอล พบว่าเชื้อ *Saccharomyces cerevisiae* TISTR5055 มีประสิทธิภาพในการผลิตสูงกว่าเชื้อ *Candida shehatae* TISTR5843 และการหมักร่วมของเชื้อทั้งสองสายพันธุ์ สำหรับการผลิตกรดอะซิติกแบบสองขั้นตอน (two-stage fermentation) และการหมักแบบร่วม (co-culture fermentation) โดยเชื้อ *S. cerevisiae* TISTR5055 และ *Acetobacter aceti* ในไฮโดรไลเสตจากกากลำต้นปาล์มน้ำมัน พบว่า การเติมสารอาหาร (YM nutrients) มีผลต่อการผลิตเอทานอลทั้งการหมักแบบร่วม (4.01 กรัมต่อลิตร ที่ 12 ชั่วโมง) และการหมักแบบสองขั้นตอน (4.01 กรัมต่อลิตร ที่ 18 ชั่วโมง) แต่ไม่มีผลต่อการผลิตกรดอะซิติก และพบว่าการหมักไฮโดรไลเสทจากกากลำต้นปาล์มน้ำมันที่ไม่เติมสารอาหารแบบการหมักร่วม เชื้อผลิตกรดอะซิติกได้สูงสุด (2.12 กรัมต่อลิตร ที่ 24 ชั่วโมง) คิดเป็น 1.7 เท่า และ 4 เท่า เมื่อเทียบกับการหมักแบบสองขั้นตอน (1.13 กรัมต่อลิตร)

เมื่อศึกษาผลของความเข้มข้นของเอนไซม์จากเชื้อ *T. koningiopsi*s TM3 ต่อการย่อยน้ำทิ้งโรงงานสกัดน้ำมันปาล์ม (POME) และกากลำต้นปาล์มน้ำมัน ที่อุณหภูมิ 40 และ 50 องศาเซลเซียส ก่อนการนำไปผลิตก๊าซชีวภาพ และใช้เป็นสารหมักร่วม (co-substrate) พบว่า การใช้เอนไซม์ 15 ยูนิตต่อกรัมของแข็งระเหยได้ และบ่มที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 18 ชั่วโมง POME ให้ปริมาณน้ำตาลทั้งหมดสูงสุด เท่ากับ 27.10 กรัมต่อลิตร (กลูโคส 16.69 กรัมต่อลิตร, ไซโลส 4.39 กรัมต่อลิตร, เซลโลไบโอส 3.49 กรัมต่อลิตร และอะราบิโนส 2.35 กรัมต่อลิตร) คิดเป็นผลผลิต 0.35 กรัมต่อกรัมของแข็งระเหยง่าย ส่วนไฮโดรไลเสทจากกากลำต้นปาล์มน้ำมันมีปริมาณน้ำตาลทั้งหมด 23.28 กรัมต่อลิตร (กลูโคส 19.41 กรัมต่อลิตร ไซโลส 1.09 กรัมต่อลิตร เซลโลไบโอส 2.18 กรัมต่อลิตร และอะราบิโนส 0.60 กรัมต่อลิตร) ในสภาวะเดียวกัน เมื่อนำไฮโดรไลเสทจากน้ำทิ้งโรงงานสกัดน้ำมันปาล์มไปผลิตก๊าซชีวภาพ พบว่า ศักยภาพการผลิตก๊าซมีเทน (1,243 มิลลิลิตรมีเทนต่อกรัมของแข็งระเหยได้) เพิ่มขึ้น 15.3 เปอร์เซ็นต์ เมื่อเทียบกับน้ำทิ้งโรงงานสกัดน้ำมันปาล์ม (1,078 มิลลิลิตรมีเทนต่อกรัมของแข็งระเหยง่าย) ในขณะที่ศักยภาพการผลิตก๊าซมีเทนจากกากลำต้นปาล์มน้ำมันสูงกว่าการผลิตโดยใช้ไฮโดรไลเสทจากกากลำต้นปาล์มน้ำมันเล็กน้อย (3.7 เปอร์เซ็นต์) (1,402 และ 1,350 มิลลิลิตรมีเทนต่อกรัมของแข็งระเหยได้ ตามลำดับ) การหมักร่วมระหว่างไฮโดรไลเสตทั้งสองแหล่งให้ผลผลิตการผลิตก๊าซมีเทนดีที่สุด (1,340 มิลลิลิตรมีเทนต่อกรัมของแข็งระเหยง่าย) และพบว่าเชื้ออาร์เคีย (archaea) เด่นที่มีบทบาทและสำคัญต่อการผลิตก๊าซมีเทน คือ *Methanoculleus* sp. และ *Methanosarcina* sp. ซึ่งผลการทดลองนี้ชี้ให้เห็นว่าการย่อยด้วยเอนไซม์และการหมักร่วมของน้ำทิ้งโรงงานสกัดน้ำมันปาล์มกับกากลำต้นปาล์มน้ำมันสามารถปรับปรุงการผลิตก๊าซชีวภาพได้

Thesis Title Production of Cellulase and Xylanase from Oil Palm Trunk and Frond and Their Applications

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ABSTRACT

Oil palm trunk (OPT) and oil palm frond (OPF) are abundant biomass in Southern Thailand. The major chemical composition of OPT varied along its height and found to accumulate starch along its height with the highest quantity at the top end part (1 m long) of 3.79 % (w/w of the top end part). After extraction of sugars, the sap from the bottom part and middle part of OPT had the highest glucose (20.13 and 12.74 g/L, respectively) and fructose (3.04 and 6.02 g/L, respectively), respectively. The OPF sap contained the highest glucose concentration (25.42 g/L) followed by fructose (3.66 g/L).

The ground OPT and OPF fibers were fermented at various temperatures for 15 days. The maximum carboxymethyl cellulase (CMCase) (0.48 Unit/gds) was obtained from fermentation of OPT at room temperature for 15 days while the maximum xylanase activity (0.44 Unit/gds) was achieved from fermentation of OPF at room temperature for 3 days (0.44 Unit/gds). There was no enzyme activity after fermentation of OPT and OPF at 50 ˚C. The microbial community analysis of the natural fermentation of OPT and OPF revealed high diversity of bacteria with low diversity of yeasts and fungi. The total of 20 fungal strains were isolated and only eight of them could grow on agar plate containing OPT and OPF residues (after sugar extraction). They were encoded as the isolate TT1, TT2, TT3, TT4, TT5, TM1, TM2 and TM3. They were compared for their ability to produce cellulase and xylanase under SSF and SmF. The isolates TT1, TM3 and TT2 produced the highest enzyme activity and identified as *Ceratocystis paradoxa*, *Trichoderma koningiopsis* and *Hypocrea nigricans*, respectively. Time-courses of enzymes production from these strains were conducted. Oil palm trunk residues (OPTr) was a better substrate for enzymes production than oil palm frond residues (OPFr). *C. paradoxa* TT1 gave the

highest CMCase (18.16 Unit/gds) in SmF while *T. koningiopsis* TM3 exhibited the highest xylanase (56.46 Unit/gds) and FPase (2.13 Unit/gds) production in SSF.

The inoculums of the two newly isolated strains *C. paradoxa* TT1 and *T. koningiopsi*s TM3 were prepared in packed dried form with the quantity. The inoculums contained of 1.2×10^9 and 1.6×10^8 CFU per g dry weight, respectively. After storage at room temperature and 4 °C for 6 months, only the inoculum of *T. koningiopsis* TM3 remained at the same level (approximately 10^8 CFU per g dry weight). In contrast, the survival of the formulated TT1 decreased sharply to 30-45% of their original values. Lignocellulolytic enzymes production from the formulated inoculums and spore suspension inoculum of *C. paradoxa* TT1 and *T. koningiopsi*s TM3 (individual and mixed inoculum) were compared with the mixed cultures from the Land Development Department (Super LDD1). Results in SSF and SmF revealed that the formulated inoculums TM3 and TT1 produced the lignocellulolytic enzymes higher than the Super LDD1 and similar to that of the spore suspension form. The formulated inoculum TM3 produced the lignocellulolytic enzymes higher than the formulated inoculum TT1. Oil palm biomass (OPTr, OPFr, EFB, decanter cake and plam pressed fiber (PPF)) were used as substrates for production of enzymes under SSF and POME under SmF by the formulated inoculum *T. koningiopsi*s TM3. The maximal CMCase and xylanase activities (4.44 and 63.17 Unit/gds, respectively) were obtained when OPTr was used as a carbon source.

Characterization of the enzymes revealed the optimum temperature for CMCase and xylanase at 50 °C while the optimum pH of CMCase and xylanase were in the pH range of 4.4-4.8 and 4.8-5.6, respectively. Thermal stability study revealed that CMCase retained more than 75% of their activities after 5 h incubation at 40 °C and lower than 50% of its activity at room temperature (30±2 °C), 50 and 60 °C. The xylanase exhibited lower thermal stability as it only preserved 75% of its activities at temperature below 40 °C and lost 73% and 79% of its activity within 1 h of incubation at 50 °C and 60 °C, respectively, while CMCase lost only 21% and 31% of its activities under the same condition. The crude enzyme obtained above from the formulated *T. koningiopsis* TM3 was precipitated by using acetone. The activity of CMCase and xylanase increased 6 and 6.8 fold, respectively (3.22 and 54.14 Unit/ml, respectively) with the recovery yields about 60 and 68%, respectively.

The concentrated enzymes were used to hydrolyze OPTr by using the enzymes concentrations in the range of 0-40 Unit/g OPT and incubated at 50 °C for 24 h. Enzymatic hydrolysis of OPTr revealed that the maximum reducing sugars of 11.92 g/L with the yield of 0.48 g/g were obtained by hydrolyzing with 25 Unit/g of the enzymes at 50 °C for 15 h. For ethanol production from the OPTr hydrolysate (without nutrients added), *Saccharomyces cerevisiae* TISTR5055 was more efficient than *Candida shehatae* TISTR5843 and the co-cultures. Two-stage process and simultaneous fermentation using the co-cultures of *S. cerevisiae* and *Acetobacter aceti* were compared. Supplementation of YM nutrients to the OPTr hydrolysate exhibited strong influence on ethanol production (4.10 g/L at 12 h in two-stage process and 4.01 g/L at 18 h in simultaneous fermentation) but not acetic acid production. Without nutrients addition, the maximum acetic acid concentration and productivity (2.12 g/L at 24 h) were achieved from the simultaneous fermentation of the co-cultures which were 1.7 folds and 4 folds higher than those from the two-stage process (1.23 g/L at 54 h).

The efficacy of concentrated enzymes in hydrolyzing sterilized POME and OPTr at 40 and 50 °C was evaluated prior to methane fermentation and its co-digestion. The maximum sugars concentrations of POME hydrolysate were obtained from enzymatic hydrolysis using 15 Unit/g TVS at 50 °C for 18 h incubation (glucose 16.69 g/l, xylose 4.39 g/l, cellobiose 3.49 g/l and arabinose 2.35 g/l) with the yield of 0.3521 g/g TVS. The OPTr hydrolysate had the total sugar concentration of 23.28 g/L (19.41 g/l glucose, 1.09 g/l xylose, 2.18 g/l cellobiose and 0.60 g/l arabinose) under the same condition. Methane potential of the POME hydrolysate (1,243 ml CH_4/g VS-added) increased by 15.3% compared to the raw POME (1,078 ml CH_4/g VS-added). Meanwhile, the methane potential of raw OPTr was slightly (3.7%) higher than that of OPTr hydrolysate (1,402 and 1,350 ml CH_4/g VS-added, respectively). Co-digestion of POME hydrolysate with OPTr gave the best result of methane yield (1,340 ml CH_4/g VS-added). The dominant archaea that played an important role in methane production were *Methanoculleus* sp. and *Methanosarcina* sp.. These results indicated that enzymatic pretreatment and co-digestion of POME hydrolysate with OPTr could improve biogas yield from anaerobic fermentation.

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Tanawut Nutongkaew

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CHAPTER 1

INTRODUCTION AND LISTERATURE REVIEW

Introduction

Biological degradation of lignocellulose has attracted the interest of microbiologists and biotechnologists for many years (Doolotkeldieva and Bobusheva, 2011). Most of the old or felled oil palm trunks (OPT), around 81 million cubic meters per year, are cut and discarded or burnt at the plantation site (Noparat *et al*., 2012). Consequently, felled OPT can be regarded as one of the most unutilized biomass resources in the country. Unfortunately, the OPT structure is not strong enough for use as lumber. Thus, only the relatively strong outer part of the trunk is partially utilized for plywood manufacturing while the soft inner part is left unused although the OPT contains many simple sugars particularly xylose and glucose (Kosugi *et al*., 2010; Noparat *et al*., 2012). Oil palm fronds (OPF) are generated about 26 million ton/year in the oil palm plantation in Malaysia (Rahman *et al*., 2011).

OPT typically comprises of cellulose (29-46%), hemicelluloses (12-26%) and lignin (10-24%) (Noparat *et al*., 2011; Khalil *et al*., 2012; Ang *et al.*, 2013). In view of its high content of holocellulose (41-72%), OPT is a potential source for production of lignocellulolytic enzymes (Ang *et al*., 2013) and fermentable sugars for producing biofuels. The bioconversion of lignocellulose to fermentable sugars requires the synergistic action of complete cellulase system which act randomly on soluble and insoluble cellulose chains (Milala *et al*., 2005; Bansal *et al*., 2011; Deswal *et al*., 2011; Bansal *et al*., 2012) and xylanase for hemicellulose degradation (Kumar *et al*., 2008). The successful strategy to produce lignocellulolytic enzymes can be achieved through microbial selection and improved fermentation process conditions. These include screening for effective enzyme-producing microbes and developing pretreatments that alter the cellulose lattice structure and increase enzyme accessibility. It is therefore necessary to search for microorganisms that have a high rate of lignocellulolytic enzymes production (Sanchez, 2009). Both bacteria and fungi can use cellulose as a primary carbon source. Most bacteria are incapable of

degrading crystalline cellulose since their cellulase systems are incomplete. On the other hand, cellulolytic enzymes produced by some fungi generally involve all three types of enzymes, so are very useful in the saccharification of renewable pretreated lignocellulosic materials (Santos *et al*., 2012). In addition, several fungi can metabolize cellulose as an energy source, only few strains are capable of secreting a complex of cellulase enzymes, which could have practical application in the enzymatic hydrolysis of cellulose (Sukumaran *et al.*, 2005).

Solid-state fermentation (SSF) has received enormous attention for its biological and processing advantages compared to liquid state or submerged fermentations (Holker *et al*., 2004). To date, fermentation studies were conducted at laboratory scale with only few at pilot scale due to unresolved technological and operational constraints particularly when fungus is employed in the process. One of the constraints is the preparation of inoculums (Ang *et al*., 2013). In practice, an inoculum for SSF is prepared by growing fungus in liquid culture or by taking plugs from actively expanding end of an established culture (Matsubara *et al*., 2006; Gupte *et al*., 2007). Unlike unicellular bacteria and yeast, an inoculum preparation by growing fungus in liquid culture is troublesome due to the changes of its physiological state in liquid. The fungus tends to clump and distribute unevenly in liquid culture; thus, this complicates the quantification of fungal biomass. In view of the above mentioned issues, an alternative inoculum preparation method that can overcome the limitations is desirable. This has led to the development of a simple and effective method to preparation of fungal biomass in packed dried form to alleviate the related contamination problems and increase the production of lignocellulolytic enzymes in this work.

Hydrolysis process for producing sugars from cellulosic biomass are preferable to thermochemical process (Almeida *et al*., 2007; Hassan *et al*., 2013) but cause the further conversion of the released sugars to other by-products such as furfural and 5-hydroxymethylfurral (5-HMF) that were reported to be inhibitors and interfered with microbial fermentation (Panagiotou and Olsson, 2007; Lenihan *et al*., 2010). Enzymatic hydrolysis of lignocelluloses has been discussed extensively in the literature (Hassan *et al*., 2013; Cui *et al*., 2014; Maitan-Alfenas *et al*., 2015; Palamae *et al*., 2017). Degradation of lignocelluloses by microbial enzymes outperforms

chemical hydrolysis because enzymes display the high substrate and reaction specificity, operate under mild conditions and do not generate by-products (Micard *et al*., 1996; Zieminski *et al*., 2012). Application of enzymes have been increasingly implemented in chemical, fuel, food and textile industries as well as, in washing powder formulations and paper making (Howard *et al*., 2003; Zieminski *et al*., 2012).

Nowadays, acetic acid is an important intermediate compound for industrial production of many chemicals such as vinyl acetate polymer, cellulose acetate, terephthalic acid, dimethyl terephthalate, acetic acid esters/acetic anhydride and calcium magnesium acetate. All these products are made from petroleum-derived acetic acid (Awad *et al*., 2012). In addition, acetic acid is also one of the key intermediate used in food, detergent, and wood industries. Acetic acid bacteria were divided into five to six genera of which *Acetobacter* and *Gluconobacter* species can tolerate high concentration of acetic acid, which explain their use in vinegar production (Yamada *et al*., 2009). For industrial production, there are several species of *Acetobacter* that can be described as the main vinegar producer (Awad *et al*., 2012). Acetic acid bacteria could oxidize ethanol into acetic acid in the aerobic environment, which has become the main method of vinegar production in industry. However, production of acetic acid could be carried out by anaerobic fermentation in one-stage process (by *Clostridium* sp.) while aerobic fermentation using two-stage process. In two-stage processes, glucose is converted into ethanol by *S. cerevesiae* followed by conversion of ethanol into acetic acid by *Acetobacter aceti* (Patel and Pandya, 2015). This production process is very sensitive to the chemical composition of the production medium and the cultivation conditions applied (Awad *et al*., 2012). Carbon source plays important role for bacterial growth and acetic acid production. Sugars such as: arabinose, xylose, ribose, glucose, galactose, mannose, melibiose, and trehalose can be fermented by most of the *Acetobacter* strains (Kadere *et al*., 2008). For cultivation conditions, the oxygen requirement for *Acetobacter* conversion makes the processes energy intensive. In addition, cultivation mode used (batch, fed-batch) could affect the product concentration. In repeated fed-batch fermentation, the acetic acid concentration was about 80 g/L but the number of viable cells at this product concentration was relatively low (Ito *et al*., 1991; Awad *et al*., 2012)

In Southeast Asia, particularly in Malaysia, Indonesia and Thailand, effluent from palm oil mills referred to as palm oil mill effluent (POME) can be converted into biogas. This can be used to generate electric power through gas turbines or gas-fired engines. Raw POME contains a considerable amount of oil and fatty acids which all contribute to its high oxygen demand. Hence, it has to be treated in a series of open oxidation ponds, for the organic matter to be biodegraded to a much lower oxygen demand before being discharged (Alias and Tan, 2005). Different pretreatment methods have been proved effective for improving the biodegradability of lignocellulosic materials (Chen *et al*., 2005; O-Thong *et al*., 2012). Previous research showed that stream pretreatment (Bruni *et al*., 2010), steam treatment with NaOH presoaking (Wang *et al*., 2009), alkaline hydrolysis with NaOH (Sun *et al*., 2002), mechanical treatment (milling) (Hartmann *et al*., 2000; Bruni *et al*., 2010) and enzymatic pretreatment (Zieminski *et al*., 2012) could significantly improve biodegradability and enhance biogas production of biofibers. Therefore, pretreatments facilitating the accessibility of holocellulose (cellulose and hemicelluloses) could result in the increase of biogas production (Zieminski *et al*., 2012). Thus, raw POME can be digested by enzymes, including cellulase and xylanase to available nutrients for supporting the bacterial growth (Zieminski *et al*., 2012).

This study aims to isolate, select and identify the fungal strains for lignocellulolyic enzymes production from OPT residues (OPTr) through solid-state fermentation (SSF) compared to submerged fermentation (SmF). The selected fungi were formulated and used for production of enzymes using oil palm biomass as a carbon source. Then, the mixed enzymes were precipitated and applied for saccharification of OPTr and its hydrolysate was used as feedstock for production of ethanol and acetic acid, as well as for pretreatment of POME and OPTr prior to co-digestion for enhancing the efficiency of biogas production.

Literature review

1. Lignocellulosic biomass

1.1 Composition of lignocellulosic biomass

Lignocellulose is a renewable organic material and is the major structural component of all plants. Lignocellulose consists of three major components: cellulose, hemicellulose and lignin (Figure 1.1). In addition, small amounts of other materials such as ash, proteins and pectin can be found in lignocellulosic residues, in different degrees based on the source (Sánchez, 2009). Cellulose, the major constituent of all plant material and the most abundant organic molecule on the Earth, is a linear biopolymer of anhydroglucopyranose-molecules, connected by β-1,4-glycosidic bonds. Coupling of adjacent cellulose chains by hydrogen bonds, hydrophobic interactions and Van der Waal's forces leads to a parallel alignment of crystalline structures known as micro fibril (Zhang *et al*., 2007). Hemicelluloses, the second most abundant component of lignocellulosic biomass are heterogeneous polymers of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. Composition of hemicelluloses is very variable in nature and depends on the plant source (Dashtban *et al*., 2009). Lignin, the third main heterogeneous polymer in lignocellulosic residues, generally contains three aromatic alcohols including coniferyl alcohol, sinapyl and *p*-coumaryl. Lignin acts as a barrier for any solutions or enzymes by linking to both hemicelluloses and cellulose and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. Not surprisingly, lignin is the most recalcitrant component of lignocellulosic material to degrade (Himmel *et al*., 2007; Sánchez, 2009). Lignocellulosic wastes are produced in large amounts by different industries including forestry, pulp and paper, agriculture and food, in addition to different wastes from municipal solid waste (MSW) and animal wastes (Dashtban *et al*., 2009). These potentially valuable materials are treated as waste in many countries in the past and still are today in some developing counties, which raises many environmental concerns (Palacios-Orueta *et al*., 2005).

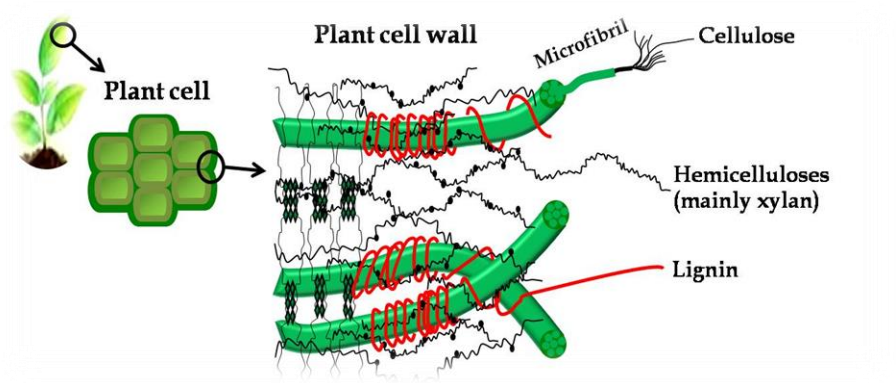

Figure 1.1. Structure of lignocellulosic biomass
Source: Ratanakhanokchai *et al*., 2013

1.2 Oil palm and chemical composition

Worldwide 42 countries cultivate *Elaeis guineensis* (oil palm tree) on about 27 million acres. Oil palm is one of the most valuable plants in Malaysia, Indonesia and Thailand. Oil palm tree generally has an economic life span of about 25 years and it contributes to a high amount of agricultural waste in countries (Abdul *et al*., 2012). The oil palm tree is ≈ 7–13 m in height and 45–65 cm in diameter, measuring 1.5 m above the ground level and one of the commercial crop in Thailand. Oil palm industries generate abundant amount of biomass say in millions of tons per year which when properly used will not only be able to solve the disposal problem but also can create value added products from this biomass. Oil palm biomass (OPB) is an agricultural by-product periodically left in the field during the replanting, pruning and milling processes of oil palm. Oil palm biomass is classified as lignocellulosic residues that typically contain 50% cellulose, 25% hemicellulose and 25% lignin in their cell wall (Alam *et al*., 2009). It is well know that chemical constituents of oil palm biomass significantly vary due to their diverse origins and types (Chew and Bhatia, 2008). The biomass from oil palm residue include the oil palm trunk (OPT), oil palm frond (OPF), kernel shell, empty fruit bunch (EFB), presses fruit fiber (PFF) and palm oil mill effluent (POME). Oil palm fronds accounts for 70% of the total oil palm biomass produced, while the EFB accounts for 10% and OPT accounts for only about 5% of the total biomass produced (Figure 1.2) (Abdul *et al*., 2012). They also stated that 89% of the total oil palm biomass produced annually used as fuel, mulch and fertilizer. Despite this enormous production, oil comprises only a small fraction of

the total biomass produced by the plantation. The remaining biomass is an immense amount of lignocellulosic materials in the form of fronds, trunks and empty fruit bunch. As such, the oil palm industry must be prepared to take advantage of the situation and utilize the available biomass in the best possible manner (Basiron and Yusof, 2007). Oil palm biomass waste can create substantial environmental problems when simply left on the plantation fields. Oil palm industries generate massive quantities of oil palm biomass such as oil palm trunk (OPT), oil palm frond (OPF) and oil palm empty fruit bunch (EFB). The OPF and OPT generated from oil palm plantation while the oil palm EFB from oil palm processing. Oil palm frond (OPF) is one of the most abundant by-products of oil palm plantation in Thailand. Oil palm fronds are available daily throughout the year when the palms are pruned during the harvesting of fresh fruit bunches for the production of oil. OPF contains carbohydrates as well as lignocellulose. Oil palm frond, consisting of leaflets and petioles, is a by-product of the oil palm industry and their abundance has resulted in major interest in their potential use for livestock feed. Oil palm tree discarded for replantation after 25-30 years of oil production. Related to the large production of main products from oil palm in Thailand, there is abundance of oil palm trunk. A large quantity of cellulosic raw material generated in the form of felled trunks during replanting can be utilized. Oil palm trunk obtained from oil palm tree and it consists of vascular bundles and parenchayma. Up to now, there is no economic value of oil palm trunk from the structural point of view and ultimately it becomes a hazardous material to farmers. To increase the added value of these residues, several investigations have been carried out to produce hybrid plywood, polymer composites, particle boards, paper, pulp, furniture, bio fuels etc. from oil palm biomass. At present, most of the oil palm biomass are disposed-off at the oil palm plantation or burned at the mills to produce oil palm ash (Abdul *et al*., 2012). Thus, finding useful utilization of the oil palm biomass will surely alleviate environmental problems related to the disposal of oil palm wastes.

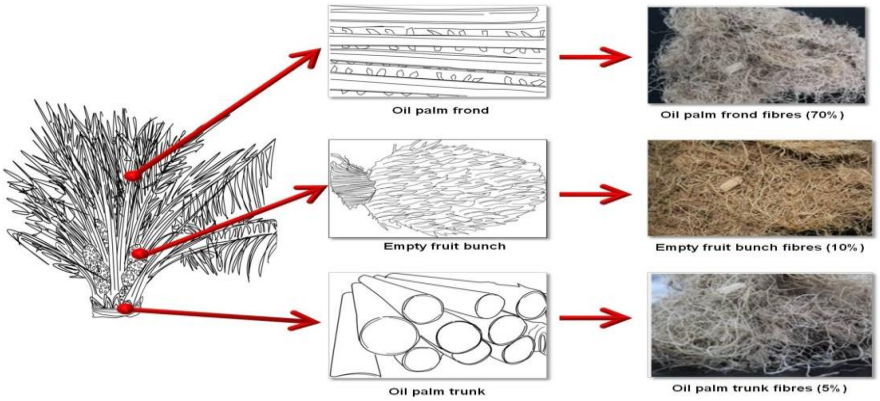

Figure 1.2. Oil palm biomass and oil palm biomass fibers form oil palm tree
Source: Abdul *et al*., 2012

Oil palm biomass is classified as lignocellulosic residues that typically contain 50% cellulose, 25% hemicellulose and 25% lignin in their cell wall (Alam *et al*., 2009). It is well know that chemical constituents of oil palm biomass significantly vary due to their diverse origins and types (Chew and Bhatia, 2008). Table 1.1 shows the chemical composition of different oil palm biomass (Abdul *et al*., 2012). Oil palm EFB fibres are lignocellulosic fibres where the cellulose and hemicellulose are reinforced in a lignin matrix similar to that of other natural fibres. These oil palm empty fruit bunch consist of high cellulose content and is a potential natural fibre resources, but its applications account for a small percentage of the total biomass productions. High cellulose content and high toughness value of oil palm EFB fibres make it suitable for application in polymer composites (Sreekala *et al*., 2004; John *et al*., 2008). The cell wall of OPF also consists of cellulose, hemicellulose and lignin. In addition to these main components, ash, glucose and xylose are also present in cell wall of oil palm fibres. It was revealed that oil palm fibre from oil palm frond contain highest composition of hemicellulose compared to coir, pineapple, banana, and even soft and hardwood fibres (Abdul *et al*. 2006). Researchers reported that chemical composition of EFB and OPF are quite comparable with coir but lower in cellulose content as compared to jute and flax fibres. Oil palm trunk fibre is strong and high content of lignin (23%) as lignified cellulose fibres retain their strength better than delignified fibres. The chemical compositions of a lignocellulosic fibre vary according to the species, growing conditions, method of fibre preparations and many other factors (Bledzki and Gassan 1999; Abdul *et al*., 2012).

Table 1.1. Chemical composition of oil palm biomass

Composition	Oil palm biomass chemical composition (wt%)		
	Oil palm EFB	**Oil Palm Frond**	**Oil Palm Trunk**
Cellulose	43-65	40-50	29-37
Hemicellulose	17-33	34-38	12-17
Holocellulose	68-86	80-83	42-45
Lignin	13-37	20-21	18-23
Xylose	29-33	26-29	15-18
Glucose	60-66	62-67	30-32
Ash	1-6	2-3	2-3

Source: Abdul *et al*., 2012

Ang *et al*. (2013) conducted production of cellulase and xylanase by *Aspergillus fumigatus* SK1 using untreated oil palm trunk (OPT) as a carbon source under solid-state fermentation (SSF). OPT used in current study contained 45.81% (w/w) cellulose, 17.74% (w/w) hemicellulose, 24.49% (w/w) lignin and 11.96% (w/w) extractives (gums, resins, pitch, waxes and many other) on dry weigh basis. The highest activities of extracellular cellulase and xylanase were produced at 80% moisture level and initial pH 5. The cellulase and xylanase activities obtained were 54.27, 3.36, 4.54 and 418.70 U/g of substrate for endoglucanase (CMCase), exoglucanase (FPase), β-glucosidase and xylanase, respectively (Ang *et al*., 2013).

Sabiha-Hanim *et al*. (2011) conducted effect of autohydrolysis and enzymatic treatment on oil palm (*Elaeis guineensis* Jacq.) frond fibres for xylose and xylooligosaccharides production. The composition of oil palm frond used in this study was as follows: cellulose, 44.0%; hemicellulose, 30.4%; lignin, 15.4%; ethanol-toluene extractives, 4.1%; ash, 3.2%; others (by difference), 15.5%, on the basis for dried oil palm frond weight.

Noparat *et al.* (2011) conducted isolation and characterization of high hydrogen-producing strain *Clostridium beijerinckii* PS-3 from fermented oil palm sap. Felled oil palm trunk (OPT) (25 years old) is an abundant biomass in Southern Thailand. The OPT composition was 31.28-42.85% cellulose, 19.73-25.56% hemicellulose, 10.74-18.47% lignin, 1.63-2.25% protein, 1.60-1.83% fat, 1.12-1.35%

ash and trace amount of minerals (0.01-0.40%). Oil palm sap extracted from OPT was found to contain 15.72 g/L glucose, 2.25 g/L xylose, and 0.086 g/L arabinose.

2. Lignocellulose-degrading microorganisms and lignocellulolytic enzymes

2.1 Microorganisms

Both bacteria and fungi can use cellulose as a primary carbon source. Most bacteria are incapable of degrading crystalline cellulose since their cellulase systems are incomplete. On the other hand, cellulolytic enzymes produced by some fungi generally involve all three types of enzymes, so are very useful in the saccharification of renewable pretreated lignocellulosic materials. Fungal strains that produce cellulases are mainly comprised of *Trichoderma, Aspergillus, Penicillium* and *Fusarium genera*. *Trichoderma reesei* is the most widely employed fungus for the production of cellulolytic enzymes and has been extensively studied (Stockton *et al*., 1991; Brijwani *et al*., 2010). Strains of *Trichoderma* can accumulate high activities of endo- and exo-glucanase, but are poor in β-glucosidase, whereas the strains of *Aspergillus* are high in β-glucosidase activity (Brijwani *et al*., 2010). Cellulolytic microorganisms are known as true cellulolytic microorganisms, which are able to degrade natural cellulose. Free cellulases can be produced by fungi or bacteria and fungi enzymes dominate commercial applications due to their high levels of expression and secretion (Santos *et al*., 2012).

While several fungi can metabolize cellulose as an energy source, only few strains are capable of secreting a complex of cellulase enzymes, which could have practical application in the enzymatic hydrolysis of cellulose. Besides *T. reesei*, other fungi like *Humicola, Penicillium* and *Aspergillus* have the ability to yield high levels of extracellular cellulases. Aerobic bacteria such as *Cellulomonas, Cellovibrio* and *Cytophaga* are capable of cellulose degradation in pure culture. However, the microbes commercially exploited for cellulase preparations are mostly limited to *T. reesei, H. isolens, A. niger, Thermomonospora fusca, Bacillus* sp. and a few other organisms (Table 1.2) (Sukumaran *et al*., 2005).

Table 1.2. Major microorganisms employed in cellulase production

Major group	Microorganism	
	Genus	**Species**
Fungi	*Aspergillus*	*A. niger, A. nidulans, A. oryzae* (recombinant)
	Fusarium	*F. solani, F. oxysporum*
	Humicola	*H. isolens, H. grisea*
	Melanocarpus	*M. albomyces*
	Penicillium	*P. brasilianum, P. occitanis, P. decumbans*
	Trichoderma	*T. reesei, T. longibrachiatum, T. hazianum*
Bacteria	*Acidothermus*	*A. cellulolyticus*
	Bacillus	*Bacillus* sp., *Bacillus subtilis*
	Clostridium	*C. acetobutylicum, C. thremocellum*
	Pseudomonas	*P. cellulosa*
	Rhodothermus	*R. marinus*
Actinomycetes	*Cellulomonas*	*C. fimi, C. bioazotea, C. uda*
	Streptomyces	*S. drozdowiczii, Streptomyces* sp., *S. lividans*
	Thermononospora	*T. fusca, T. curvata*

Source: Sukumaran *et al*., 2005

Lignocellulolytic enzymes-producing fungi are widespread and include species from the ascomycetes (e.g. *T. reesei*), basidiomycetes including white-rot fungi (e.g. *P. chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*) and finally a few anaerobic species (e.g. *Orpinomyces* sp.) which degrade cellulose in gastrointestinal tracts of ruminant animals (Yoon *et al*., 2007; Ljungdahl, 2008). Biomass degradation by these fungi is performed by complex mixtures of cellulases (Bayer *et al*., 1998), hemicellulases (Ljungdahl, 2008) and ligninases (Sánchez, 2009), reflecting the complexity of the materials. Cellulases and most hemicellulases belong to a group of enzymes known as glycoside hydrolases (GH). Currently more than 2500 GH have been identified and classified into 115 families (Cantarel *et al*., 2009). Interestingly, the same enzyme family may contain members from bacteria, fungi and plants with several different activities and substrate specifications.

2.2 Microbial community

Only an estimated 20% of the naturally occurring microorganism have been isolated and characterized so far. Selective enrichment cultures fail to mimic the conditions that particular microorganisms require for proliferation in their natural habitat. Furthermore, many microorganisms are bound to sediment particles and are thus not detected by conventional microscopy (Muyzer *et al.,* 1993).

At present, different molecular fingerprinting methods are available for studies microbial community, e.g., Denaturing Gradient Gel Electrophoresis (DGGE), terminal-Restriction Fragment Length Polymorphisms (t-RFLP) and Ribosomal Intergenic Spacer Analysis (RISA). The most common and powerful 16S rDNA fingerprinting based technique is DGGE. Muyzer *et al*. (1993) developed DGGE method that has potential to study the microbial flora quickly. Clone library technique, one of 16S rDNA based method has been widely used for microbial community studies. Sequencing of the clone libraries generated from environmental DNA has advantages over DGGE, as it provides precise identification and quantification of the phylotypes present in samples. However, clone library approach can be laborious in producing a number of sequences large enough to cover a whole community and limited by the difficulty to compare libraries and in determining if they are significantly different (Hur and Chun, 2004). Finally, a specific culture medium for the isolation of interested bacterial strains can be designed based on results obtained from community analysis. This procedure enables to isolate the true microbial key players in biological system or novel species in natural environment samples.

The comparative microbial community analysis will provides an accelerated approach to understanding community structure and function. The identification of unique or numerically dominant strains or groups under defined or controlled conditions is also possible. Therefore, tools for identification of the microbes present in the enzymes production process are necessary. It was recommended that the step to overcome such instability and to provide high enzymes-production efficiency, the insight into the enzymatic fermentation microbiology and factors involved in the stabilization/destabilization of the process should be further investigated.

Baharuddin *et al*. (2009) studied observation and identification of enteric microorganisms, biochemical changes and cellulase profiles during the co-composting of EFB with partially treated POME in pilot scale. DGGE technique was used to characterize microbial communities and diversity during the composting process. The results indicated that the composting process of EFB with partially treated POME was dominated by uncultured bacteria species. The dominant bacterial group changed from phylum *proteobacteria* in the thermophilic stage to phylum *chloroflexi* in the maturing stage. The maximum cellulase activity for CMCase, FPase and β-glucosidase were 13.6, 4.1 and 20.3 U/gds, respectively at day 30 of composting.

2.3 Lignocellulolytic enzymes

A cellulolytic enzyme system is a complex system of enzymes composed of endoglucanase (endo-1,4-β-D-glucanase, EC 3.2.1.4), exo-glucanase (1,4-β-D-glucan-cellobiohydrolase, EC 3.2.1.91) and β-glucosidase (β-D-glucoside glucanohydrolase, cellobiase, EC 3.2.1.21) that acts synergistically to degrade cellulosic substrate (Brijwani *et al*., 2010). Cellulolytic enzymes are central to biomass processing for the production of fuel ethanol and bio-products. Solid-state fermentation (SSF) presents many advantages including high volumetric productivity and relatively high concentration of the enzymes produced. Also, it will involve a lower capital investment and lower operating cost (Cen and Xia, 1999; Brijwani *et al*., 2010). Another important feature of SSF is that it utilizes heterogeneous products of agriculture (mainly agricultural residues) and by-products of agro-based industries (Brijwani *et al*., 2010). In solid-state fermentation of cellulase production, cellulosic substrate acts as both the carbon source and as an inducer for cellulase production (Cen and Xia, 1999; Brijwani *et al*., 2010).

Cellulose is a homopolysaccharide composed of β-D-glucopyranose units, linked by β-(1⟶4)-glycosidic bonds. Cellobiose is the smallest repetitive unit of cellulose and can be converted into glucose residues. The enzyme, which governs the hydrolysis of cellulose is known as ‘‘cellulase’’. Unlike most of the enzymes cellulase is a complex of enzymes that work synergistically to attack native cellulose. Cellulase is a family of at least three groups of enzymes: firstly endoglucanases

(EC 3.2.1.4) which act randomly on soluble and insoluble cellulose chains; Endoglucanases (EG) are also referred to as carboxymethylcellulases (CMCase), named after the artificial substrate used to measure the enzyme activity. EG initiate cellulose breakdown by attacking the amorphous regions of the cellulose, making it more accessible for cellobiohydrolases by providing new free chain ends. This has been shown by the effect of the enzyme on carboxymethylcellulose and amorphous cellulose. Fungal EGs are generally monomers with no or low glycosylation and have an open binding cleft. They mostly have pH optima between 4.0 and 5.0 and temperature optima from 50 to 70 °C (Percival *et al*., 2006; Dashtban *et al.,* 2009); secondly exoglucanases (cellobiohydrolases EC 3.2.1.91) that act to liberate cellobiose from the reducing and non-reducing ends of cellulose chains. Cellobiohydrolases (CBH) preferentially hydrolyze β-1,4-glycosidic bonds from chain ends, producing cellobiose as the main product. CBHs have been shown to create a substrate-binding tunnel with their extended loops which surround the cellulose. Similar to EGs, CBHs are monomers with no or low glycosylation with pH optima mostly between 4.0 and 5.0, but the temperature optima are wider, from 37 to 60 °C (Rouvinen et al., 1994; Divne et al., 1994) and finally, β-glucosidases (EC 3.2.1.21) which liberate glucose from cellobiose. β-glucosidases (BGL) have been isolated from many different fungal species including ascomycetes such as *T. reesei*, and basidiomycetes such as white-rot and brown-rot fungi. β-glucosidases hydrolyze soluble cellobiose and cellodextrins to glucose, and are thus competitively inhibited by glucose (Henrissat *et al*., 1991). The cellulases give us an opportunity to reap the tremendous benefits of biomass utilization in an eco-friendly manner (Himmel *et al*., 1999; Deswal *et al*., 2011). The cellulose-hydrolyzing enzymes (i.e. cellulases) are divided into three major groups: endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases. Total cellulose activity was determined as filter paper (FPase) activity by using Whatman No. 1 filter paper as substrate (Kumar *et al*., 2016). The endo-glucanases catalyse random cleavage of internal bonds of the cellulose chain, while cellobiohydrolases attack the chain ends, releasingcellobiose. β-glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose, for instance (Figure 1.3) (Kumar *et al*., 2008).

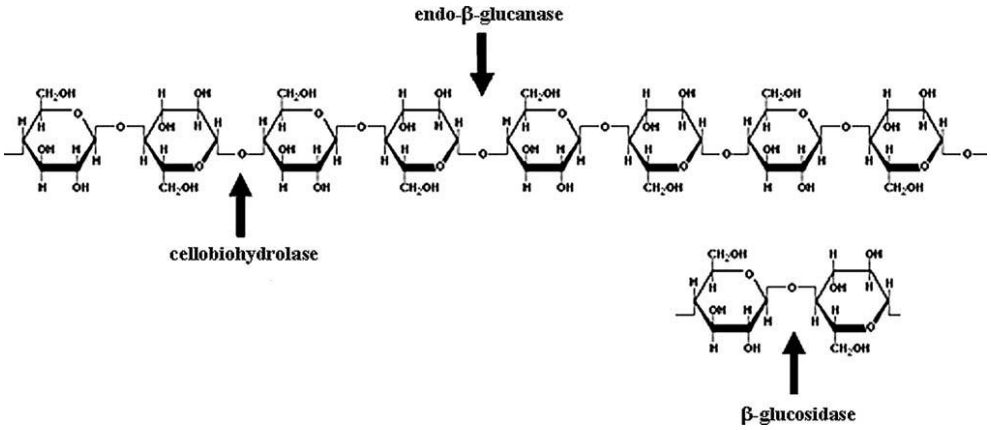

Figure 1.3. Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase and β-glucosidase

Source: Kumar *et al*., 2008

Hemicelluloses are heterogeneous polymers built up by pentoses (D-xylose, D-arabinose), hexoses (D-mannose D-glucose, D-galactose) and sugar acids. Hemicelluloses in hardwood contained mainly xylans, while in softwood glucomannans are most common. There are various enzymes responsible for the degradation of hemicellulose (Figure 1.4). In xylan degradation, for instance, endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, β-L-arabinofuranosidase and acetylxylan esterase all act on the different heteropolymers available in nature. In glucomannan degradation, β-mannanase and β-mannosidase cleave the polymer backbone. Xylanases are produced by diverse group of organisms including bacteria, algae, fungi, protozoa, gastropods and arthropods (Collins *et al*., 2005). However, xylanase produced from the filamentous fungi is very important from industrial point of view since filamentous fungi secretes much higher amount of xylanolytic enzymes into the medium than other microorganisms like bacteria or, yeast (Polizeli *et al*., 2005; Dobrev *et al*., 2007; Uday *et al*., 2016). The vast diversity of fungal species in nature is recognized as a target for screening to find out the appropriate source of enzymes with constructive and novel characteristics (Bakri *et al*., 2008). It hasbeen found that the production of xylanase enzyme is mostly car-ried out from the fungal genera of *Trichoderma, Aspergillus* and *Penicillium* in industrial scale (Bakri *et al*., 2009). Therefore, scientists are looking for new filamentous fungi which can produce higher levels of xylanse enzyme having novel characteristics. Like cellulose,

hemicellulose is also an important source of fermentable sugars for biorefining applications. Xylanases are being produced and used as additives in feed for poultry and as additives to wheat flour for improving the quality of baked products at the industrial scale (Kumar *et al*., 2008).

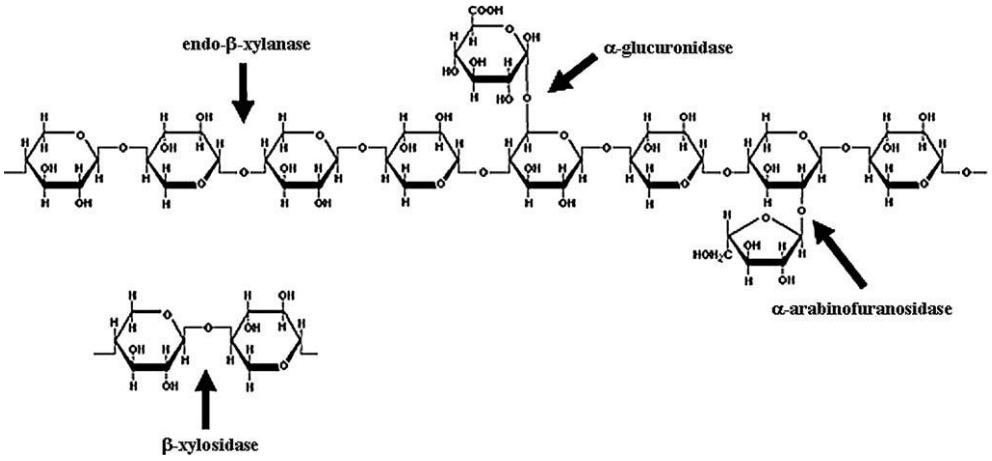

Figure 1.4. Polymeric chemical structure of hemicellulose and targets of hydrolytic enzymes involved in hemicellulosic polymer degradation

Source: Kumar *et al*., 2008

3. Inoculum preparation for enzyme production

Inoculum preparation is another important aspect in fermentation. There are several ways of preparing fungal inoculum for fermentation. To identify the most suitable type of inoculum to be employed in SSF, both the nature of fungi involved and the purpose of studies have to be taken into consideration. The commonly applied inoculum preparation methods for SSF include spore suspension, mycelia disc, mycelia suspension and pre-inoculated substrate (Yoon *et al*., 2014).

Spore suspension can be prepared by washing the surface of fungi cultured on Petri dish with sterilized water or salt solution (Hong *et al*., 2011, Dong *et al*., 2013). In the preparation of spore suspension, the fungal biomass is macerated and spore suspension it then obtained after filtration of the washing liquid. The number of spore count in the suspension can be adjusted with the aid of hemocytometer and the inoculum density can then be adjusted by adding water or salt solution into the suspension to achieve the desired spore concentration (Jha *et al*.,

1995; Shi *et al*., 2009). Spores suspension with concentration of approximately 10^6 cm^{-3} is often used as the inoculum for SSF (Khan *et al*., 2007; Huang *et al*., 2010). Besides the ease of inoculum preparation, spore suspension can also be stored for a longer duration and thus, mishandling during transfer of inoculums can be prevented. The drawback of this method is that the spore counting process is time consuming and it might not be suitable for some of the fungi that are sporeless or lack of spore such as the mutants of *Pleurotussajor-caju* (Ravishankar *et al*., 2006), *Pleurotus eryngii* (Obatak *et al*., 2003) and *Lentinus edodes* (Hasebe *et al*., 1991; Yoon *et al*., 2014). Fungal growth might also be slower if the spore suspension was used for inoculation due to its longer lag phase.

Inoculum in the form of mycelia disc is prepared by cutting the agar plug from the periphery of the actively grown fungi (Philippoussis *et al*., 2011). The mycelia disc can be directly used to inoculate the substrate. Mycelia disc inoculation is a more convenient method compared to spore suspension, but it might not be advisable to be used in comparison study involving different types of fungi. This is because the growth rate of different fungi might be different and hence, the mycelia density of the fungi grown on the agar plug varies with the types of fungi. This contributes to the difficulties in determining the density of fungi and a fair comparison for cellulase production for different fungi cannot be established. For white-rot fungi (WRF) or brown-rot fungi (BRF) that is sporeless or lack of spores, mycelia suspension appears as a preferable choice of inoculum in SSF. Inoculation of substrate by mycelia suspension can greatly eliminate the lag phase experienced by cultivating the fungi from its spore suspension. As a result, mycelia suspension inoculation was employed by many researchers and it has become the most popular choice of inoculum preparation method in cellulase production via SSF (Heidorne *et al*., 2006; Deswal *et al*., 2011). However, this method involves many preparation steps that are tedious and time consuming. First, the mycelia mat or mycelia disc from an agar plate with actively grown fungi needs to be transferred into a liquid medium before incubating it for 5-7 days (Elisashvili *et al*., 2009). After the incubation period, washing and homogenization of the fungal pellets is performed (Heidorne *et al*., 2006; Deswal *et al*., 2011).

Some researchers used pre-inoculated substrate as the inoculum for SSF (Reddy *et al*., 2003; Lechner *et al*, 2006; Xu *et al*., 2009). In general, this type of inoculum was prepared by transferring the mycelia disc onto the cooked or autoclaved wheat grains. It was then incubated at room temperature for a period of time ranges from 6 to 21 days, depending on the amount of wheat grain and mycelia disc used (Kumaran *et al*., 1997; Velazquez-Cedeno *et al*., 2002 Reddy *et al*., 2003). Calcium carbonate was added to the cooked wheat grain before inoculation to adjust the pH of the wheat grain into a range which is suitable for the particular fungus to grow (Kumaran *et al*., 1997; Yoon *et al*., 2014). By using this inoculum preparation method, the inoculum size is relatively difficult to be quantified and this might hinder the work which involves direct comparison of the performance of different fungi in cellulase production. On the other hand, one of the advantages associated with this method is that the substrate with grown fungus after SSF can be blended with a portion of fresh inoculum and use to re-inoculate a new batch of substrate. This can be seen as a step to minimize the use of fresh inoculums and also the waste generated from the SSF process.

Other than the abovementioned inoculum preparation methods, a user-friendly and cost-effective inoculum preparation method developed specifically for solid-state fermentation called Cellophane Film Culture (CFC) technique has been reported (Ang *et al*., 2013). This technique employed agar plated overlaid with cellophane film to ease the separation of viable fungal biomass, which is used subsequently as inoculum in fermentation. Similar to inoculum preparation via mycelia suspension, inoculum prepared from CFC technique can be quantified. It is reported that the technique has added advantages such as it requires less stringent handling condition and has lower risk of contamination during the inoculum preparation process compared to spore and mycelia suspension methods. In addition, inoculum prepared from CFC technique exhibits homologous morphology which permits the quick colonization following inoculation on the solid substrate (Ang *et al*., 2013).

Besides the types of inoculum as mentioned, cellulase production is also affected by the inoculum size applied (Zhang *et al*., 2012). Colonization of fungi on lignocellulosic substrate might take a relatively longer time if a low dosage of

inoculum is used. This might correspondingly raise the risk of contamination where other fast growing fungi might colonize the substrate in a faster rate compared to that of the intended microbial species. Higher inoculum size might accelerate the fungal growth rate but at the same time increase the rate of nutrient depletion. Upon nutrients depletion, the growth of the fungi is affected and this might not be helpful in improving the yield of cellulase (Kumaran *et al*., 1997; Yoon *et al*., 2014).

Cho and Lee (1999) conduct the formulation of a biocontrol agent by entrapping biomass of *Trichoderma viride* in Gluten matrix. The release of active fungi from formulated preparations to soil was governed by the soil pH, the moisture content of 5-20%, all formulated agent could generate 10^6-10^7 colony forming unit g^{-1} soil in the second week. The use of formulated preparations reduced the amount of biomass required, compared with nonformulated fungi as biocontrol agents.

Elzein *et al*. (2004) conduct the effects of inoculum type and propagule concentration on shelf life of Pesta formulations containing *Fusarium oxysporum* Foxy 2, a potential mycoherbicide agent for *Striga* spp. The lack of an adequate shelf-life in mycoherbicidal products has been an obstacle to their commercialization. Therefore, experiments were conducted to study the effect of inoculum type and concentration on the viability of the encapsulated propagules of *Fusarium oxysporum*, abbreviated as Foxy 2, in Pesta granules during storage. Pesta granules were made with different inocula of Foxy 2, including: microconidia, a mixture of mycelia and microconidia, and fresh as well as dried chlamydospore-rich biomass, each with three different inoculum concentrations. All granular preparations (0.5–2 mm, particle size) were stored at 4 °C or at room temperature (21±3 °C) for 1 year. All Pesta granules containing chlamydospore-inoculum retained higher viability (up to 100%) than those with mycelial and/or microconidial inocula, irrespective of inoculum concentration and storage temperature. Microconidial and mycelial preparations were not viable after 1 year at room temperature. Throughout the year, the viability of fresh and dried chlamydospore-rich biomass was not significantly affected by the concentration of chlamydospores in the formulation at 4 °C or room temperature. Thus, 85–100% viability of Foxy 2 propagules can be achieved in Pesta granules (0.5–2 mm) for at least 1 year by formulating chlamydospore-rich biomass and storing at a temperature

of 4 °C. This information has significant implications in enhancing shelf-life of Foxy 2 products thereby helping us to overcome this obstacle to commercialization.

Wijesinghe *et al.* (2011) development of a formulation of *Trichoderma asperellum* to control black rot disease on pineapple caused by *Thielaviopsis paradoxa*. A local isolate of *Trichoderma asperellum* was tested for its antagonistic activity against Thielaviopsis paradoxa (telemorph = Ceratocyctis paradoxa). The highest antagonistic activity was achieved when the concentration of *T. asperellum* conidia was 1 x 10^7 conidia/mL. The highest biomass and number of colony forming unit/mL of the *T. asperellum* peaked at 144 h after incubation in yeast waste residue medium. The minimum inhibition concentration value of the formulation was observed as 1% on growth of *Th. paradoxa* incubated at 28 °C for 10 d. In the soil fungicide-screening test, the effect of concentrations 100-1600 mg/mL on mycelia growth was not significant ($P < 0.05$). Complete mycelia growth inhibition occurred at concentration above 52,600 mg/mL. Results of the fruit application tests clearly showed that all treated fruits were free of disease at the end of the incubation period. No significant differences ($P > 0.05$) in pH, total soluble solids and titratable acidity were observed between fruits treated with formulation of *T. asperellum* and the control formulation treated pineapples.

Ang *et al.* (2013) study development of a novel inoculum preparation method for solid-state fermentation—Cellophane film culture (CFC) technique. This study report a user-friendly technique in the preparation of fungal inoculum intended for solid state fermentation (SSF)—cellophane film culture (CFC) technique. This technique uses cellophane film overlaid agar plates to facilitate the separation of fungal biomass. The findings showed that inoculums of *P. sajor-caju* produced is viable, and it was confirmed by the presence of laccase enzyme activity in SSF of rice husk. The correlation between fungal dry and wet weights ($r^2 = 0.9329$) provides an accurate estimation of fungal dry weight from its wet weight during inoculum preparation. Besides, this technique does not require a strict sterile handling condition and possesses lower risk of contamination compared with liquid culture and agar plugs approaches. In consideration of large scale inoculum preparation, this newly developed technique is comparatively more cost-effective, which further suggests its potential in inoculum preparation from mycelial fungi for

4. Production of lignocellulolytic enzymes under solid- and submerged fermentation

4.1 Methods for lignocellulolytic enzyme production

The two main strategies for the production of cellulases are solid state fermentation (SSF) and submerged fermentation (SF), which differ with respect to environmental conditions and forms of conduction. One of the most exalted parameters in differentiating these types of processes is unquestionably the analysis of the volume of water present in the reaction (Mazutti *et al*., 2010; dos Santos *et al*., 2012). The activity level of water for the purpose of ensuring growth and metabolism of cells, on the other hand, does not exceed the maximum binding capacity of the water with solid matrix. The static tray bioreactor, also known as a koji bioreactor is the commonly used bioreactor for SSF. In a tray bioreactor, the substrate is placed in trays and incubated in a controlled atmosphere room or chamber (Brijwani *et al*., 2010). Solid-state fermentation (SSF) conditions have shown to be potential for enzyme production by the filamentous fungi (Hölker *et al*., 2004). The commercial production of enzymes is carried out through SSF for its obvious advantages over liquid cultivation (Hölker *et al*., 2004; Deswal *et al*., 2011). The substrate used in SSF for cellulase production is detrimental in economizing the enzyme production process. Several factors are responsible for limiting the growth of microorganisms. Operating conditions like temperature, pH and moisture content are very important for microbial growth and efficient cellulolytic enzyme system production during solid-state fermentation (Wen *et al*., 2005). Also, successful scale-up strategy demands optimization of critical parameters that influence microbial growth and product formation. Often optimization of multiple parameters is an arduous and time consuming task.

4.2 Comparison between solid state fermentation (SSF) and submerged fermentation (SmF) methods

Lignocellulolytic enzymes are produced using the SmF method traditionally, in which the cultivation of microorganisms occurs in an aqueous solution containing nutrients. An alternative to this traditional SmF method is the SSF method, which involves the growth of microorganisms on solid materials in the

absence of free liquid. Since SSF involves relatively little liquid when compared with SmF, downstream processing from SSF is theoretically simpler and less expensive (Table 1.3) (Sadhu *et al.,* 2013).SSF has three advantages viz. i) lower consumption of water and energy, ii) reduced waste stream and iii) more highly concentrated product (Zhuang *et al*., 2007; Sadhu *et al*., 2013). Moreover, the biosynthesis of cellulases in SmF process is strongly affected by catabolic and end product repressions. On the overcoming of these repressions to significant extent in SSF system, is of economic importance. The amenability of SSF technique to use up to 20-30% substrate, in contrast to the maximum of 5% in SmF process, has been documented (Pamment *et al*., 1978; Sadhu *et al*., 2013).

Table 1.3. Comparison of characteristics for solid and submerged fermentation methods

Factor	**Solid-state fermentation**	**Submerged fermentation**
Water	Limited consumption of water and no effluent	High volumes of water consumed and effluent discarded
Mechanical agitation	Static conditions preferred	Good homogenization
Scale up	New design equipment needed	Industrial equipment available
Energy	Low energy consuming	High energy consuming
Equipment Volume	Low volumes and lost costs	High volumes and high costs
Concentration	100-300 g/L	30-80 g/L

Source: Sadhu *et al.,* 2013

Currently, cellulases, xylanase and pectinase contributed to almost 20% of world enzyme market (Polizeli *et al*., 2005; Ang *et al*., 2013). High production cost and low production yields had cause the bottleneck for industrial enzymes applications (Kang *et al*., 2004), thus alternate enzyme production method using cheaper ingredient with higher yield is the main goal of current study. Commercially, most of cellulases and xylanase enzymes are produced through submerged fermentation (SmF)due to easier controlled and maintained fermentation factors (Tolan and Foody, 1999; Ang *et al.*, 2013). However, the filamentous fungi which considered as strong cellulases and xylanase secreting strains perform better using SSF since solid medium could simulate fungi natural habitat (Ang *et al*., 2013) . Furthermore, SSF was more advantageous since it has greater volumetric productivities, higher product stability, low contamination risk and lower instrumental costs (Ang *et al*., 2013). Another advantage is the use of cheap solid agro-lignocellulose wastes which acts as carbon and energy source and further reduce the need of expensive nutrient medium.

Dhillon *et al.* (2011) conducts value-addition of agriculture wastes for augmented cellulase and xylanase production through solid-state tray fermentation employing mixed-culture of fungi. Solid-state fermentation (SSF) was performed to evaluate the potential of agricultural residues for the production of cellulase and hemicellulose using individual and mixed culture *Aspergillus niger* and *Tricoderma reseei*. The maximum filter paper (FP) cellulase activity of 13.57 IU/gram dry substrate (gds), 22.89 IU/gds and 24.17 IU/gds and β-glucosidase activities of 21.69 IU/gds, 13.58 IU/gds and 24.54 IU/gds were obtained with wheat bran medium at 96 h incubation period with *A. niger*, *T. reseei* and mixed-cultures of *A. niger* and *T. reseei*, respectively.

Bahrin *et al*. (2011) conducts the cellulase production on oil palm empty fruit bunch by *Botryosphaeria* sp. under solid-state fermentation. *Botryosphaeria* sp. showed the ability to produce cellulase (FPase, CMCase and β-glucosidase) from oil palm empty fruit bunch as substrate. The highest production of FPase, CMCase and β-glucosidase were 3.261 U/g, 8.134 U/g and 0.112 U/g, respectively.

4.3 Comparison of lignocellulolytic enzymes production under solid state fermentation and submerged fermentation method

The comparisons between the cellulases and hemicellulase activities production of other lignocellulosic materials in SSF and SmF system was shown in Table 1.4. The maximum CMCase and β-glucosidase activities recorded in current research were 6.67 and 39.25 fold higher respectively compared to enzymes produced by *Botryosphaeria* sp. using OPEFB in SSF (Bahrin *et al.*, 2011). Furthermore, SK1 showed high potential due to higher CMCase (3.21 fold), FPase (3.42 fold) and xylanase (7.2 fold) activities compared to SSF of wheat straw with similar fungi species (*A. fumigatus*). On other hand, SK1 CMCase activity was 1.4 fold higher compared to *A. fumigatus* MS16 (Sohail *et al*., 2009) and 3.6 fold higher compared to *Myrothecium verrucaria* TISTR 3225 (Prasertsan *et al*., 1992) which both cultivated in pure carboxymethylcellulose (CMC) as substrate in SmF system. The ability of *Aspergillus* sp. to secrete high concentration of xylanase has been reported (Polizeli *et al*, 2005; Sohail *et al*., 2009) and *Aspergillus niger* was commonly classified as strong xylanase producer (Ibrahim, 1998; Ang *et al*., 2013). In current research, *A. fumigatus* SK1 was a higher xylanase producer compared to *A. niger* USM Al 1 (12 fold) and *A. niger* ATTC 6275 (1.5 fold) where both cultivated using optimized palm kernel cake SSF method (Kheng *et al*., 2005). Solid-state fermentation which provides a natural environment conditions suitable for growing filamentous fungi was proven to be a better method to produce cellulases and xylanase (Singhania *et al*., 2010). Crude xylanase activity of strain SK1 was much higher (2.7 fold) compared to *A. niger* MS80 which cultivated in pure birchwood xylan as substrate using SmF method (Sohail *et al*., 2009). Current research also revealed that untreated substrate preserved large amount of xylan which remained intact and hidden under the lignocellulosic structure and this could stimulate the production of xylanase, compared to the acid-base pretreated substrate where their hemicellulose layers might lost along with the lignin (O'Dwyer *et al*., 1934; Ang *et al*., 2013).

Table 1.4. Cellulases and xylanase production from different fungi and substrate under solid state fermentation (SSF) and submerged fermentation (SmF)

				Enzyme activities*				
Organisms	**System**	**Reactor**	**Substrate**	**CMCase**	**FPase**	**β-Glucosidase**	**Xylanase**	**Reference**
Aspergillus fumigatus SK1	SSF	Flask	Oil palm trunk	54.27	3.36	4.51	418.70	Ang *et al*., 2013
Botryosphaeria sp.	SSF	Flask	Empty fruit bunch	8.13	3.26	0.11	-	Bahrin *et al*., 2011
A. niger USMAl 1	SSF	Flask	Palm kernel cake	-	-	-	35.00	Kheng *et al*., 2005
A. niger ATTC 6275	SSF	Flask	Palm kernel cake	23.80	-	-	282.90	Prasertsan *et al*, 1992
A. fumigatus	SSF	Flask	Wheat straw	16.90	0.98	11.80	56.40	Shenef *et al*., 2010
A. fumigatus SK1	SmF	Flask	Oil palm trunk	0.43	0.03	0.04	3.35	Ang *et al*., 2013
A. niger MS80	SmF	Flask	Birchwood xylan	-	-	-	1.24	Sohail *et al*., 2009
A. fumigatus MS19	SmF	Flask	CMC	0.31	-	0.39	-	Sohail *et al*., 2009
A. niger EFB 1	SmF	Rotary drum	Empty fruit bunch	0.13	0.05	0.16	-	Noratiqah *et al*., 2012

*U/g (SSF);U/ml (SmF)

Source: Ang *et al*., 2013

5. Precipitation of enzymes (proteins) and characterization of enzymes

Protein samples commonly contain substances that interfere with downstream applications. Several strategies exist for eliminating these substances from samples. Small soluble substances may be removed and the samples exchanged into appropriate buffers by dialysis or gel filtration (desalting columns). Pierce offers a variety of dialysis and desalting products for performing such buffer exchanges with small or large sample volumes (see Related Pierce Products). Another strategy for removing undesirable substance is to add a compound that causes protein to precipitate. After centrifugation to pellet the precipitated protein, the supernatant containing the interfering substance is removed and the protein pellet is re-dissolved in buffer compatible with the downstream application.

Enzymes are optimally active at a specific pH and temperature. These reactor conditions must be optimised to achieve optimal hydrolysis of substrates. The situation can be further complicated in SSF and CBP processes where conditions must also be optimal for the microorganisms involved in saccharification and fermentation. Where enzymes are not operating under optimal conditions, higher enzyme loadings may be required in order to achieve the same level of hydrolysis efficiency and this will affect the overall cost of the process (Van Dyk and Pletschke, 2012)

Faulds *et al*. (2008) investigated the effect of pH on solubilisation of brewer's spent grain over a range of pH 3.2–11.2. An enzyme mixture from *Trichoderma* (Depol 686, Biocatalysts) was efficient at low pH, while an enzyme mixture from Humicola (Depol 740, Biocatalysts) was effective over the entire pH range. In the Depol 686 mixture, side-chain cleaving enzymes such as arabinofuranosidase lost activity at higher pH levels and cellulase activity was absent at pH 7.5. In the Humicola mixture (Depol 740), optimum activities were between pH 6 and 8, with maximum solubilisation occurring at pH 9.

When dealing with lignocellulose substrates that are insoluble, suspension and mixing of the substrate in the assay or reactor may have an impact on hydrolysis. Particularly in large reactions, mass transfer limitations become important (Chundawat *et al*., 2008). Chundawat *et al*. (2008) indicated that increased agitation had an impact on Avicel conversion. Samaniuk *et al*. (2011) even referred to high intensity mixing as having a synergistic effect with enzyme hydrolysis. However,

other authors have argued that intensity of agitation had no effect as long as the solids remained suspended. Compounds such as glycerol may also be added to reduce settling of particles (Chundawat *et al*., 2008). While Chundawat *et al*. (2008) recommended mixing speeds of up to 400 rpm, Champagne and Li (2009) indicated that mixing above 200 rpm resulted in decreased hydrolysis as enzyme activity is lowered. Merino and Cherry (2007) have also indicated that the type of mixing could have an impact on hydrolysis. When comparing mixing of reactions in an orbital shaker as opposed to mixing by tumbling, more efficient hydrolysis was achieved through tumbling. They pointed out that this could be an important factor in hydrolysis of less severely pretreated substrates where hydrolysis rates are often slower. Mixing has also been investigated with respect to improvement of hydrolysis at high substrate loadings which is further discussed in Section 6.12. Some authors indicated that more intense mixing could improve hydrolysis at high substrate loadings (Wang *et al*., 2011) while others contradict this (Kristensen *et al*., 2009). Although mixing had an effect, intense or continuous mixing was not required to overcome mass-transfer limitations for efficient hydrolysis, but mixing obtained in flask studies was not considered adequate (Roche *et al*., 2009). Stability of enzymes at various mixing intensities could be examined to determine specific characteristics of enzyme.

Adeleke *et al*. (2012) investigated some properties of cellulase purified from the culture supernatant of *Bacillus coagulans Co4*, isolated from cocoa pod dumpsite were investigated for possible biotechnological applications. The crude cellulase was purified to apparent homogeneity using a combination of acetone precipitation, CM Sepharose CL-6B ion exchange chromatography and gel filtration on Sephadex G-100. The molecular and thermodynamic properties of the purified enzyme were studied following standard procedures. The specific activity of the purified cellulase rose from 0.10 to 47 units/mg of protein, at the end of purification. The molecular weight was found to be 14.5 kDa; and an apparent Km value of 0.18±0.06 mg/ml of carboxylmethylcellulose. The optimum pH and temperature were 7.5 and 60^{o}C respectively. The cellulase retained 40% residual activity when heated at 60^{o}C for 40 minutes. On the basis of these properties, it is concluded that the purified

cellulase is moderately thermostable and may have applications in the bioconversion of agricultural wastes into economically useful products.

Singh and Sharma (2012) using central composite design (CCD) concerning the purification of cellulase from the *Bacillus* sp. *JS*14 in a solvent extraction was established with Response surface methodology (RSM). Solvent concentration, pH, temperature and retention time were selected as process variables to evaluate the purification impact factor in solvent precipitation, including the purification fold and % recovery. An experimental space with 13 purification fold and 23 recovery percentage recovery is achieved through the optimized condition based on the model. The molecular weight of the purified enzyme was estimated to be 32.5 KDa. Optimum activity of purified enzyme was at pH and temperature 6.5 and 40°C respectively. Enzyme showed maximum activity with carboxymethyl cellulose as substrate with compare to rice husk, wheat straw and sucrose. The purified cellulase activity was inhibited by Na^{+}, Cl^{-}, Mg^{2+} Tween 80 and EDTA

6. Application of cellulolytic enzymes

Cellulolytic enzymes have been commercially available for more than 30 years, and these enzymes have represented a target for both academic as well as industrial research. Basic and applied studies on cellulolytic enzymes have demonstrated their biotechnological potential in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textile, and laundry. In the present paper, the potent industrial applications of cellulases have been critically reviewed. Microbial cellulases find applications in various industries as shown in Table 1.5 (Kuhad *et al*., 2011).

Table 1.5. Applications of cellulases in various industries

Industry	Applications
Agriculture	Plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence onmineral fertilizers
Bioconversion	Conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein, and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high quality fodder
Detergents	Cellulase-based detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; antiredeposition of ink particles
Fermentation	Improved malting and mashing; improved pressing and color extraction of grapes; improved aroma of wines; improved primary fermentation and quality of beer; improved viscosity and filterability of wort; improved mustclarification in wine production; improved filtration rate and wine stability
Food	Release of the antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruitjuices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture,flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits
Pulp and paper	Co-additive in pulp bleaching; biomechanical pulping; improved draining; enzymatic deinking; reduced energyrequirement; reduced chlorine requirement; improved fiber brightness, strength properties, and pulp freenessand cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, andsanitary paper
Textile	Biostoning of jeans; biopolishing of textile fibers; improved fabrics quality; improved absorbance propertyof fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics;restoration of colour brightness
Others	Improved carotenoids extraction; improved oxidation and colour stability of carotenoids; improved olive oilextraction; improved malaxation of olive paste; improved quality of olive oil; reduced risk of biomass waste;production of hybrid molecules; production of designer cellulosomes

Source: Kuhad *et al*., 2011

7. Ethanol production

Bioethanol is also known as ethyl alcohol or chemically C_2H_5OH or EtOH. It can be used directly as pure ethanol or blended with gasoline to produce "gasohol" (Staniszewski *et al*., 2007). It can be used as a gasoline improver or octane enhancer and in bioethanol-diesel blends to reduce the emission of exhaust gasses (Pejin *et al*., 2009). Bioethanol offers several advantages over gasoline such as higher octane number (108), broader flammability limits, higher flame speeds and increased heats of vaporization (Balat *et al*., 2009). In contrast to petroleum fuel, bioethanol is less toxic, readily biodegradable and produces lesser air-borne pollutants. A variety of feedstocks from the first, second and third generation has been used in bioethanol production. The first-generation bioethanol involves feedstocks rich in sucrose (sugar cane, sugar beet, sweet sorghum and fruits) and starch (corn, wheat, rice, potato, cassava, sweet potato and barley). Second-generation bioethanol comes from lignocellulosic biomass such as wood, straw and grasses. Third-generation bioethanol has been derived from algal biomass including microalgae and macroalgae (Nigam *et al*., 2011). Microorganisms such as yeasts play an essential role in bioethanol production by fermenting a wide range of sugars to ethanol. They are used in industrial plants due to valuable properties in ethanol yield (> 90.0% theoretical yield), ethanol tolerance (> 40.0 g/L), ethanol productivity (> 1.0 g/L/h), growth in simple, inexpensive media and undiluted fermentation broth with resistance to inhibitors and retard contaminants from growth condition. As the main component in fermentation, yeasts affect the amount of ethanol yield (Dien *et al*., 2005). Since thousands of years ago, yeasts such as *S. cerevisiae* have been used in alcohol production especially in the brewery and wine industries. It keeps the distillation cost low as it gives a high ethanol yield, a high productivity and can withstand high ethanol concentration (Kasavi *et al*., 2012). Nowadays, yeasts are used to generate fuel ethanol from renewable energy sources. Certain yeast strains such as *Pichia stipitis* (NRRL-Y-7124), *S. cerevisiae* (RL-11) and *Kluyveromyces fagilis* (Kf1) were reported as good ethanol producers from different types of sugars (Mussato *et al*., 2012).

S. cerevisiae is the most commonly employed yeast in industrial ethanol production as it tolerates a wide range of pH (Lin *et al*., 2012) thus making

the process less susceptible to infection. Baker's yeast was traditionally used as a starter culture in ethanol production due to its low cost and easy availability. However, baker's yeast and other *S. cerevisiae* strains were unable to compete with wild-type yeast which caused contamination during industrial processes. Stressful conditions like an increase in ethanol concentration, temperature, osmotic stress and bacterial contamination are the reasons why the yeast cannot survive during the fermentation (Basso *et al*., 2008). There are common challenges to yeasts during sugar fermentation which are rise in temperature (35–45 °C) and ethanol concentration (over 20%) (Tofighi *et al*., 2014). Yeasts growth rate and metabolism increase as the temperature increases until it reaches the optimum value. Increase in ethanol concentration during fermentation can cause inhibition to microorganism growth and viability. Inability of *S. cerevisiae* to grow in media containing high level of alcohol leads to the inhibition of ethanol production (Fiedurek *et al*., 2011). The other problems in bioethanol fermentation by yeast are the ability to ferment pentose sugars. *S. cerevisiae* is the most commonly used in bioethanol production. However, it can only ferment hexoses but not pentoses (Kumar *et al*., 2009). Only some yeast from genera *Pichia, Candida, Schizosaccharomyces* and *Pachysolen* are capable of fermenting pentoses to ethanol (Mussato *et al*., 2012). The problems of pentose fermentation can be solved by using hybrid, genetically engineered or co-culture of two yeast strains. Hybrid yeast strains are used simultaneously to ferment pentose and hexose sugars to ethanol. The hybrid strain has been developed by fusing protoplast of *S. cerevisiae* and xylose-fermenting yeasts like *P. tannophilus, C. shehatae* and *P. stipitis* (Kumari *et al*., 2013). Genetically engineered *S. cerevisiae* and co-culture of two strains have been developed to produce bioethanol from xylose with high yield. Genetic engineering use recombinant DNA technology to up-regulate the stress tolerance genes in order to overcome the inhibitory situations. Xylose reductase and xylitol dehydrogenase genes from *S. stipitis* were introduced into *S. cerevisiae* to develop strain with the ability of fermenting xylose. The engineered yeast strains can convert cellulose to ethanol more rapidly compared to unmodified yeast strains. Co-culture process simultaneously culture and grow two different yeasts in the same reactor (Tanimura *et al.,* 2012). Co-culture shows better ethanol production as compared to its pure culture (Nuwamanya *et al.,* 2012). In co-culture, pentose

utilizing yeasts like *Pichia* fermentans and *Pichia stipitis* are combined together with *S. cerevisiae* so that hexose and pentose sugars can be efficiently utilized (Singh *et al*., 2014; Karagoz *et al*., 2014).

8. Acetic acid production

Acetic acid (CH_3COOH) is one of the simplest organic carboxylic acid. This colourless weak acid is characterized by distinctive sour taste and pungent smell. Nowadays, this acid is considered as one of the key intermediate for many industries including: chemical, detergent, woo and food industries (Awad *et al*., 2012). Currently, the production of acetic acid is carried out by chemical means using petrochemical feedstock or by the traditional approach of fermentative alcohol conversion using specific type of acetic acid bacteria. Among different chemical methods used, methanol carboxylation is the dominant production technology and accounting for over 65% of global capacity followed by ethylene oxidation, and alkane oxidation processes. Nowadays, acetic acid is an important as intermediate compound for the industrial production of different chemicals such as vinyl acetate polymer, cellulose acetate, terephthalic acid, dimethyl terephthalate, acetic acid esters/acetic anhydride and calcium magnesium acetate. All these products are made from petroleum-derived acetic acid (Kim *et al*, 2002; Awad *et al*., 2012). In spite of the fact that biological process for acetic acid production account for only 10% of global market production, it remain important process as many countries law stipulate that food grade vinegar must come from biological origin (fermentation). Therefore, optimization of biological process for acetic acid production is one of the most important industrial research and subject for study by many researcher groups using either free or immobilized cell systems (Nishiwaki and Dunn, 2005; Kocher *et al*., 2006; Jimennez-Hornero *et al*., 2009). For this bioprocess, there are several bacteria which can contribute to the production of acetic acid. Acetic acid bacteria were divided into five to six genera of which *Acetobacter* and *Gluconabacter* species can tolerate high concentration of acetic acid, which explain their use in vinegar production (Yamada *et al*., 2010). For industrial production, there are several species of *Acetobacter* that can be described as the main vinegar producer such as, *A. aceti, A. pateurianus, A. peroxydans, A. orleaniensis, A. lovaniensis, A. estuniensis, A.*

malorum, A. cerevisiae and A. oeni. Therefore, *Acetobacter* is usually used in the production of vinegar from ethanol through acetaldehyde by consumed oxygen. This production process is very sensitive for cultivation conditions applied and the chemical composition of the production medium (Awad *et al*., 2012). Carbon source used plays important role for bacterial growth and acetic acid production. It has been reported that, sugars such as: arabinose, xylose, ribose, glucose, galactose, mannose, melibiose, and trehalose can ferment by most of the *Acetobacter* strains (Kadere *et al*., 2008). However, the oxygen requirement for *Acetobacter* conversion makes the processes energy intensive. Other research also found that, the maximum production of acetic acid was achieved when cultivation medium was kept at 30 °C (Park *et al*., 1992). Nevertheless, the study was examined on dilution rates of bioreactor. However, the study which had been done by Zahoor (2006) revealed that, *Acetobacter aceti* cells can grew in culture medium at temperature between 28 °C and 34 °C. Higher temperature up to 37 °C resulted in complete cell death (Zahoor *et al*., 2006).

Production of acetic acid can be carried out by aerobic and anerobic fermentation. Anaerobic process is one process carried out by *Clostridium*. Aerobic fermentation is two stage processes. Glucose is converted into ethanol by *Saccharomyces cerevisiae*. And second stage is ethanol is converted into acetic acid by *acetobacter aceti*. The fermentation is usually initiated by yeasts which break down glucose into ethyl alcohol with the liberation carbon dioxide gas (Eq. 1). Following on from the yeasts, *acetobacter aceti* oxidize the alcohol to acetic acid and water (Eq. 2). The *acetbacter aceti* are dependent upon the yeasts to produce an easily oxidisable substance (ethyl alcohol) not possible to produce vinegar by the action of one type of micro-organism alone (Patel *et al*., 2015).

Yeast reaction:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Ethanol}}{2CO_2 + 2CH_3CH_2OH} \quad (1)$$

Acetobacter aceti reaction:

$$\underset{\text{Ethanol}}{CH_3CH_2OH + O_2} \longrightarrow \underset{\text{Acetic acid}}{CH_3COOH + H_2O} \quad (2)$$

Kadere (2008) investigated the occurrence and identified the dominant spoilage genera of acetic acid bacteria in coconut wine, by plating the dilution series previously pre-enriched in a basal medium onto GYP agar, followed by physiological and biochemical tests. Both *Acetobacter* and *Gluconobacter* strains were Gram variable, oxidase negative and catalase positive. All Acetobacter strains over-oxidized ethanol to acetic acid and finally to CO_2 and H_2O, while *Gluconobacter* were unable to oxidize acetic acid to CO_2 and H_2O. *Acetobacter* and *Gluconobacter* alike showed positive growth at 25, 30 and 40°C and also at pH 7.0 and 4.5, while there was no growth at 45°C, pH 2.5 and 8.5. *Acetobacter* strains oxidized both lactate and acetate while *Gluconobacter* oxidized lactate only. Both genera were unable to liquefy gelatin. *Acetobacter* showed negative growth at 15°C and also in peptone medium, while *Gluconobacter* showed positive growth both in peptone medium and at 15°C.

The production of acetic acid is mainly carried out using submerged fermentation system and the standard strain *A. aceti*. The highest acetic acid production (53 g/l after 144 h fermentation) was produced in medium composed of glucose (100 g/l), yeast extract (12 g/l) and peptone (5 g/l). Further optimization in the production process was achieved by process scaling up to 16-L stirred tank bioreactor. Maximal acid production of about 76 g/l was achieved in non pH controlled culture (Awad *et al*., 2012).

9. Biogas production

9.1 Biogas

With fossil fuel supplies depleting and oil prices rising, the search is on for a carbon-neutral fuel as an alternative source that is sustainable and efficient. Biogas is another energy source that is used as car fuel, or for production of heat or electricity in different countries (Sims, 2003; Taherzadeh and Karimi, 2008). Biogas produced from agriculture, industrial and municipal waste waters, food industries and municipal garbage, not only fits this criteria, it is also readily available and holds promise for the future. Therefore, biogas could be regarded as an alternative and affordable green fuel that deserves study. Biogas constitutes mainly methane (55-65%) and carbon dioxide (30-45%) and may contain traces of gases (H_2S, H_2 and N_2) (Kapdi and Vijay, 2005). Biogas power has a higher potential in the Asian countries

due to the availability of palm oil residue industrial wastewater and livestock manure. The rise of intensive livestock production with focus more on cattle farming is causing major environmental damage around the world (Nasir *et al*., 2012) (Table 1.6 and 1.7).

The prospects of biogas power generation are possibly to be high in India, China, Malaysia, Thailand, Indonesia and the Philippines because of their favorable renewable energy policies and targets (Nasir *et al*., 2012). In South East Asia, particularly in Malaysia, Indonesia and Thailand, effluent from palm oil mills referred to as palm oil mill effluent (POME) can be converted into biogas. This in addition can be used to generate electric power through gas turbines or gas-fired engines. Raw POME contains a considerable amount of oil and fatty acids which all contribute to its high oxygen demand. Hence, it has to be treated in a series of open oxidation ponds, for the organic matter to be biodegraded to a much lower oxygen demand before being discharged (Alias and Tan, 2005). POME has the ability to support bacterial growth with the waste biodegradation because of its high nutrient content (Nasir *et al*., 2012).

Table 1.6. Property of biogas

Property of biogas	Value
Heating value (CH_4 60%)	21.5 /m^3
Proper high velocity	25 cm/s
Combustion air	650 ˚C
Heat capacity	1.6 kJ/m^3-˚C
Density	1.15 kg/m^3

Source: Nasir *et al*., 2012

Table 1.7. Physical and chemical character of methane

Property of methane	Value
Molecular formula	CH_4
Molar mass	16.042
Boiling point 14.696 psia (760 mm Hg.)	161.49 ˚C
Melting point 14.696 psia (760 mm Hg.)	182.48 ˚C
Specific gravity 15.5 ˚C (760 mm Hg.)	1.47 L/g
Heating value: 15.5 ˚C (760 mm Hg.)	38130.71 kJ/m^3
Octane	130
Combustion temperature	650 ˚C

Source: Nasir *et al*., 2012

9.2 Process of biogas production

Anaerobic digestion for biogas production has become a worldwide focus of research, because it is an attractive waste treatment practice where both pollution control and energy recovery can be attained (Ounnar *et al*., 2012). Anaerobic digestion is a naturally occurring process, by which anaerobic micro-organisms convert biodegradable organic matter into biogas in the absence of oxygen (Nasir *et al*., 2012). In addition, a nutrient-rich digestate is also produced which offer either fertilizer or soil conditioner properties (Nasir *et al*., 2012). Therefore, it is expected that the nutrients within the POME to produce a viable additive for biogas production.

Biological treatment in wastewater processes has been accepted as an effective way to remove dissolved and biodegradable constituents by utilizing variety of microorganisms, principally bacteria (Sankaran *et al*., 2010). Since POME contains high level of organic matters and thus, adoption of anaerobic digestion in the first stage of the treatment process is a necessity to convert the bulk of the wastes to biogas (biomethane).

The metabolic reactions that occur during anaerobic digestion of substrates involve four important stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Demirel and Scherer, 2008). The operational efficiency of an anaerobic digestion system primarily depends on the structure of microbial

community present in the system (Weiland, 2010). In addition, environmental factors such as temperature and pH play a significant role in determining the performance and fate of the microbial community in anaerobic digesters (Weiland, 2010). Figure 1.5 shows the main pathways of an anaerobic digestion and the descriptions for each stage are given in the following section.

Hydrolysis: The first step of an anaerobic digestion process is hydrolysis in which organic polymers (carbohydrates, proteins and lipids) are hydrolyzed to their respective organic monomers. For example, carbohydrates are converted to sugar or alcohols, proteins to amino acids and lipids to fatty acids. This is carried out by several hydrolytic enzymes such as cellulases, cellobiase, xylanase, amylase, lipase and protease secreted by hydrolytic microbes (Weiland, 2010). The organic monomers will then be utilized either as substrates by fermentative organisms (amino acids and sugars) or by anaerobic oxidizers (fatty acids) (Demirel and Scherer, 2008).

Acidogenesis: The second step is acidogenesis (also referred to as fermentation), in which the hydrolyze products are degraded further to simpler organic products such as acetate, hydrogen (H_2) and carbon dioxide (CO_2). These final products of fermentation will eventually become the precursors of biomethane formation.

Acetogenesis: During acidogenesis process, not only acetate, H_2 and CO_2 are produced, but complex intermediary products such as propionate, butyrate, lactate and ethanol will be produced simultaneously. Such intermediary products will be converted to simpler organic acid, CO_2 and H_2 by acetogenic bacteria.

Methanogenesis: The final step of anaerobic digestion is methanogenesis inwhichmethane is produced by two groups of bacteria (methanogens), namely acetotrophic methanogens and hydrogenotrophic methanogens. Acetotrophic methanogens convert acetate to biomethane (CH_4) and CO_2 whereas hydrogenotrophic methanogens use H_2 as electron donor and CO_2 as electron acceptor to produce biomethane (Demirel and Scherer, 2008). In addition, many H_2-using methenogens can also use formate as an electron donor for the reduction of CO_2 to biomethene (Demirel and Scherer, 2008). These bacteria are

highlysensitive to oxygen; oxygen is a deadly poison that kills all methanogens even at low concentration.

Biogas production from activated sludge is an old and almost established process. It has also recently been produced on industrial scales from municipal solid waste (MSW) and some homogeneous wastes such as manures. Forestry and agriculture residues and MSW are by nature heterogeneous in size, composition, structure, and properties. Sugars, starches, lipids and proteins present in MSW are among the materials easily degradable by microorganisms, while some other fractions such as lignocelluloses and keratin are more difficult to degrade (Buffiere *et al*., 2006; Taherzadeh and Karimi, 2008). Biological degradations of these polymers are carried out by several enzymes such as amylase, cellulase, protease, keratinase and lipase, before further fermentation or digestion to e.g. ethanol or biogas. However, these polymers should be accessible to the enzymes for biodegradation (Taherzadeh and Karimi, 2008).

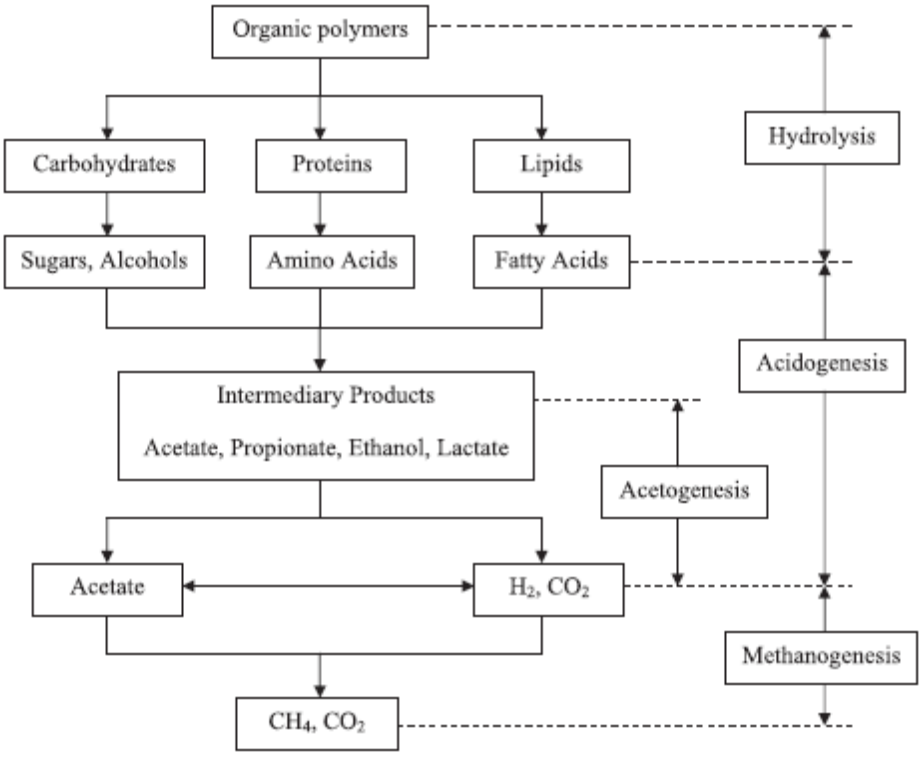

Figure 1.5. Anaerobic digestion of organic matter to methane.

Source: Lam and Lee, 2011

Chaikitkaew *et al*. (2015) conducted the biogas production form biomass residues of palm oil mill by solid state anaerobic digestion. The results shown that empty fruit bunches (EFB) can converted to methane with the maximum methane potential of 144 ml CH_4/g VS at F/I ratio of 2:1 corresponding to cumulative methane production of 2180 ml and 89% biodegradability.

Suksong *et al.* (2015) investigated the biohythane production from co-digestion of palm oil mill effluent (POME) with solid residues (empty fruit bunches (EFB) and decanter cake (DC)) by two-stage solid state anaerobic digestion process. The methane yield from POME mixed with 10% DC was 391 ml CH_4/g VS and from POME mixed with 10% EFB was 240 ml CH_4/g VS.

Zieminski *et al.* (2012) study the effect of enzymatic pretreatment of sugar beet pulp and spent hops prior to methane fermentation, causing their partial saccharification, positively affected outcomes f anaerobic fermentation. The observed increase in the yield of biogas production (by 19 and 13% from hydrolysates of sugar beet pulp and spent hops, respectively, versus relevant controls. The highest yield of biogas was obtained from the enzymatic hydrolysate of sugar beet pulp (184 ml/d from 1 g COD at fermenter loading with organic matter of 5.43 g COD/L.d.

O-thong *et al.* (2012) conducted the effect of pretreatment methods for improved biodegradability and biogas production of EFB and its co-digestion with POME. The maximum methane potential of POME was 502 ml CH_4/g VS-added corresponding to 98% biodegradability. Meanwhile, the maximum methane potential of EFB was 202 ml CH_4/g Vs-added corresponding to 38% biodegradability. Co-digestion of EFB and POME enhanced microbial biodegradability and the methane yield was 276-340 ml CH_4/g VS-added for co-digestion of EFB with POME at mixing ratios of 0.4:1-2.3:1. The best improved was achieved from co-digestion of treated EFB by NaOH presoaking and hydrothermal treatment with POME, which resulted in 98% improvement in methane yield comparing with co-digestion untreated EFB. The maximum methane production of co-digestion treated EFB with POME was 82.7 m^3 CH_4/ton of mixed treated EFB and POME (6.8:1), corresponding to methane yield of 392 ml CH_4/g VS-added.

Objectives of Research Work

1. To study effect of incubation temperature on enzyme production and microbial community profile during natural fermentation of ground OPT and OPF.
2. To isolate, select and identify of fungal strains for cellulose and xylanase enzymes production.
3. To compare on enzymes production from the selected fungal strains, the mixtures, the formulated inoculums and Super LDD1
4. To characterize of the crude enzymes from the formulated inoculum cultivated under SSF.
5. To apply crude enzymes for production of sugars from OPT residues (OPTr) and used for production of ethanol and acetic acid.
6. To apply crude enzymes for hydrolysis palm oil mill effluent (POME) and OPTr for biogas production from co-digestion of POME or POME hydrolysate with OPTr or OPTr hydrolysate in batch fermentation.

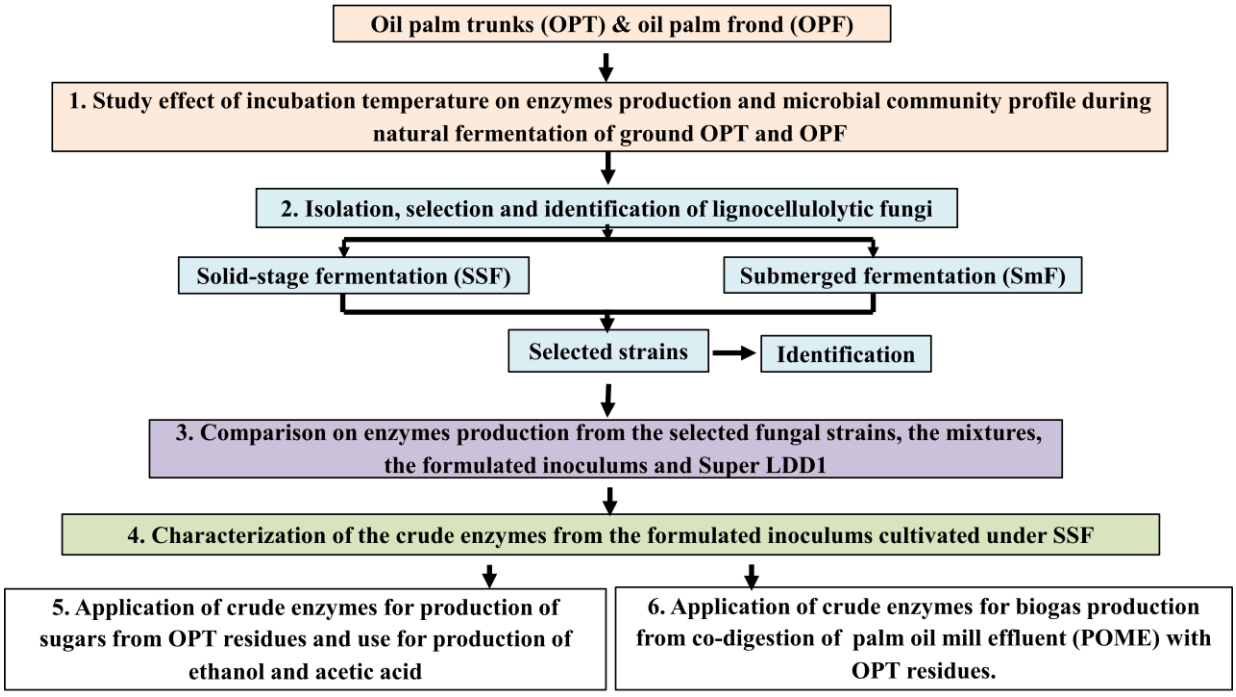

Figure 1.6 Flow chart of experimental procedure of this study

CHAPTER 2

MATERIAILS AND METHODS

1. Materials

1.1. Microorganisms and inoculums preparation

The fermentative yeast *Saccharomyces cerevisiae* TISTR5055 and *Candida shehatae* TISTR5843 from the culture collection of Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand were grown at room temperature (30±2 °C) in sterilized yeast malt broth on a rotary shaker (150 rpm) for 24 h. The culture broth was centrifuged, then, the sediment was dissolved in sterile distilled water to obtain the optimum concentration of the inoculum (OD600 = 0.5).

Acetobacter aceti was obtained from the culture collection of Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The strain was grown in sterilized nutrient broth (NB) on a rotary shaker (150 rpm) at room temperature (30±2 °C) for 24 h. Growth of the culture was measured as optical density at 600 nm and the initial inoculum concentration was adjusted using sterile distilled water to an absorbance of 0.5 for all experiments.

The mixed-culture inoculum “Super LDD1” was provided by the Land Development Department, Ministry of Agriculture, Thailand. It is generally used as the seed culture for composting in the solid-state fermentation. The Super LDD1 contained the mixed cultures of aerobic cellulose decomposing fungi (*Corynascus* sp., *Scytalidium* sp., *Chaetomium* sp., *Scopulariopsis* sp., *Helicomyces* sp. and *Trichoderma* sp.), bacteria (*Bacillus* sp.) and actinomycete (*Streptomyces* sp.) (Leaungvutiviroj *et al*., 2007). These mixed cultures were used as reference inoculum.

1.2. Culture medium

The modified Czapek-Dox medium (CDM) was used for isolation of lignocellulolytic fungi. It contained (g/L): 3 g $NaNO_3$, 1 g Na_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.005 g yeast extract and pH 6.5-6.8 (Singh *et al*., 2010) with OPT residues (OPTr) or OPF residues (OPFr) (1 kg) as the carbon source.

For selection of lignocellulolytic fungal isolates, the modified CDM agar plates was used with ground OPTr or OPFr (1% (w/v)) as the carbon source and 1.6 % (w/v) agar.

Mandel mineral salt medium (MMS) was used for fungal cultivation and spore preparation. It contained (g/L): $(NH_4)_2SO_4$ 1.4, $NaNO_3$ 5.0, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.3, $CaCl_2 \cdot 2H_2O$ 0.15, $ZnSO_4 \cdot 7H_2O$ 0.0025, $MnSO_4 \cdot 6H_2O$ 0.0014, $FeSO_4 \cdot 7H_2O$ 0.0025, $CoCl_2 \cdot 6H_2O$ 0.0014 and pH 6.5-6.8 (Mandel *et al*., 1996) with OPTr or OPFr (1 kg) as the carbon source.

Potato dextrose agar (PDA) was used for fungal growth and spore preparation. It contained (g/L): 4 of potato extract (equivalent to 200 of infusion from potatoes), 20 dextrose, 15 agar and final pH 5.6 ± 0.2 (HiMedia Laboratories Pvt. Ltd.).

Yeast malt (YM) broth was used for yeast cultivation and inoculums preparation. It contained (g/L): 5 peptic digest of animal tissue, 3 yeast extract, 3 malt extract, 10 dextrose and final pH 6.2±0.2 (HiMedia Laboratories Pvt. Ltd.).

Nutrient broth (NB) was used for bacteria cultivation and inoculums preparation. It contained (g/L): 10 peptone, 10 beef extract and 5 sodium chloride and pH after sterilization 7.3±0.1 (HiMedia Laboratories Pvt. Ltd.).

1.3. Preparation of ground OPT and OPF

Oil palm trunk (OPT) from old oil palm tree (approximately 25 years old) and oil palm frond (OPF) were obtained from oil palm plantation at Khao Phanom District, Krabi Province, Thailand. The OPT (9 m long from tip of OPT and weighed 1,786 kg) was cut into five parts based on its height by chainsaw; top end (a), top (b), middle (c), bottom (d), and bottom end (e) pieces, as shown in Figure 2.1. It was also cut into plank form (1.5 inch thick × 4 inch wide × 2 m long). Every OPT

parts and OPF were ground (using a grinder) (Figure 2.2) and determined for cellulose, hemicellulose, lignin (Lin *et al*, 2010), moisture content and ash (AOAC, 1990). Starch content of the top end and top parts were determined by adding water into each part in the ratio 1:1 (w/v), left for an hour then mixed well before leaving overnight (Noparat *et al*., 2011).

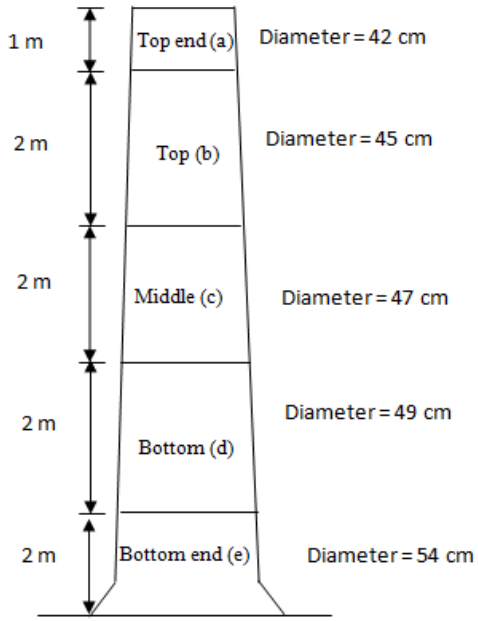

Figure 2.1 Diagrams of oil palm trunk preparation for determination of its composition.

1.4. Preparation of oil palm sap (OP sap), OPT and OPF residues

Each part of OPT and OPF were ground and soaked in distilled water (ratio 1:1, w/v) for 1 h, then pressed through a screw press to obtain oil palm sap (OP sap) and residues. The OP sap was centrifuged to obtain a clear solution for determination of glucose, fructose, cellobiose and arabinose (Noparat *et al*., 2011). The OPTr and OPFr were sun-dried for 1 day, then dried in an hot air oven (68˚C) for 3 days. They were kept as feedstock for further studies (Figure 2.3). The OPT part with high holocellulose and low lignin content would be selected as feedstock for production of lignocellulolytic enzymes.

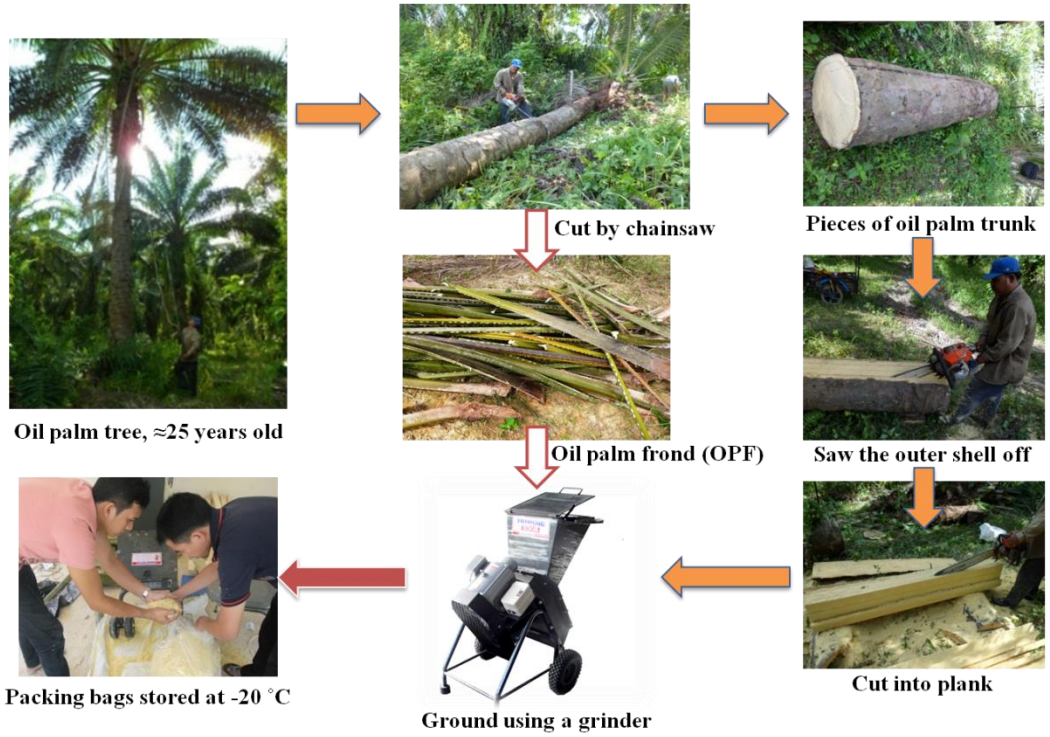

Figure 2.2 Oil palm trunk and oil palm frond preparation for determination of its compositions and used for extraction of sugar by pressed through a screw press to obtain oil palm sap and residues.

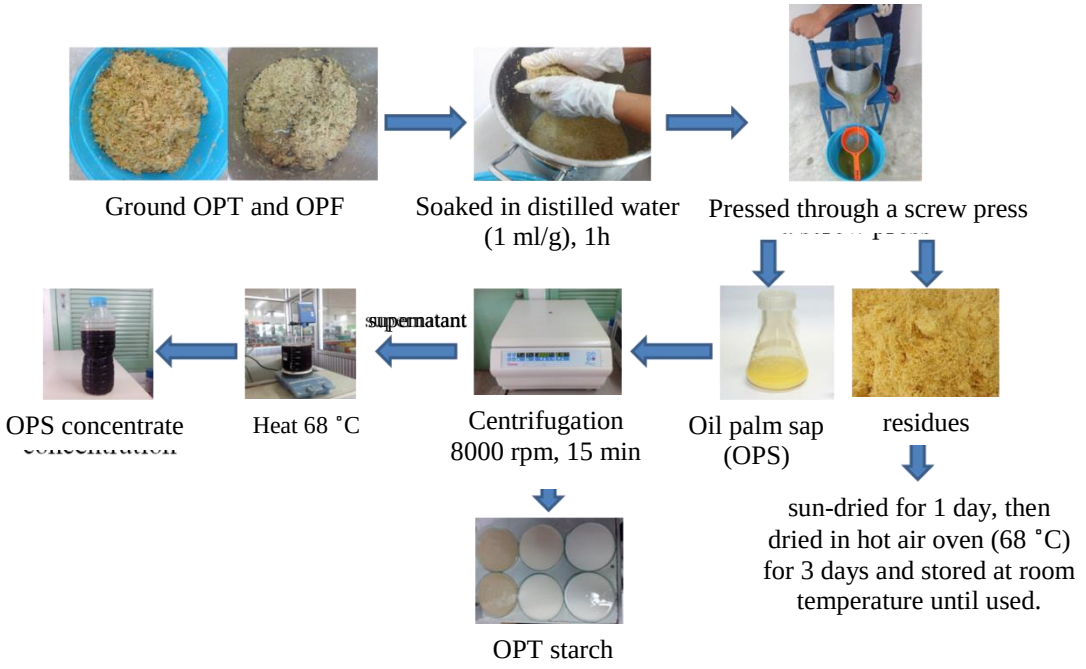

Figure 2.3 Preparation of oil palm sap (OPS), OPS concentrate, OPT starch from oil palm trunk (OPT) and oil palm frond (OPF) and their residues

1.5. Oil palm wastes

Decanter cake (DC) was the solid residue discharged from the decanter which is the 3-phase separator. Empty fruit bunches (EFB) was taken from a rotary drum thresher after the palm fruits had been removed from the sterilized fresh fruit bunches (FFB) and passed through a shredder machine. Palm pressed fiber (PPF) was obtained after the crude oil was separated from the sterilized fruit by means of a screw-press. These raw materials were collected from the palm oil mill in Southern Thailand and sun-dried for 1 day, then dried in hot air oven (68˚C) for 3 days and stored at room temperature until used.

Samples of palm oil mill effluent (POME) were taken from Nam Hong Palm Oil Co., Ltd. in Krabi Province, Thailand. Chemical oxygen demand (COD), total solid (TS), volatile solid (VS), suspended solid (SS), alkalinity and pH were determined by Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

2. Analytical methods

2.1. Enzyme activities assay

Crude enzymes were extracted from solid-state fermentation by adding 0.1% Tween 80 (10 ml/g of fermented substrate). The mixture was shaken (150 rpm) for 30 mins (Dhillon *et al.,* 2011) after which it was filtered and centrifuged at 4000 rpm for 10 mins to obtain a clear supernatant (Bahrin *et al*., 2011) and a liquot of the supernatant was diluted to assay the enzyme activities.

Carboxymethyl cellulase (CMCase) was assayed in the reaction containing 1 % (w/v) of carboxymethyl cellulose (CMC) in 50 mM citrate buffer pH 4.8 (0.5 ml) and appropriate diluted enzyme (0.5 ml). After 30 min incubation at 50 °C, reducing sugar was measured by 3,5-dinitrosalicylic acid (DNS) method (Miller *et al*., 1959) with glucose was used as a standard (Bailey *et al*., 1992; Ncube *et al*., 2012). One unit (U) of CMCase activity is defined as the amount of enzymes that liberates 1 µmol of glucose per minute.

Xylanase activity was assayed in the reaction containing 1 % (w/v) of oat spelt xylan in 50 mM citrate buffer pH 4.8 (0.5 ml) and appropriate diluted enzyme (0.5 ml). After 10 min incubation at 50 °C, reducing sugar was measured by

3,5-dinitrosalicylic acid (DNS) method (Miller *et al*., 1959) with xylose as a standard (Bailey *et al*., 1992; Ncube *et al*., 2012). One unit (U) of xylanase activity is defined as the amount of enzymes that liberates 1 µmol of xylose per minute.

Exoglucanase (FPase) assay was carried out by incubating 0.5 ml suitably diluted crude enzyme with 1 ml citrate buffer (50 mM, pH 4.8) containing Whatman Filter paper (No.1) strip (1 cm × 6 cm, 50 mg) and incubation at 50 ˚C for 60 min. Then, reducing sugar was measured by 3,5-dinitrosalicylic acid (DNS) method (Miller *et al*., 1959) with glucose was used as a standard. One unit of FPase activity correspondent to 1 µmole of glucose released per minute (Ang *et al*., 2013).

2.2. Reducing sugar estimation by nitrosalicylic acid (DNS) method

The reducing sugar was determined using DNS method. DNS reagent of 3 ml was added to 1 ml of sample in a capped test tube, then heat the mixture at 90º C for 5 min to develop the red-brown color. After cooling to room temperature in a cold water bath, the absorbance was recorded with a spectrophotometer at 520 nm (Miller, 1959).

2.3. Determination of sugars concentration

The concentrations of hexose sugars (glucose) and pentose sugars (xylose, arabinose) were determined using high performance liquid chromatography (HPLC) (Agilent 1200) equipped with a HPX-87H (300 mm × 7.8 mm) column (Bio-Rad, Hercules, CA) and a refractive index detector. The sample was diluted with deionized water, filtered through 0.22 µm, 13 mm Nylon membrane filter (Sartorius, Goettingen, Germany) and then injected in the chromatograph under the following conditions: column temperature at 65 °C, 5 mM sulfuric acid as mobile phase at a flow rate of 0.7 ml/min, and an injection volume of 20 µl. The concentration of these compounds was calculated using calibration curves obtained from standard solutions (Noparat *et al*. 2012). Data shown were the average of three replicated assessments.

2.4. The microbial community analysis

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was used to analyze microbial community structure in methane production stage as previously described by Kongjan *et al*. (2011) and Hniman *et al*.

(2011). Most of the bands were excised from the gel and re-amplified. After reamplification, PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by database searches in Gen Bank using BLAST (Muyzer and Smalla, 1998; Mamimin *et al*., 2015).

2.5. Determination of colony forming units (cfu)

For the measurement of cfu, serial dilutions were made on Petri dishes containing a selective medium consisting of PDA (39 g/L), Triton X-100 (1 ml/L) and cholorotetracycline-HCl (20 mg/L), followed by incubation at room temperature for 4 days (Cho and Lee, 1999).

2.6 Determination of the biogas composition

The biogas composition (CH_4, CO_2 and N_2) were analyzed by gas chromatography (GC) (Hansen *et al*., 2004) using a GC-8A Shimadzu Gas Chromatograph equipped with Shin-Carbon ST 100/200 Restek (2 m × 0.25 mm ID) and thermal conductivity detector (TCD). The flow rate of carrier gas (Argon) was 2.3 ml/min (100 kPa) and the injector temperature was 100 ˚C. Methane accumulation and yield were presented as ml CH_4 and ml CH_4/g VS of initial mixed substrate of control, respectively.

2.7. Statistical analysis

The data presented were analyzed using SPSS (SPSS Inc., version 15.0). One-way analysis of variance (ANOVA) was carried out to compare the means of different treatment where significant *F* value was obtained. Differences between individual means were tested using Duncan's Multiple Range Test (DMRT) at 0.05 significant levels. The data was performed in triplicate.

3. Methods

3.1. Effect of incubation temperature on enzyme production and microbial community profile during natural fermentation of ground OPT and OPF

3.1.1. Chemical compositions of OPT and OPF

The OPT (9 m long from tip of OPT) was cut into five parts based on its height by chainsaw. It was also cut into plank form (1.5 inch thick × 4 inch wide × 2 m long). Every OPT parts and OPF were ground and determined for cellulose, hemicellulose, lignin (Lin *et al*, 2010), moisture content and ash (AOAC, 1990). Each part of OPT and OPF were ground and soaked in distilled water (ratio 1:1, w/v) for 1 h, then pressed through a screw press to obtain oil palm sap (OP sap + starch) and residues. The OP sap was centrifuged to obtain a clear solution for determination of glucose, fructose, cellobiose and arabinose (Noparat *et al*., 2011).

3.1.2. Effect of incubation temperature on enzyme production profile and microbial community profile during natural fermentation of ground OPT and OPF

The ground OPT and OPF (3 g each) were added into each test tube (16 mm × 150 mm) and incubated at room temperature (30±2 °C), 40 °C and 50 °C for 30 days. Samples every 3 days were taken for analysis of carboxymethyl cellulase (CMCase) and xylanase activity. The samples with the lowest and the highest enzyme activity as well as samples at the beginning and the end of fermentation period were analyzed for microbial community by polymerase chain reaction-denaturing gel electrophoresis (PCR-DGGE) method (Muyze *et al*., 1993; Hniman *et al*., 2011). The samples with the highest enzymes activities were selected for further studies (step 3.2).

3.2. Isolation, selection and identification of high enzyme-producing fungi from OPTr under SSF and SmF

The selected part of OPT, OPF and OPT plank were naturally fermented at room temperature and samples were taken when growth of microorganisms appeared. All samples were added with 0.1% Tween 80 (10 ml/g of fermented substrate) and the mixture was shaken (150 rpm) for 30 min (Dhillon *et al*.,

2011), then 0.1 ml diluted culture (10^{-6}-10^{-7}) was inoculated onto PDA plates using spread-plate technique. After 3 day incubation, the fungal isolates from the PDA plates were restreaked until the pure culture were obtained, then maintained on PDA slant and stored at 4 °C for further studies.

Selection of high enzyme-producing fungi on oil palm biomass plates was conducted. All fungal isolates were grown on the modified CDM plates containing ground OPTr or OPFr (1% w/v each) as a carbon source. The plates were incubated at room temperature (30±2 ˚C) for 5 days. Fungal isolates that grew on oil palm biomass plates were selected and subcultured on PDA slants and incubated at room temperature for 5 days. Spore suspension was prepared by adding 10 ml of 0.1% sterile Tween 80 solution on PDA slant, scrapped and mixed gently. The spore concentration was adjusted to 10^6 spores/ml and used as an inoculum for comparison on enzymes production.

Selection of high enzyme-producing fungi was conducted by cultivation under SSF with OPTr or OPFr as a carbon source with the addition of modified CDM solution (5 ml/g dry substrate (gds)). After sterilization, spore suspension containing 10^6 spores/g dry substrate (gds) was inoculated and added distilled water to adjust the moisture content to 60 % (Prasertsan *et al*., 1997; Prasertsan *et al*., 2001; Delabona *et al.*, 2012), then, incubated at the room temperature for 4 days under constant shaking (150 rpm). Crude enzymes were extracted by adding 0.1% Tween 80 (10 ml/g of fermented substrate). The mixture was shaken (150 rpm) for 30 min (Dhillon *et al.*, 2011) after which it was filtered and centrifuged at 4000 rpm for 10 min to obtain a clear supernatant (Bahrin *et al*., 2011). Aliquots of the supernatant was diluted appropriate concentration for determination of CMCase, xylanase activities (Bailey *et al*., 1992; Ncube *et al*., 2012) and FPase activity (Ang *et al*., 2013). For submerged fermentation (SmF), spore suspension (10^6 spores/gds) was inoculated into the modified CDM solution containing 5% (w/v) of OPTr and OPFr and incubated at room temperature (30±2 °C) for 4 days under constant shaking (150 rpm). Samples were taken for determination of CMCase, xylanase and FPase activity followed the procedure as described above.

The isolates producing the highest CMCase, xylanase and FPase activity under SSF and SmF using OPTr as a carbon source were identified by

18S rRNA gene sequence and NCBI BLAST search. The sequence was amplified by PCR using primer pair and the PCR product was eluted using gel extraction kit. After re-amplification, the PCR product was purified and sequenced using reverse primer by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). The closest matches for partial 18S rRNA gene sequences were identified. This was done using the ribosomal database project (http:// rdp.cme.msu.edu/) with SeqMatch program and basic local alignment search tool (BLAST) with the nucleotide database in the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3. Enzymes production from the selected and identified fungal strains and the mixedcultures

The selected and identified fungal strains, possessing the highest CMCase, xylanase and FPase from either SSF or SmF, were cultivated on PDA plates at room temperature for 5 days. Spore suspension was prepared to obtain 10^6 spores/ ml and used as an inoculum (Ncube *et al*., 2012).

The inoculum of each strain and mixed strains (ratio 1:1:1, v/v/v) were grown under SSF and SmF in modified MMS solution (5 ml) with each of OPTr and OPFr (5 g) with either OPTr or OPFr (5 g) in 250 ml Erlenmeyer flasks with condition as descended above (section 3.2). Spore suspension containing 10^6 spores/gds was inoculated and incubated at room temperature (30±2 °C) for 7 days. Samples were taken at regular intervals of 24 h and analyzed for CMCase, xylanase and FPase activities.

3.4. Enzymes production from the formulated inoculum

3.4.1 Formulation of the inoculums and effect of the storage temperature

The two selected fungal isolates possessing the highest cellulase and xylanase activities were grown on PDA for 5 days at room temperature (30±2 °C). Spores were harvested by adding 10 ml of 0.1% sterile Tween 80 solution and using sterile spatula to gently remove the spores from the agar surface. A standard spore count was done using a haemacytometer. The spore suspension was adjusted to 1×10^6

spores/ml and added 10% glycerol solution to obtain an inoculum (Cho and Lee, 1999). The ground OPTr were mixed with sterilized broken rice in the ratio of 1:3 (ground OPTr : sterilized broken rice). The substrate mixture was thoroughly mixed, then sterilized at 121 ˚C for 15 min and cooled. After that, the spore suspension was inoculated into the substrate mixture and mixed well before incubating at the optimum temperature for 4 days, then spreaded on a plate and drying at 37°C for 4 days (Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Thailand). The formulated inoculum in packed dried form were determined for the number of colony forming units (cfu) (Cho and Lee, 1999).

The formulated inoculum in packed dried form were packed in plastic bottle and determined for the number of colony forming units (cfu) (Cho and Lee, 1999). They were stored at room temperature and 4 ˚C for 6 months. The inoculum sample (0.5 g each) was taken each month for determining the viable count as number of colony forming units (cfu).

3.4.2. Enzyme production from the formulated inoculum and "Super LDD1" under SSF and SmF

The formulated dried inoculum was tested for enzymes production efficiency and compared with the packed dried reference inoculum "Super LDD1". Both inoculums were cultivated under SSF and SmF with sterilized OPTr as a carbon source in 250 ml Erlenmeyer flasks with condition as described above (section 3.3) and incubated at the optimum temperature for 3 and 4 days. Samples were taken and analyzed for lignocellulolytic enzymes activity including CMCase, xylanase and FPase activity.

3.4.3. Enzymes production from the formulated inoculum using different oil palm biomass as carbon sources

Enzymes production from the formulated inoculum was conducted using oil palm trunk residues (OPTr), oil palm fronds residues (OPFr), empty fruit bunches (EFB), decanter cake (DC) and palm pressed fibers (PPF) as a carbon source under SSF and using palm oil mill effluent (POME) as a carbon source under SmF. The substrate was supplemented with modified MMS solution and mixed thoroughly before autoclaved at 121 ˚C for 15 min. After cooling, the medium was inoculated

with the formulated inoculum (10%) and incubated at optimum temperature for 4 days. Samples were taken and enzymes were extracted from the culture broth by adding 0.1% Tween 80 (10 ml/g of fermented substrate). The mixture was shaken (150 rpm) for 30 minute (Dhillon *et al.*, 2011) after which it was filtered and centrifuged at 4000 rpm for 10 minute to obtain a clear supernatant (Bahrin *et al*., 2011). The supernatants were analyzed for CMCase and xylanase activities.

3.5. Characterization of the crude enzymes from the fresh formulated inoculum cultivated under SSF

3.5.1. Production of the lignocellulolytic enzymes by SSF

Enzyme production from OPTr was conducted in plastic bags (9 × 14 inch) containing one kg of OPTr and MMS medium in the ratio 1:1 (w/v) and added distilled water to adjust the moisture content to 60% (Delabona *et al*., 2012). After thoroughly mixed, they were plugged loosely with a stopper of cotton wool, and autoclaved at 121 ˚C for 15 min. After cooling, the medium was inoculated with 10% of the formulated inoculum of *Trichoderma koningiopsi*s TM3 and incubated at the optimum temperature for 5 days. Samples were taken for extraction of enzymes by adding 0.1% Tween 80 (5 ml/g of fermented substrate). The mixture was shaken (150 rpm) at room temperature for 30 min after which it was centrifuged at 4000 rpm for 10 min, then the supernatant was analyzed for activity of CMCase and xylanase (Bailay *et al*., 1992; Ncube *et al*., 2012).

3.5.2. Characterization of the crude enzymes

The crude enzymes from *Trichoderma koningiopsi*s TM3 were characterized on the optimum temperature, pH and thermal stability for the enzymes activity. The optimum temperature was determined by assaying at different reaction temperatures (30 to 60 ˚C) in 50 mM citrate buffer (pH 4.8). The temperature presented the maximum activity was selected and used in the assay to find the optimum pH. The pH profile was assayed in 50 mM citrate buffer with the pH range 3.6-6.0. The thermostability was determined by incubating the crude enzymes at the temperature range of 30-60 °C for 5 h without respecting substrate. The residual activity of each enzyme was measured subsequently at the time interval

(0, 1, 2, 3, 4 and 5 h) using standard assay method under the optimum temperature and pH.

3.6. Application of the enzymes for sugars production from OPT residues and use for production of ethanol and acetic acid

3.6.1. Precipitation of the crude enzymes

The enzymes in the supernatant were precipitated by adding cold acetone at the ratio of 1:4 (supernatant: acetone) and allowed precipitation to occur at -20 °C overnight (Adeleke *et al.*, 2012). After centrifugation, the precipitate was dissolved in minimal amount of 50 mM citrate buffer (pH 4.8). The concentrated enzymes were assayed for CMCase and xylanase activity, expressed as unit per ml.

3.6.2. Effect of enzyme concentration on sugar production from OPTr

The 2.5 % (w/v) of ground OPTr was added into citrate buffer (50 mM, pH 4.8) and sterilized (121 ˚C for 15 min). After cooling, the enzymes (step 3.6.1) at various concentrations (0-40 unit/g OPTr) were added and incubated at 50 °C for 24 h under constant shaking (150 rpm) condition. Samples were taken at time interval for 24 h and determined for sugar concentration using HPLC (Noparat *et al*., 2012). The enzymes concentration giving the highest sugar production was selected for further studies.

3.6.3. Ethanol production using sugars from hydrolyzing the OPT residues with and without nutrients supplementation

The inoculum was prepared by cultivation of each *Saccharomyces cerevisiae* TISTR5055 and *Candida shehatae* TISTR5843 in sterilized YM medium at the room temperature (30±2 °C) on a rotary shaker (150 rpm) for 24 h. The culture broth was centrifuged at 4000 rpm for 10 min, then, the sediment was dissolved in sterile distilled water and the absorbance was measured at 600 nm of 0.5 before using as the inoculum. The 10% inoculum of *S. cerevisiae* TISTR5055, *C. shehatae* TISTR5843 and co-culture (ratio 1:1, v/v) was inoculated to 90 ml OPTr hydrolysate (from section 3.6.2). The cultures were incubated at room temperature (30±2 °C) on a shaker (150 rpm) for 36 h. Samples were taken every 6 h and after centrifugation, the

supernatant was analyzed for sugars and ethanol concentration using HPLC (Noparat *et al*., 2012). The yeast strain giving the highest ethanol concentration was selected for further studies.

3.6.4. Acetic acid production from OPTr hydrolysate by two-stage fermentation and co-cultures (*S. cerevisiae* and *A. aceti*)

The inoculums were prepared by cultivation of *S. cerevisiae* TISTR5055 and *A. aceti* in sterilized YM medium and NB medium, respectively, at room temperature on a rotary shaker (150 rpm) for 24 h. The culture broth was centrifuged, then, the sediment was dissolved in sterile distilled water to obtain the optimum concentration of the inoculum (OD_{600} = 0.5).

For two-stage fermentation, the 10% (v/v) starter culture of *S. cerevisiae* TISTR5055 (OD_{600} = 0.5) was inoculated into OPTr hydrolysate, with and without addition of nutrients and incubated at room temperature (30±2 °C) for 24 h. Then, the starter culture (10%, v/v) of *A. aceti* (OD_{600} = 0.5) was inoculated and incubated at room temperature (30±2 °C) for 36 h (until 60 h). Samples were taken at time interval. Then the supernatant was analyzed for sugars, ethanol and acetic acid using HPLC (Noparat *et al*., 2012).

For co-culture fermentation, the mixed starter culture (10%, v/v) of *S. cerevisiae* TISTR5055 and *A. aceti* (OD_{600} = 0.5, 1:1 ratio (v/v)) was inoculated into 250 Erlenmeyer flask containing the OPTr hydrolysate, with and without addition of nutrients in YM medium. They were incubated at room temperature for 36 h. Samples were taken at time interval and the supernatant was analyzed for sugars, ethanol and acetic acid using HPLC (Noparat *et al*., 2012).

3.7. Application of crude enzymes to increase biogas production from co-digestion of POME or POME hydrolysate with OPTr or OPTr hydrolysate

3.7.1. POME, anaerobic seed sludge and inoculum preparation

Palm oil mill effluent (POME) was taken from Nam Hong Palm Oil Co., Ltd. in Krabi Province, Thailand. Chemical oxygen demand (COD), total solid (TS), volatile solid (VS), suspended solid (SS), alkalinity and pH were determined by Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

Seed sludge was taken from covered lagoon of biogas production system from Nam Hong Palm Oil Co., Ltd. in Krabi Province, Thailand. An inoculum for biogas production was prepared by mixing seed sludge with POME in the volume ratio of 4:1 and acclimatized by adding POME (in the same ratio) every day for 5 day at 37 °C incubation. After the biogas production decreased and the separation appeared in the reactor, the clear supernatant was decanted. POME was then added into the seed sludge in the volume ratio of 4:1, giving the total volume of 5 L in 7 L reactor. The fermentation was conducted until no biogas.

3.7.2. The efficiency of crude enzymes for saccharification of POME and OPTr

Hydrolysis of lignocellulose in the raw POME using the concentrated enzymes from xylanase producer (*Trichoderma koningiopsi*s TM3) was tested. Enzymatic hydrolysis of total solid (TS) in the POME was carried out in sterilized POME by addition of the various concentrated enzymes at 0-15 Unit/g TS and incubation at 40 and 50 °C under constant shaking (150 rpm) for 18 h. The sample was taken every 3 h to analyze for sugars concentration. The hydrolysate with the highest sugar concentration was selected for biogas production in further studies.

For hydrolysis of OPTr, 2.5% (w/v) of the ground OPTr in the citrate buffer (50 mM, pH 4.8) was autoclaved at 121 ˚C for 15 min. After cooling, the enzymes at various concentrations (0-15 unit/g OPTr) were added and incubated at 40 and 50 °C under constrant shaking (150 rpm) for 18 h. The sample was taken every 3 h and analyzed for sugars concentration. The hydrolysate with the highest sugar concentration was selected for biogas production in further studies.

3.7.3. Biogas production of POME or POME hydrolysate with and without OPTr or OPTr hydrolysate

The biodegradability and biogas potential of POME, POME hydrolysate, OPTr, OPTr hydrolysate, co-digestion of POME with OPTr, co-digestion of POME with OPTr hydrolysate, co-digestion of POME hydrolysate with OPTr and co-digestion of POME hydrolysate with OPTr hydrolysate were determined in batch fermentation in 120 ml glass serum bottles under mesoplilic condition. In case of co-digestion, each condition was tested at the mixing ratios of 1:1. In each bottle, 80 ml

of inoculum (section 3.7.1) and 20 ml of substrate mixture were added and incubated at 37 °C for 30-45 days. The amount of biogas produced was measured by using the water replacement method, every day in the first 7 days and every 3 days thereafter and analyzed for gas composition by gas chromatography (GC). The effluent samples were analyzed for COD, sugars and pH every 3 day.

CHAPTER 3

RESULTS AND DISCUSSION

1. Effect of incubation temperature on enzyme production and microbial community profile during natural fermentation of ground OPT and OPF

1.1. Chemical composition of OPT and OPF

The chemical composition of cross section of OPT was previously reported (Noparat *et al*., 2011). Therefore, this research work emphasized on the chemical composition of OPT along its height as well as the sugar composition of OPT sap and OPF sap (Table 3.1). The oil palm was found to accumulate starch along its height with the highest quantity (2.57 kg) at the top end part (1 m long and weighed 67.85 kg) or calculated to be 3.79% (w/w of the top end part). Therefore, the top end part contained the highest starch content followed by the top part (2.78%). The middle part of the whole log of OPT (2.5 m long and 36-41 cm in diameter) was reported to contain 3.5% of dried solid OPT disc (Yamada *et al*., 2010). On the contrary, the moisture content of OPT was highest at the bottom part (about 65%) and decreased along its height to the top end part (about 53%). This was lower than that reported by Noparat *et al*. (2011) (about 75%) which may be due to the time of sampling and the age of oil palm. In addition, not only moisture content, the total amount of lignocelluloses of OPT was also highest at the bottom part (87.71%) and decreased along its height (59.10, 37.16 and 26.15%, respectively). The holocellulose content of OPT (13-55% cellulose and 12-20% hemicellulose) was comparable to that reported by Ang *et al*. (2013) (45.81% cellulose, 17.74% hemicelluloses) but the lignin content was lower (1.30-13.00% compared to 24.49%, respectively). Based on the composition of high holocellulose with low lignin content, the top and middle parts of felled OPT were considered to be good feedstock for production of cellulase and xylanase enzymes.

The sap of OPT and OPF contained glucose as the dominant sugar with the highest value in the OPF (25.42 g/L) followed by bottom part and middle part of OPT (20.13 g/L and 12.74 g/L, respectively) (Table 3.2). The average glucose

concentration of these two parts of OPT (16.44 g/L) was similar to that reported by Noparat *et al*. (2011) (15.72 g/L). In addition, the OPT sap had fructose content (3.04-6.02 g L^{-1}) similar to the Malaysian oil palm sap (3.07 g L^{-1}) (Kosuki *et al*. 2010) and OPF sap in this study (3.66 g L^{-1}). Arabinose was present in trace amount only in the sap from top end part (0.84 g L^{-1}) and top part (0.43 g L^{-1}) with none in the saps from the other two parts of OPT and OPF. In contrast, the sap of Raphia palm (*Raphia hookeri*) contained sucrose as the dominant sugar (Obahiagbon and Osagie, 2007). The discrepancy may be due to the difference in varieties, species and/or cultivating conditions.

Table 3.1 Chemical composition of ground oil palm trunk (OPT) and oil palm frond (OPF)

Composition	**OPT**				**OPF**
	Top end (a)	**Top (b)**	**Middle (c)**	**Bottom (d)**	
Moisture (%)	52.58±0.33	53.84±0.52	56.04±1.02	64.92±1.47	69.42±0.58
Cellulose (%)	12.98±0.92	23.41±1.40	36.60±1.17	55.04±0.50	44.78±0.57
Hemicellulose (%)	11.87±0.95	12.25±1.76	16.80±1.00	19.67±1.42	17.92±1.36
Lignin (%)	1.30±0.43	1.50±0.62	5.70±0.66	13.00±0.09	12.00±0.24
Ash (%)	1.49±0.34	1.75±0.06	1.95±0.03	1.95±0.03	2.84±0.11
Starch (%)	3.79	2.78	trace	trace	trace

Table 3.2 Chemical composition of saps from ground oil palm trunk (OPT) and oil palm frond (OPF)

Composition	OPT sap				OPF sap
	Top end (a)	Top (b)	Middle (c)	Bottom (d)	
Cellobiose (g/l)	0.27	0.28	0.95	1.33	1.12
Glucose (g/l)	4.96	3.91	12.74	20.13	25.42
Fructose (g/l)	3.04	4.63	5.06	6.02	3.66
Arabinose (g/l)	0.84	0.43	0.00	0.00	0.00
Acetic acid (g/l)	1.55	1.49	0.58	1.14	0.00
Total sugar (g/l)	9.11	9.25	18.75	27.48	30.20

1.2. Effect of incubation temperature on enzyme production profile during natural fermentation of ground OPT and OPF

Temperature is one of the most important physical variable affecting solid-state fermentation (Krishna *et al*., 2005) and the optimal temperature can maximize the rate of enzymatic reaction (Ran *et al*., 2012). The OPT and OPF were naturally fermented at room temperature (30±2 °C), 40 and 50 °C for 30 days and the microbial activities were detected by lignocellulosic enzymes production. Therefore, activity of CMCase and xylanase profiles throughout the process could reflect the microbial activity (Fig. 3.1). The enzymes activity increased rapidly on the first day of natural fermentation and increased dramatically during the first 6 h at room temperature with the CMCase and xylanase activity of OPT fermentation (0.48 and 0.16 Unit/gds, respectively) and OPF fermentation (0.24 and 0.23 Unit/gds, respectively) (Fig. 3.1A). The maximum CMCase (0.48 Unit/gds) was obtained at 15 days incubation of OPT while the maximum xylanase activity (0.24 Unit/gds) was achieved at 6 days incubation of OPF. After 9 days, the CMCase and xylanase activity began to decrease. At 40 ˚C (Fig. 3.1B, D), the maximum CMCase and xylanase activity of 0.25 and 0.44 Unit/gds were achieved at 3 days incubation of OPF. There

was no activity at 50 °C for both OPT and OPF. Similarly, the maximum xylanase production from *A. niger* FGSCA733 in SSF (6087 IU/g of *Jatropha curcas* seed cake) was obtained at 25 °C after 72 h whilst the highest cellulase production (3974 IU/g) was obtained at 40 °C and decreased at 45 °C (Ncube *et al.*, 2012). This suggested that a desirable fermentation temperature should be a compromise between optimum temperature for enzymes production and microbial growth.

1.3. Microbial community profile during natural fermentation of ground OPT and OPF

Sequence based surveys of bacterial diversity from samples taken from natural fermentation of ground OPT and OPF at room temperature (30±2 °C) and 40 °C at 6, 9, 15, 21 h and the end of fermentation period (30 h). Microbial community structure was investigated by polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences from DNA extracted from microorganism in the natural fermentation of ground OPT and OPF, followed by PCR-DGGE and sequencing. Diversity and abundance of more than 15 unique 16S rRNA gene phylotypes were obtained. Bacterial community developed from the natural fermentation of OPT and OPF was comprised of acetic acid bacteria, lactic acid bacteria and starch degradation bacteria constituted the major groups in these communities. The acetic acid bacteria were *Gluconobacter mesenteroides*, *Gluconobacter oxydans* and *Ameyamaea chiangmaiensis*. The lactic bacteria were *Leuconostoc mesenteroides* and *Weissella confuse*. Besides, starch degradating bacteria (*Bacillus* sp.) could be detected because the composition of OPT and OPF contained a lot of starch, thus *Bacillus* sp. was able to produce the enzyme amylase to convert starch into sugars. PCR-DGGE analyses of 16S rRNA genes in the samples from natural fermentation of ground OPT and OPF showed a diversity of bacteria (Fig. 3.2, Table 3.3).

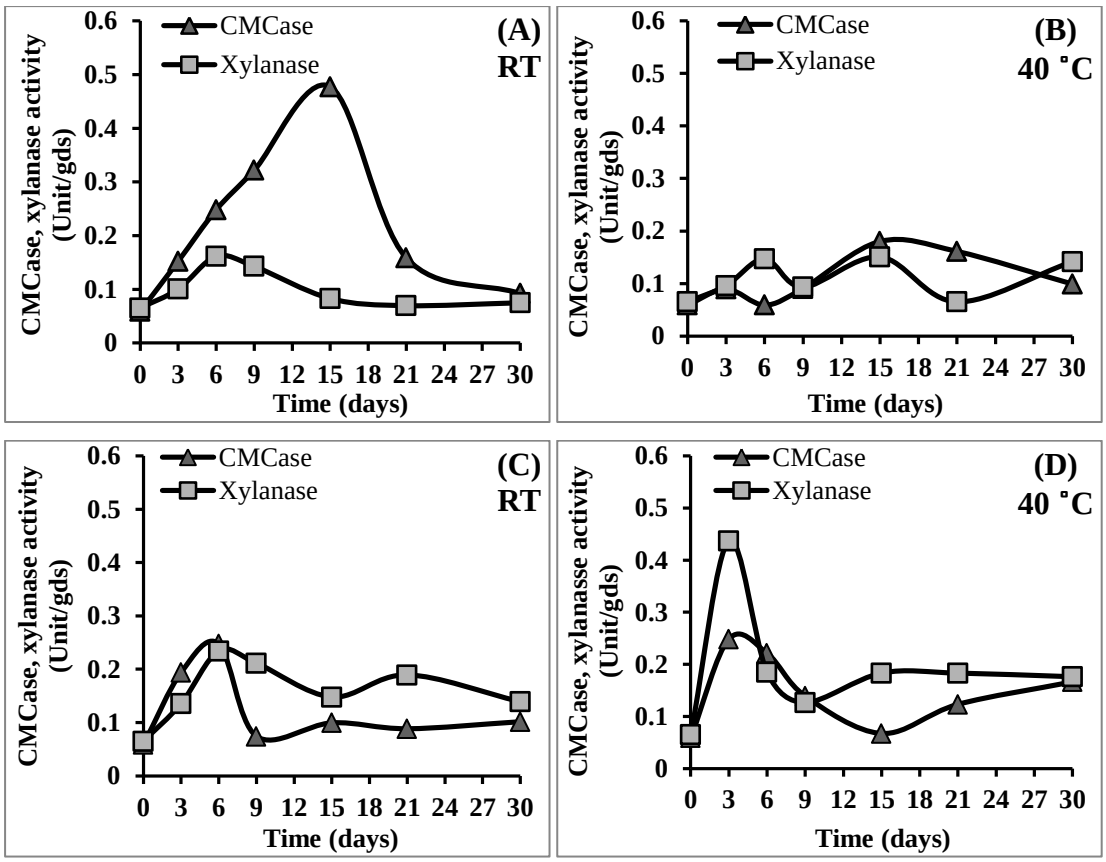

Figure 3.1 Profile of carboxymethylcellulase (CMCase) and xylanase activity during natural fermentation at room temperature (RT) (30±2 ˚C) (A, C) and 40 ˚C (B, D) using oil palm trunk (OPT) (A, B) and oil palm frond (OPF) (C, D) as substrate for 30 days.

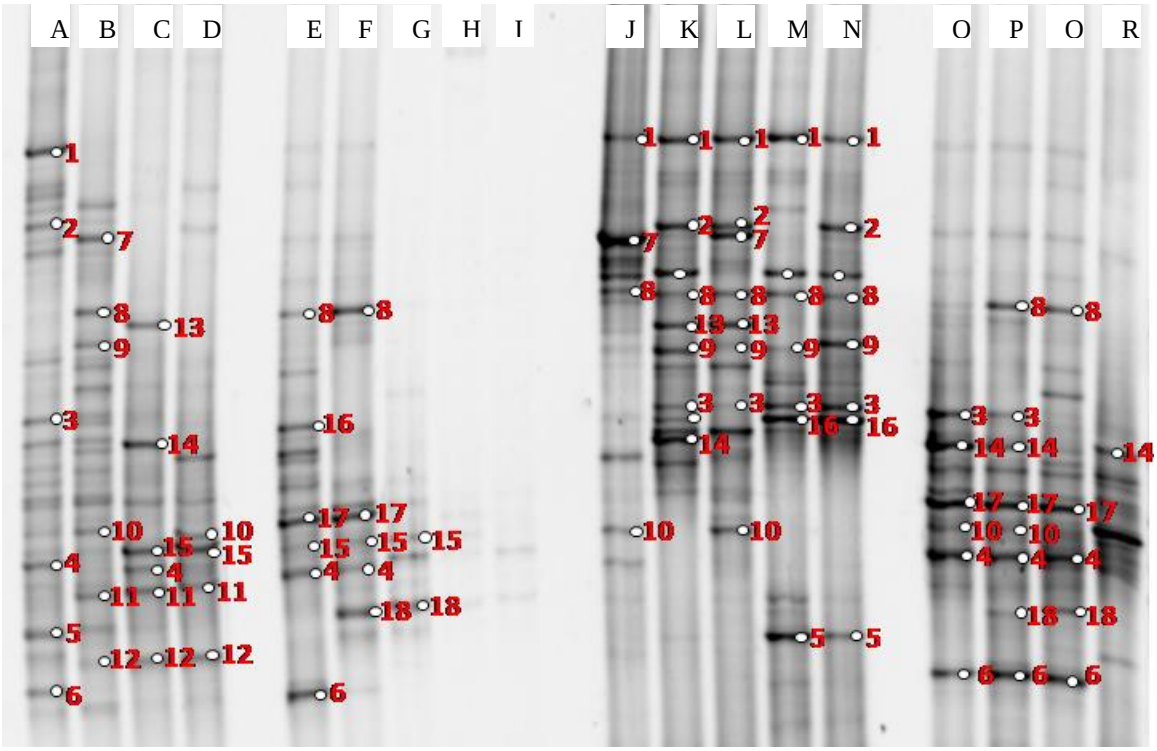

Figure 3.2 Bacteria community profile determined with PCR-DGGE of 16S rRNA genes fragments from natural fermentation of oil palm trunk (OPT), oil palm frond (OPF) at room temperature (30±2 °C) of OPT at 6, 9, 15 and 21 days (Land A-D) and OPF at 6, 9, 15, 21 and 30 days (Land E-I) and natural fermentation at 40 °C of OPT at 6, 9, 15, 21 and 30 days (Land J-N) and OPF at 6, 9, 15 and 21 days (Lane O-R).

DGGE with fungi and yeast diversity from samples of natural fermentation of ground OPT and OPF at room temperature (30±2 °C) and 40 °C with the lowest and highest enzyme activity as well as at beginning and the end of fermentation period. Microbial community structure was illustrated by polymerase chain reaction (PCR) amplification of 18S rRNA gene sequences from DNA extracted from microorganism in the natural fermentation of ground OPT and OPF, followed by PCR-DGGE and sequencing. Diversity and abundance for low unique 18S rRNA gene phylotypes were obtained. Yeasts were comprised of *Kluyveromyces marxianus*, *Candida* sp., *Pichia kudriavzevii* and *Candida tropicalis*. Yeast were common in natural fermentation in environmental. Fungi was low diversity in natural fermentation of OPT and OPF. Fungi community in natural fermentation of OPT and OPF were *Hexagonia hirta* and *Pycnoporus* sp. (Fig. 3.3, Table 3.4).

Table 3.3 Band of bacteria from Figure 3.2 sequence identified using the ribosomal database project with SeqMatch program and basic local alignment search tool (BLAST)

Band number	Description	% sequence identity (base)	Accession no.
1	*Weissella confuse*	100 (129)	NR113258
2	*Leuconostoc mesenteroides*	93 (127)	NR118557
3	*Bacillus thermoamylovorans*	93 (146)	NR117028
4	*Gluconobacter mesenteroides*	96 (110)	NR117735
5	*Ameyamaea chiangmaiensis*	100 (110)	NR112682
6	*Rhodococcus erythropolis*	100 (129)	NR074622
7	*Lysinibacillus xylanilyticus*	98 (147)	NR116698
8	*Bacillus circulans*	98 (149)	NR118445
9	*Virgibacillus marismortui*	96 (130)	NR028873
10	*Clostridium beijerinckii*	99 (109)	NR074434
11	*Microbacterium saccharophilum*	98 (116)	NR114342
12	*Cellulosimicrobium cellulan*	98 (112)	NR119095
13	*Agrococcus terreus*	93 (88)	NR116650
14	*Bacteroides xylanolyticus*	100 (129)	NR104899
15	*Microbacterium oxydan*	99 (118)	NR044931
16	*Bacillus thermoamylovorans*	98 (146)	NR029151
17	*Microvirga aerilata*	97 (117)	NR114298
18	*Gluconobacter oxydans*	99 (105)	NR074252

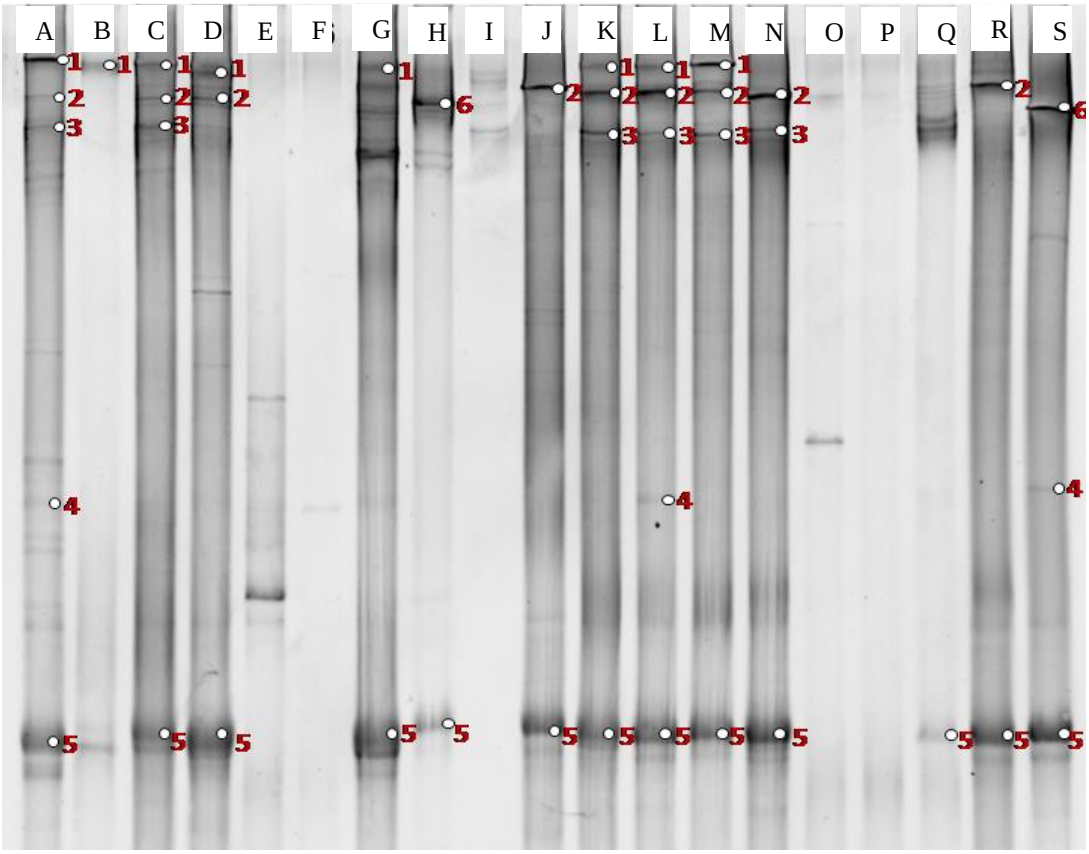

Figure 3.3 Fungi and yeast community profile determined with PCR-DGGE of 18S rRNA genes fragments from natural fermentation at room temperature (30±2 °C) of OPT at 6, 9, 15 and 21 days (Land A-D) and OPF at 6, 9, 15, 21 and 30 days (Land E-I) and natural fermentation at 40 °C of OPT at 6, 9, 15, 21 and 30 days (Land J-N) and OPF at 6, 9, 15, 21 and 30 days (Lane O-S)

Table 3.4 Band of fungi and yeast from Figure 3.3 sequence identified using the ribosomal database project with SeqMatch program and basic local alignment search tool (BLAST)

Band number	**Description**	**% sequence identity (base)**	**Accession no.**
1	*Kluyveromyces marxianus*	100 (285)	AP012217
2	*Candida* sp.	100 (474)	AY242226
3	*Pichia kudriavzevii*	100 (277)	JN941108
4	*Candida tropicalis*	96 (130)	KJ647397
5	*Hexagonia hirta*	100 (250)	AY336759
6	*Pycnoporus* sp.	100 (159)	GU182936

2. Isolation, selection and identification of high enzyme-producing fungi from OPTr under SSF and SmF

The natural fermented OPT (15 days) and OPF (6 days) at room temperature and the samples scrapped from surface of OPT plank after the natural fermentation at room temperature for 3 days were inoculated into 0.1% Tween 80 (10 ml/g of fermented substrate). The mixture was shaken (150 rpm) for 30 min (Dhillon *et al*., 2011), then diluted and 0.1 ml diluted culture (10^{-6}-10^{-7}) was inoculated onto PDA plates using spread-plate technique. Colonies were visible after 3 days incubation and the total of 20 strains were isolated. They could grow in CDM medium containing ground OPTr and OPFr as the carbon source. Eight out of the 20 fungal isolates could grow within 3 days incubation on oil palm biomass plates (data not shown). The isolates from top part of OPT encoded as TT1, TT2, TT3, TT4 and TT5 and from middle part of OPT encoded as TM1, TM2 and TM3. They were compared for their ability to produce CMCase, xylanase and FPase enzymes under SSF and SmF (Table 3.5, Fig. 3.4 and 3.5). Using OPTr as a carbon source (Fig. 3.4), the isolate TT1 exhibited the highest CMCase activity both under SSF (5.37 Unit/gds) and SmF (12.20 Unit/gds) while the isolate TM3 gave the highest xylanase activity under SSF (13.91 Unit/gds) and SmF (23.06 Unit/gds). The isolate TT2 gave the highest FPase activity (1.65 Unit/gds) under SSF but under SmF the isolates TT2, TT3 and TM1 gave similar FPase activities (1.54, 1.46 and 1.49 Unit/gds, respectively). Using OPFr as a carbon source (Fig. 3.5), the isolate TT1 also exhibited the highest CMCase activity (3.52 Unit/gds) under SmF with similar activity from the isolate TT3 (3.37 Unit/gds). The highest xylanase activity was obtained by the isolate TT2 (11.24 Unit/gds) under SmF which was similar to that of TT3 (10.36 Unit/gds). The isolate TM3 gave the highest FPase activity (1.54 Unit/gds) under SSF. Based on these results, the isolates TT1, TM3 and TT2 were selected as the best producer of CMCase (12.20 Unit/gds from SmF), xylanase (23.06 Unit/gds from SmF) and FPase activities (1.66 Unit/gds from SSF), respectively. However, all strains produced very low lignocellulolytic enzymes from the ground OPFr except FPase from the isolate TM3 (1.54 Unit/gds). This was due to the higher lignin content in the OPF (12%) than the OPT (1.5-5.7%).

The three best enzyme producer (TT1, TM3 and TT2) (Figure 3.6) cultivated on the ground OPTr and OPFr produced the highest CMCase, xylanase and FPase activities, respectively. All isolates were identified using 18S rRNA gene sequence and NCBI blast search. The results showed a 100% sequence identity of the isolate TT1 with *C. paradoxa* (KJ881375), 98% sequence identity of the isolate TM3 with *T. koningiopsis* T-404 (JQ278019) and 95% sequence identity of the isolate TT2 with *H. nigricans* NBRC 30611 (JN941681). *C. paradoxa* was reported as a sugarcane phytopathogen (Barros *et al*., 2010) and causing black seed rot disease in oil palm sprouted seeds (Eziashi *et al*., 2006) as well as bud and trunk rots affecting almost all species of palm (Garafalo and McMillan 2004; Girard and Rott 2004). *C. paradoxa* could produce xylanase and β-glucosidases enzymes (Barros *et al*., 2010). *Trichoderma* strains can accumulate high activities of endo- and exo-glucanase, but are poor in β-glucosidases (Brijwani *et al*., 2010). *T. konigiopsis* strain FCD3-1 produced the most efficient enzyme activity toward Avicel (0.37 U/ml), Filter paper (1.1 U/ml) and CMCase (5.5 U/ml), as well as β-glucosidases activity (1.18 U/ml) (Zhang *et al*., 2014). However, *T. koningiopsis* Th003 was able to induce the activity of β-1,3-glucanase and endochitanases to control different pathogens and stimulate growth in many crops (Moreno *et al*., 2009). The growth of some phytopathogenic fungi could be inhibited by the chemicals produced by *T. koningiopsis* YIM PH30002 (Chen *et al*., 2015). These chemical compounds were evaluated for their antifungal activity, nitric oxide inhibition and anticoagulant activity (Liu *et al*., 2016). *H. nigricans* is an anamorph of *Trichoderma* sp., by nature a wood decaying fungus and a common fungal species of moist forests (Myla *et al*., 2016). In addition, *Hypocrea species* was used in the biological control of plant pathogenic fungi with the ability to break down cellulosic materials. Therefore, this ability has led to the commercial exploitation of some *Hypocrea* and *Trichoderma* species in production of cellulolytic enzymes used in manufacture of denims, animal feed and bio fuels (Bhat *et al*., 1997; Myla *et al*., 2016). *H. nigricans* produced about 3 and 4 folds higher endo- and exo-1,4-β-D-glucanase under optimum condition (Myla *et al*., 2016). In addition, the isolate TT3, TT4, TT5, TM1 and TM3 were identified. The results showed that the isolates TT3, TT5 and TM1 were *T. asperellum* and the isolates TM2 and TT4 were *Aspergillus niger* and *A. tubingensis*, respectively.

Table 3.5 The sequence identity and the ability to produce CMCase, xylanase and FPase under solid-state fermentation (SSF) and submerged fermentation (SmF) of the eight selected fungal strains.

Strains	Condition	Substrate	Enzymes activity (U/gds)*		
			CMCase	Xylanase	FPase
C. paradoxa TT1	SSF	OPTr	5.37	7.11	0.09
		OPFr	2.17	5.63	0.74
	SmF	OPTr	12.19	18.33	0.72
		OPFr	3.52	2.96	0.36
T. koningiopsis TM3	SSF	OPTr	3.05	13.91	0.87
		OPFr	2.42	8.48	1.54
	SmF	OPTr	5.25	23.06	0.38
		OPFr	2.42	2.47	0.16
H. nigricans TT2	SSF	OPTr	2.83	3.71	1.66
		OPFr	0.89	1.82	0.71
	SmF	OPTr	4.83	8.57	1.54
		OPFr	0.93	11.24	0.49
T. asperellum TT3	SSF	OPTr	2.79	5.98	0.75
		OPFr	0.64	1.08	0.58
	SmF	OPTr	4.91	9.00	1.46
		OPFr	3.37	10.36	0.04
T. asperellum TT5	SSF	OPTr	2.83	6.47	0.08
		OPFr	1.11	2.48	0.79
	SmF	OPTr	4.15	10.41	0.61
		OPFr	1.32	4.19	0.14
T. asperellum TM1	SSF	OPTr	3.43	6.41	0.67
		OPFr	0.37	4.81	0.82
	SmF	OPTr	5.59	13.21	1.49
		OPFr	1.82	2.13	0.27
A.niger TM2	SSF	OPTr	2.46	5.02	0.21
		OPFr	0.46	3.29	1.12
	SmF	OPTr	1.93	4.97	0.56
		OPFr	1.46	2.69	0.56
A. tubingensis TT4	SSF	OPTr	0.90	4.14	0.37
		OPFr	0.62	1.95	0.34
	SmF	OPTr	2.27	5.77	0.77
		OPFr	1.18	5.02	0.44

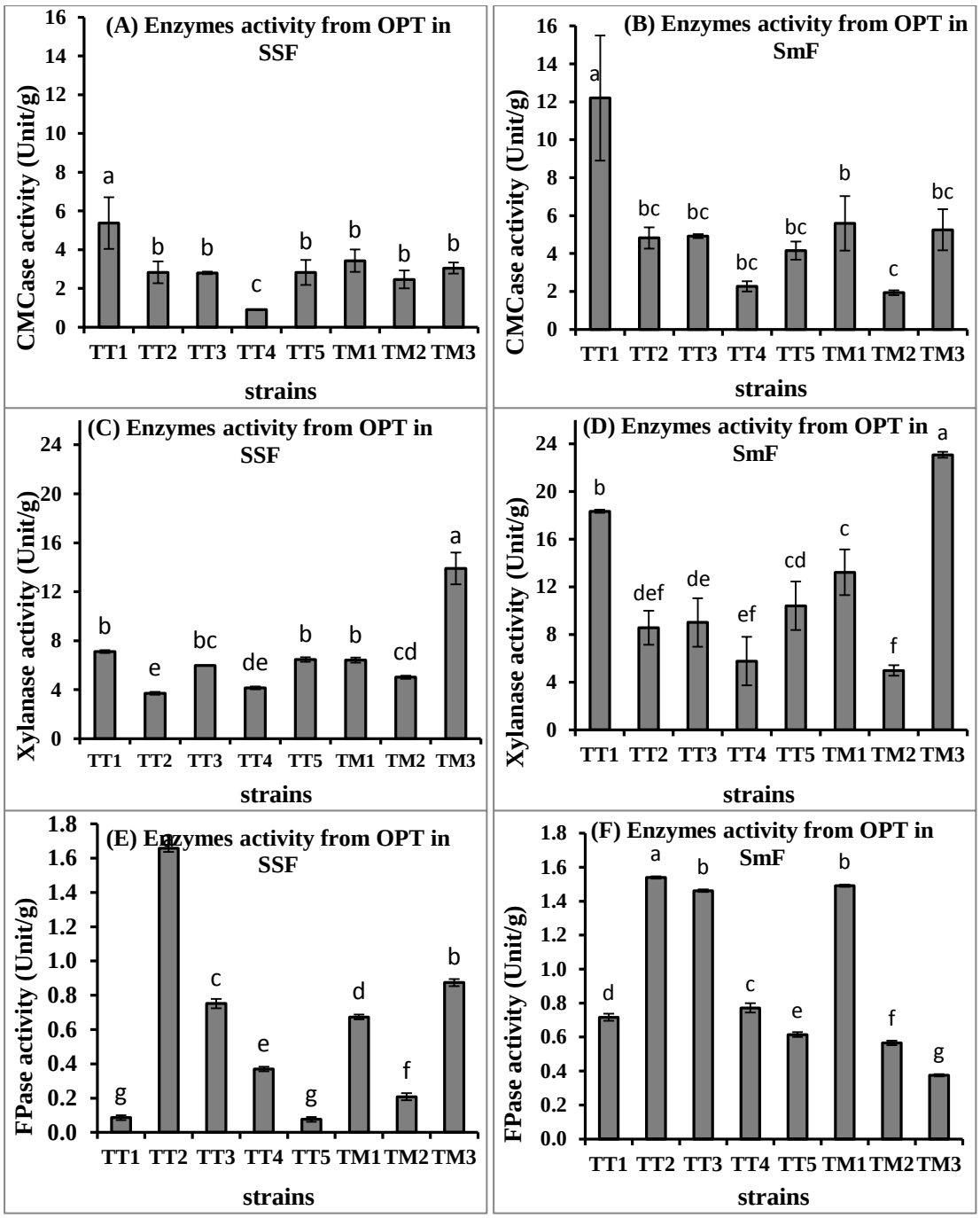

Figure 3.4 Comparison on enzymes production from the eight selected fungal strains using oil palm trunk residues (OPTr) as a carbon source under solid-state fermentation (SSF) (A, C, E) and submerged fermentation (SmF) (B, D, F) after cultivation at room temperature (30±2 °C) for 4 days

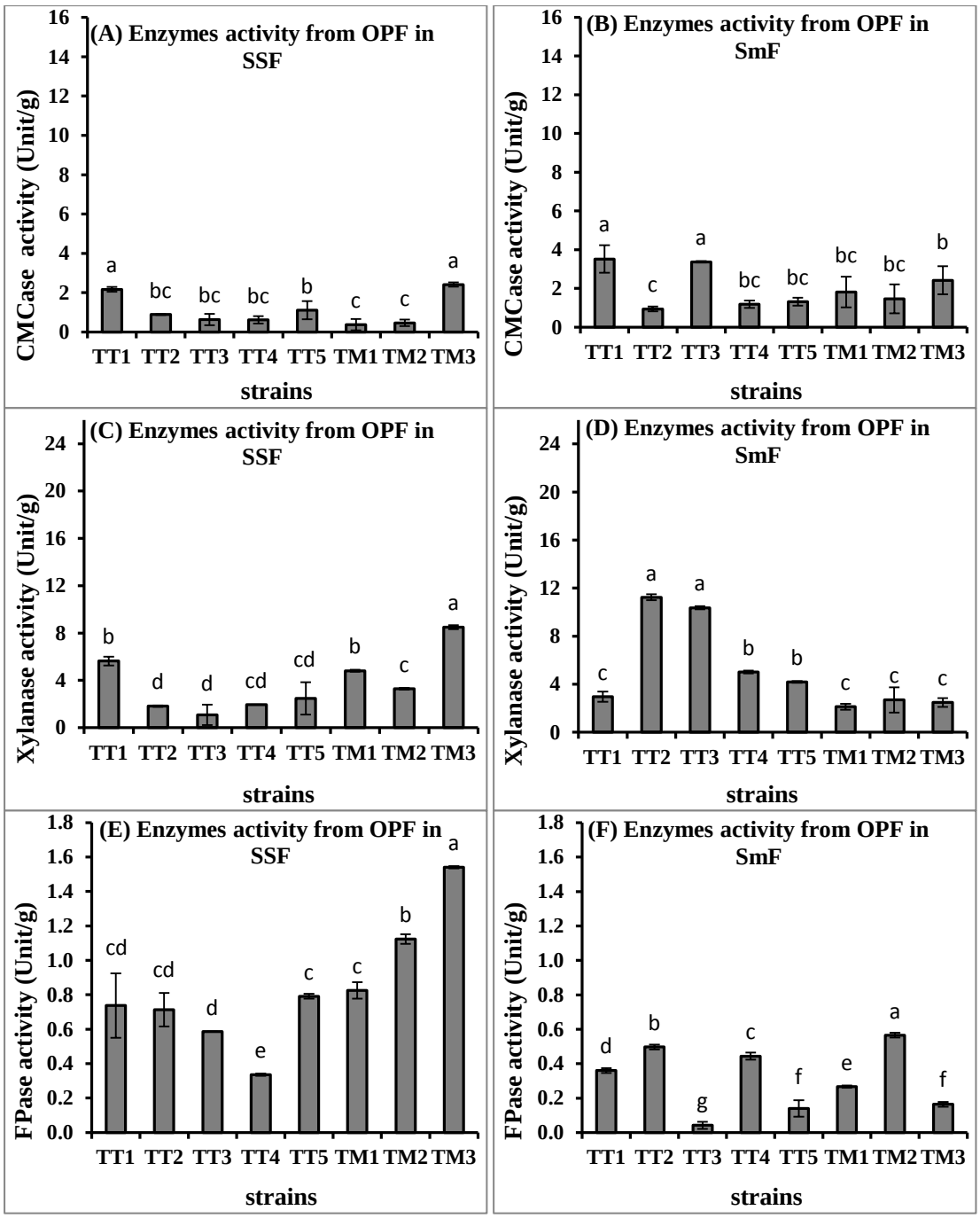

Figure 3.5 Comparison on enzymes production from the eight selected fungal strains using oil palm frond residues (OPFr) as a carbon source under solid-state fermentation (SSF) (A, C, E) and submerged fermentation (SmF) (B, D, F) after cultivation at room temperature (30±2 °C) for 4 days

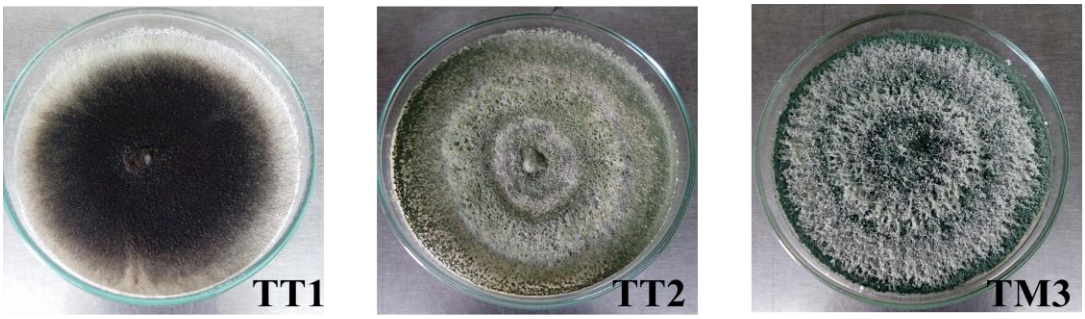

Figure 3.6 *Ceratocystis paradoxa* TT1, *Hypocrea nirgicans* TT2 and *Trichoderma koningiopsis* TM3 grown on potato dextrose agar (PDA) at room temperature (30±2 °C) for 5 days.

3. Enzymes production from the selected and identified fungal strains and the mixed-culture

Improvement in lignocellulolytic enzymes production can be achieved via the mixture of different fungi. Mixed culture is beneficial in lignocellulolytic enzymes production via SSF as the fungi are normally co-existed symbiotically on solid substrates in nature (Holker *et al*., 2004; Kitcha *et al*., 2014). Besides, mixed-culture also offers advantages such as higher productivity, adaptability and substrate utilization compared to pure and monoculture (Dashban *et al*., 2012).

Time course on enzymes production from cultivation of the three newly selected fungal strains and the mixed culture (TT1:TT2:TM3 mixed ratio of 1:1:1, v/v/v) in MMS under SSF and SmF using OPTr and OPFr as carbon source at room temperature (30±2 °C) for 7 days was conducted. Their maximum enzymes activities were summarized in Table 3.6. In general, OPTr was a much better substrate for enzymes production than OPFr from both SSF and SmF. *Ceratocystis paradoxa* TT1 showed the highest CMCase (18.16 Unit/gds) with high xylanase and FPase (36.99 and 1.64 Unit/gds) under SmF at 4 days cultivation under SmF using OPT residues as a carbon source (Fig. 3.6B). It was reported that *C. paradoxa* showed the highest xylanase activity when grown on wheat bran (12,728 IU ml^{-1}) and β-glucosidases when grown on steam-treated bagasse (1,068 IU mL^{-1}) (Barros *et al*., 2010). *Hypocrea nirgicans* TT2 (Fig. 3.7C, 3.7D, 3.8C, 3.8D) exhibited the highest CMCase, xylanase and FPase of 6.10, 21.75 and 1.70 Unit/gds at 2 days cultivation under SSF using OPT residues as a carbon source (Fig. 3.7C). *Trichoderma*

koningiopsis TM3 (Fig. 3.7E, 3.7F, 3.8E, 3.8F) gave the highest CMCase, xylanase and FPase of 7.13, 56.46 and 2.13 Unit/gds at 3 days cultivation under SSF using OPT residues as a carbon source (Fig. 3.7E). This FPase activity was lower than that of *T. konigiopsis* strain FCD3-1 (1.1 Unit/ml) (Zhang *et al*., 2014). Therefore, the strain TT1 was cellulase producer while the strain TM3 was xylanase and FPase producer. The mixed culture (TT1:TT2:TM3, 1:1:1 ratio) demonstrated the highest CMCase, xylanase and FPase activities at lower level than those from the single strain (Fig. 3.7G, 3.7H, 3.8G, 3.8H). The highest CMCase, xylanase and FPase of 6.06, 20.24 and 1.26 Unit/gds, respectively at 3 days cultivation under SSF using OPT residues as a carbon source (Fig. 3.6G), and decreased thereafter. The lower enzymes activities of the mixed cultures than those from the mono-culture may due to the undesirable competition between the fungal strains (Yoon *et al*., 2014) and may have responded differently to different substrate and growing conditions. In addition, the inoculation sequence of different fungal strains might play a significant role in stimulating the enzyme production (Lio and Wang, 2012) or imposing any significant negative effect on the growth of each other (Hu *et al*. 2011). Furthermore, *Trichoderma* species produce both volatile and non-volatile metabolites that adversely affect growth of different fungi. The *Trichoderma* metabolites had fungi static effects on the growth of *C. paradoxa* (Eziashi et al., 2006). Besides, the inoculating time of the fungal strains also has an impact on enzyme production. For examples, the co-cultivation of *Trichoderma reesei* RUT-30 and *Phanerochaete chrysosporium* exhibited the maximum cellulase activity when the inoculation time was delayed for 1.5 days which correlated to the higher saccharide yields than those from mono-culture (Yang *et al*., 2013). Similarly, the maximum cellulase activity (3.2 IU/g) was obtained when *A. oryzae* was co-cultured on soybean fiber with *T. reesei* and *P. chrysosporium* on the 36 h of incubation (Lin *et al*., 2010).

Comparison on enzymes production from the OPTr in this study to those from the other fungal strains is given in Table 3.7. It should be noted that the different values of enzyme activity were partly due to the difference in enzyme activity assay. Considering among oil palm wastes, *Ceratocystis paradoxa* TT1 produced 2.23 fold higher CMCase activities than *Botryosphaeria* sp. (8.13 Unit/g) (Bahrin *et al*., 2011) but lower than that of *A. turingensis* TSIP9 (26.10 Unit/g) (Kitcha *et al*., 2014). *Trichoderma koningiopsis* TM3 produced 1.6 folds higher xylanase (56.46 Unit/gds) than *Aspergillus niger* USM Al 1 (35 Unit/gds) (Kheng *et al*., 2005) and slightly lower than *Aspergillus fumigatus* TSIP9 (59.3 Unit/gds) (Shenef *et al*., 2010). Therefore, *Ceratocystis paradoxa* TT1 and *Trichoderma koningiopsis* TM3 were selected for future studies.

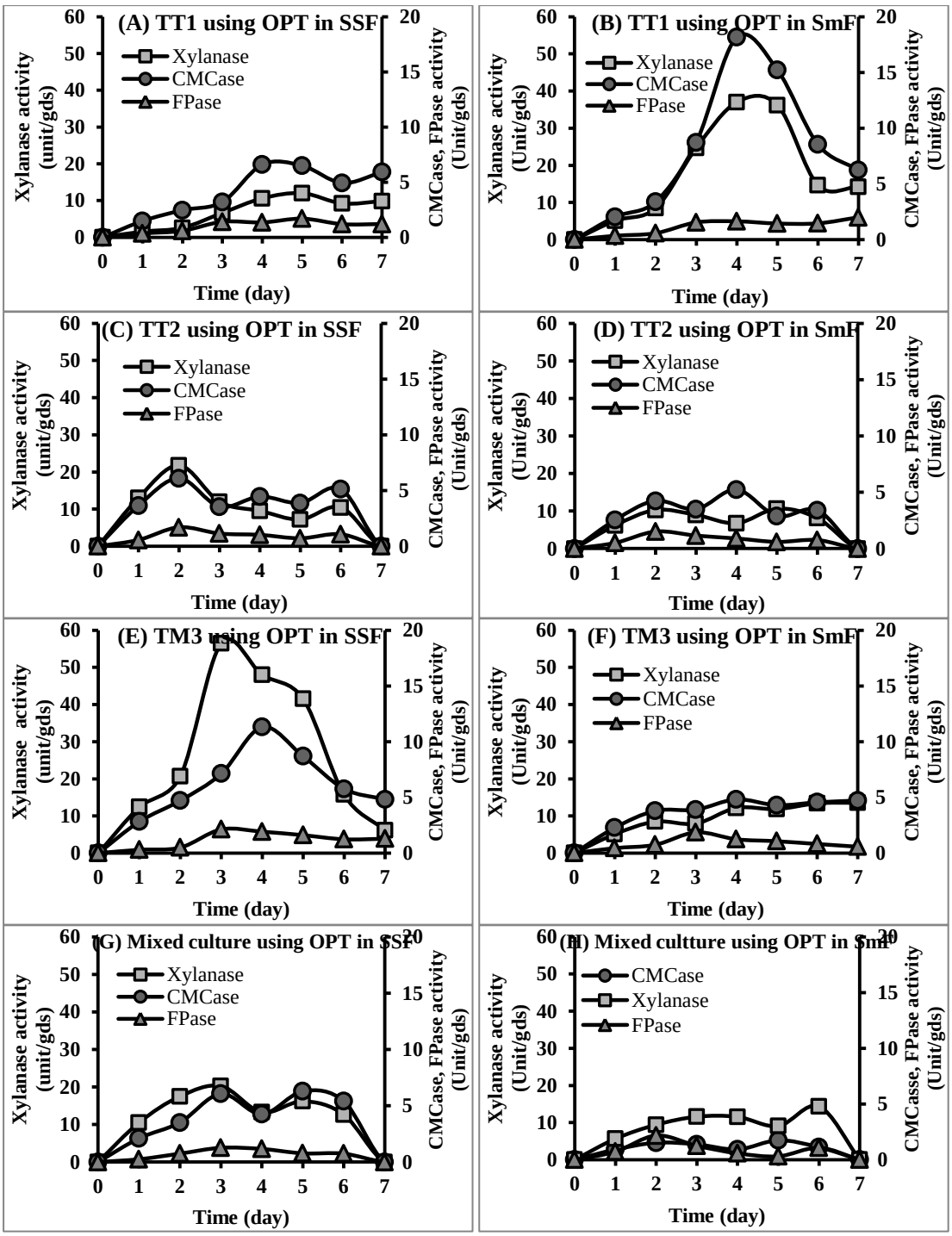

Figure 3.7 Time courses of carboxymethylcellulase (CMCase), xylanase and FPase enzymes activity of *Ceratocystis paradoxa* TT1 (A, B), *Hypocrea nirgicans* TT2 (C, D), *Trichoderma koningiopsis* TM3 (E, F) and mixed culture (TT1:TT2:TM3, 1:1:1 ratio) (G, H) cultivated in solid-state fermentation (SSF) and submerged fermentation (SmF) using oil palm trunk (OPT) residues as a carbon source for 7 days.

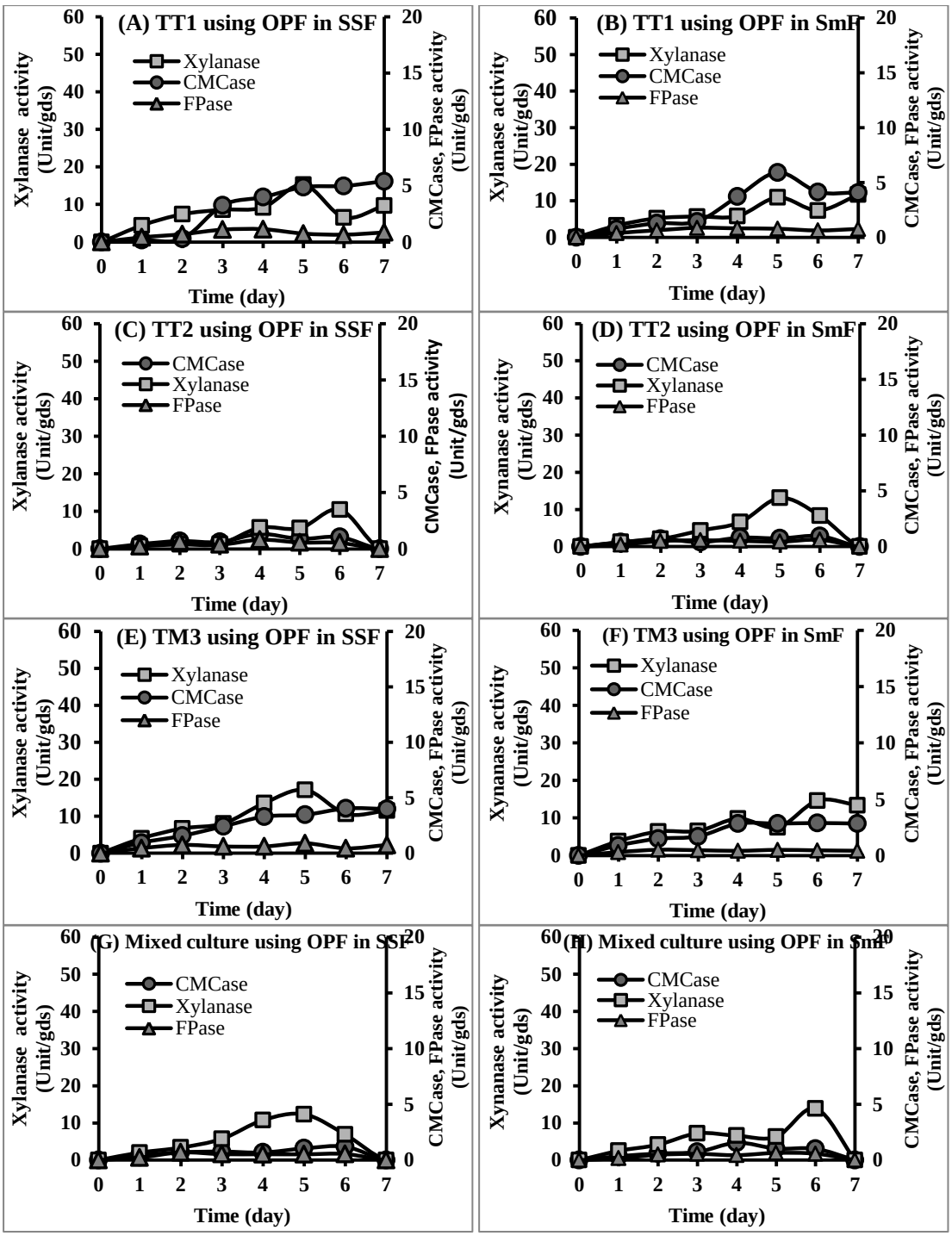

Figure 3.8 Time courses of carboxymethylcellulase (CMCase), xylanase and FPase enzymes activity of *Ceratocystis paradoxa* TT1 (A, B), *Hypocrea nirgicans* TT2 (C, D), *Trichoderma koningiopsis* TM3 (E, F) and mixed culture (TT1:TT2:TM3, 1:1:1 ratio) (G, H) cultivated in solid-state fermentation (SSF) and submerged fermentation (SmF) using ground oil palm frond (OPF) as a carbon source for 7 days

Table 3.6 The maximum enzymes activity of *Ceratocystis paradoxa* TT1, *Hypocrea nirgicans* TT2, *Trichoderma koningiopsis* TM3 and mixed culture (TT1:TT2:TM3, 1:1:1 ratio) cultivated in solid-state fermentation (SSF) and submerged fermentation (SmF) using oil palm trunk residues (OPTr) and oil palm frond residues (OPFr) as a carbon source.

Strain	Condition	Substrate	Time (days)	Enzymes activity (U/gds)*		
				CMCase	Xylanase	FPase
TT1	SSF	OPT	5	6.49±0.29	12.01±0.88	1.69±0.31
		OPF	5	4.87±0.66	15.27±0.22	0.7±0.12
	SmF	OPT	4	18.16±2.73	36.99±0.43	1.64±0.27
		OPF	5	5.92±1.01	10.92±3.29	0.78±0.14
TM3	SSF	OPT	3	7.12±0.13	56.45±0.60	2.12±0.29
		OPF	5	3.46±0.29	17.13±0.22	0.89±0.03
	SmF	OPT	4	4.82±0.15	12.16±0.22	1.24±0.04
		OPF	4	2.83±0.00	9.83±1.7	0.41±0.03
TT2	SSF	OPT	3	3.55±0.13	11.95±0.92	1.13±0.05
		OPF	4	3.98±1.18	5.65±0.22	0.82±0.04
	SmF	OPT	2	4.24±0.88	10.27±0.98	1.50±0.04
		OPF	4	2.42±1.03	6.58±2.85	0.51±0.09
Mixed TT1:TM3:TT2 (1:1:1)	SSF	OPT	3	6.06±0.31	20.24±0.52	1.26±0.05
		OPF	5	3.15±0.15	12.32±1.32	0.52±0.16
	SmF	OPT	3	4.15±0.17	11.58±1.97	1.25±0.05
		OPF	4	4.66±0.36	6.57±0.65	0.44±0.04

*U/gds = units per gram of dry fermented substrate

Table 3.7 Comparison on enzymes production from the three isolated fungal strains and their mixed culture with the other fungal strains

Enzymes	Strain	Activity (Unit/g)	Carbon source	Reference
CMCase	*Fomitopsis sp. RCK2010*	71.70	WB	Deswal *et al*., 2011
	Aspergillus fumigatus	16.90	WS	Shenef *et al*., 2010
	Botryosphaeria sp.	8.13	EFB	Bahrin *et al*., 2011
	A. tubingensis TSIP9	26.10	EFB	Kitcha *et al*., 2014
	Ceratocystis paradoxa TT1	18.16	OPT	Present work
	Hypocrea nigricans TT2	6.10	OPT	Present work
	T. koningiopsis TM3	7.13	OPT	Present work
	Mixed TT1:TT2:TM3	6.06	OPT	Present work
Xylanase	*A. niger* USM Al 1	35.00	PKC	Kheng *et al*., 2005
	Aspergillus fumigatus	56.40	WS	Shenef *et al*., 2010
	A. tubingensis TSIP9	59.30	EFB	Kitcha *et al*., 2014
	Ceratocystis paradoxa TT1	40.00	OPT	Present work
	Hypocrea nigricans TT2	21.75	OPT	Present work
	T. koningiopsis TM3	56.46	OPT	Present work
	Mixed TT1:TT2:TM3	20.24	OPT	Present work
FPase	*Thermoascus auraticus*	4.40	WS	Kalogeris *et al*., 2003
	Fomitopsis sp. RCK2010	3.50	WB	Deswal *et al*., 2011
	Aspergillus fumigates	0.98	WS	Shenef *et al*., 2010
	Botryosphaeria sp.	3.30	EFB	Bahrin *et al*., 2011
	Ceratocystis paradoxa TT1	1.64	OPT	Present work
	Hypocrea nigricans TT2	1.70	OPT	Present work
	T. koningiopsis TM3	2.13	OPT	Present work
	Mixed TT1:TT2:TM3	1.26	OPT	Present work

EFB: Empty fruit bunch WB: Wheat bran

OPT: Oil palm trunk WS: Wheat straw

PKC: Palm kernel cake

4. Enzymes production from the formulated inoculums

4.1. Formulation of the inoculums and effect of the storage temperature

Inoculum preparation is another important aspect in fermentation. There are several ways of preparing fungal inoculum for fermentation. To identify the most suitable type of inoculum to be employed in fermentation, both the nature of fungi involved and the purpose of studies have to be taken into consideration. The commonly applied inoculum preparation methods for fermentation include spore suspension, mycelia disc, mycelia suspension and pre-inoculated substrate (Yoon *et al.*, 2014). In this study, pre-inoculated substrate was chosen for inoculum preparation of *Ceratocystis paradoxa* TT1 and *Trichoderma koningiopsi*s TM3. This type of inoculum was prepared by transferring the spore suspension (10^6 spore/ml, 10% (v)) onto the sterilized cooked broken rice (70% (g)) mixed with the autoclaved OPTr (20% (g)). It was then incubated at room temperature for 3 days and dried at 40 °C for 3 days, then packed in plastic tubes (Figure 3.9). The inoculums of *Ceratocystis paradoxa* TT1 and *Trichoderma koningiopsi*s TM3 in the package dried form were found to contain microorganisms of 1.2×10^9 and 1.6×10^8 CFU per g dry weight, respectively.

The effect of the storage temperature of the formulated inoculum in the package dried form on the release of viable propagules from the formulated preparations is shown in Figure 3.12. The formulated inoculums contained microorganisms approximately 10^9 CFU per g dry weight. After storage at room temperature and 4 °C for 6 months, only the formulated TM3 could maintain at the same level. In contrast, the survival of the formulated TT1 decreased sharply to 30-45% of their original values (from 10^9 to 10^5 and 10^6 CFU per g dry weight at room temperature and 4 °C, respectively). This indicated a better storage at 4 °C than at room temperature.

Figure 3.9 The three inoculums of the highest lignocellulolytic enzyme-producing fungi in the dried form

4.2. Enzyme production from the formulated inoculums and "Super LDD1" under SSF and SmF

The formulated inoculums and spore suspension of *Ceratocystis paradoxa* TT1 and *Trichoderma koningiopsi*s TM3 were compared with Super LDD1 on enzymes production efficiency under SSF and SmF containing OPTr as a carbon source (Figure 3.10). The highest CMCase activity of 8.0 Unit/gds was obtained from the formulated TM3 at 3 days fermentation (Figure 3.10A). For xylanase activities (Figure 3.10B), the formulated TM3, spore suspension of TM3 and the mixed spore suspension of TM3:TT1 (1:1) gave similar high activities (106.3, 111.5 and 110.9 Unit/gds, respectively) at 3 days fermentation. For FPase (Figure 3.10C), the spore suspension of TM3 gave the highest FPase activity of 2.7 Unit/gds at 4 days fermentation which was similar to that obtained from Super LDD1, formulated TT1 and mixed formulated TM3:TT1 (1:1) (1.3 Unit/gds). Based on these results in SSF, the formulated TM3 produced the CMCase and xylanase activity higher than those from Super LDD1 and other spore suspension but produced very low FPase activity when compared with the spore suspension form.

The efficiency of formulated inoculums compared with Super LDD1 and spore suspension inoculum for lignocellulolytic enzymes production in SmF was

investigated (Figure 3.11). The highest CMCase activity of 6.6 Unit/gds was obtained from the spore suspension of TM3 at 4 days fermentation (Figure 3.11A). The formulated TT1, mixed spore suspension TM3:TT1 (1:1) and the spore suspension TM3 gave similar xylanase activities (27.3, 30.1 and 28.5 Unit/gds, respectively) at 4 days fermentation (Figure 3.11B). Moreover, the formulated TT1 gave the xylanase activity (27.3 Unit/gds) higher than spore suspension TT1 (18.2 Unit/gds). For FPase (Figure 3.10C), the formulated TT1 gave the highest FPase activity (2.3 Unit/gds) at 4 days fermentation. Moreover, the formulated TM3 produced very low FPase activities when compared with Super LDD1, formulated TT1 and spore suspension form, respectively. Results in SmF revealed that the formulated TT1 produced the lignocellulolytic enzyme higher than Super LDD1 and similar to spore suspension form.

Based on these results, the formulated TM3 gave the highest efficiency of the inoculum for lignocellulolytic enzymes production in SSF of oil palm trunk residues. For SmF, the formulated TT1 and spore suspension TT1 gave similar efficiency of enzymes production of oil palm trunk residues. Thus, the pre-inoculated substrate technique had many advantages such as it required less stringent handling condition and had lower risk of contamination during the inoculum preparation process compared to spore suspension method.

4.3. Enzymes production from the formulated inoculums using different oil palm biomass as carbon sources

Enzymes production from the formulated inoculum was conducted using OPTr, OPFr, EFB, DC and PPF as a carbon source under SSF and using POME as a carbon source under SmF. The highest CMCase and xylanase activity of 4.44 and 63.17 Unit/gds were obtained using OPTr as a carbon source (Figure 3.13). The formulated TM3 in OPTr produced 1.5 folds higher CMCase (4.44 Unit/gds) than that in OPF (2.80 Unit/gds). In addition, the formulated TM3 exhibited xylanase activities (63.17 Unit/gds) in PPF similar to that in OPTr as a carbon source. The formulated TM3 cultivated under SSF using different oil palm biomass produced fairly good amount of lignocellulolytic enzymes.

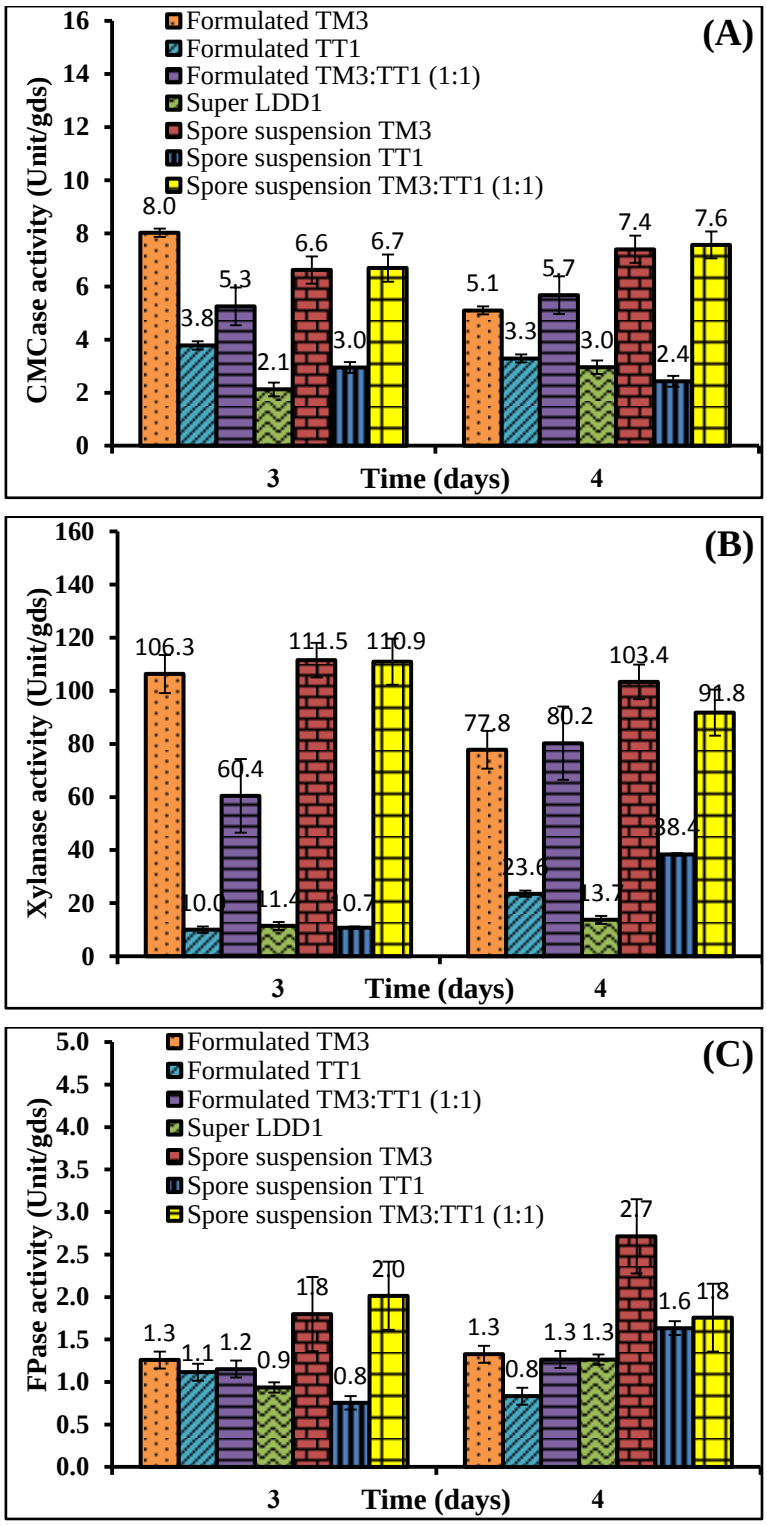

Figure 3.10 The efficiency of the three formulated inoculums (strain TM3, TT1 and the mixed culture) compared with Super LDD1 and spore suspension inoculum for CMCase (A), xylanase (B) and FPase (C) enzymes production in solid-state fermentation.

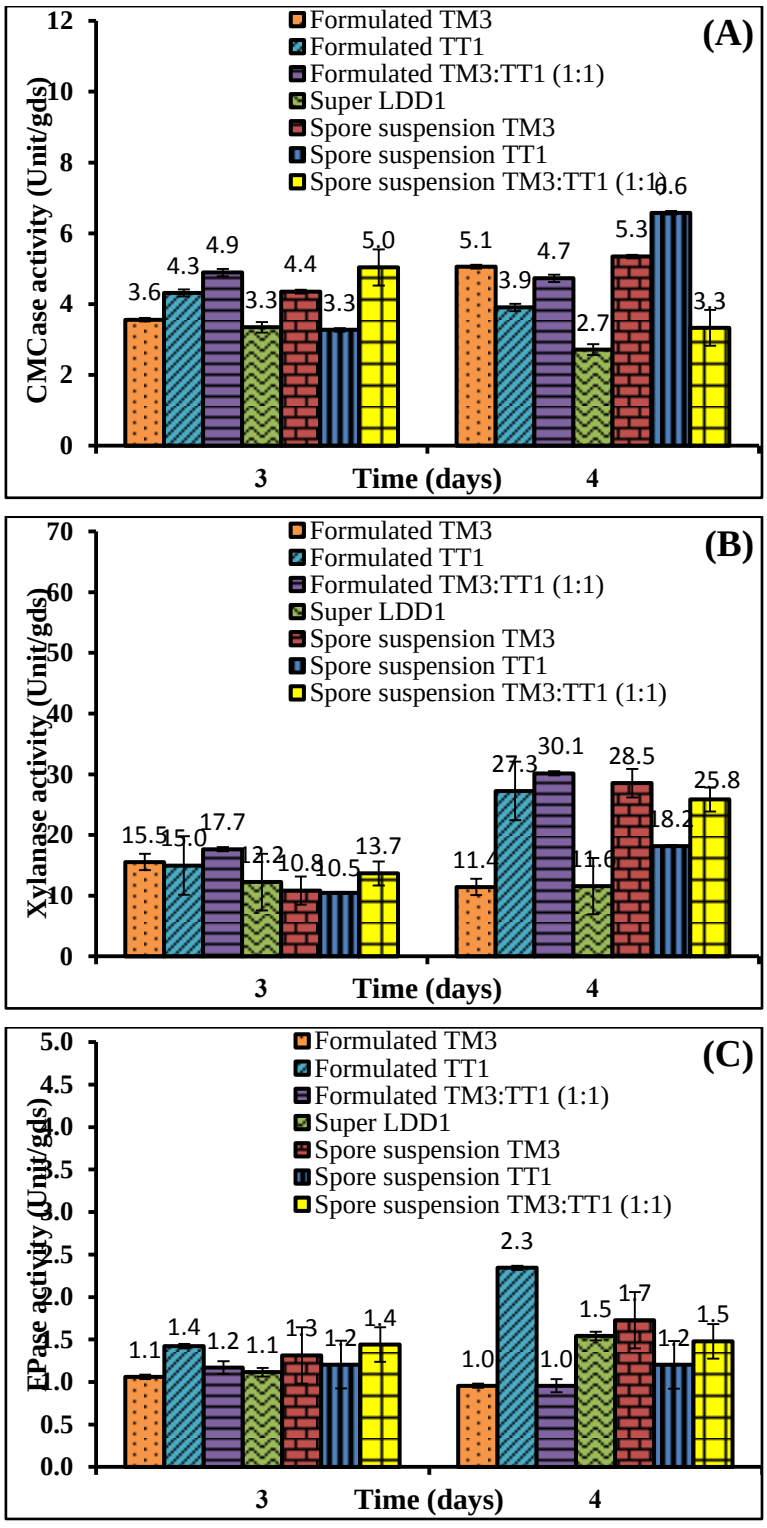

Figure 3.11 The efficiency of the three formulated inoculums (strain TM3, TT1 and mixed culture) compared with Super LDD1 and spore suspension inoculum for CMCase (a), xylnase (B) and FPase (C) production in submerged fermentation

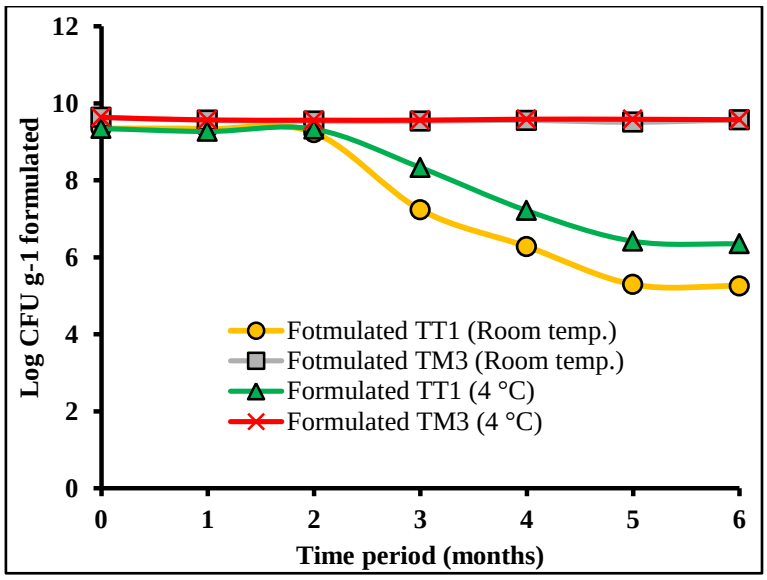

Figure 3.12 Effect of storage temperature on the survival of the formulated inoculum of *Ceratocystis paradoxa* TT1 and *Trichoderma koningiopsis* TM3 in the package dried form.

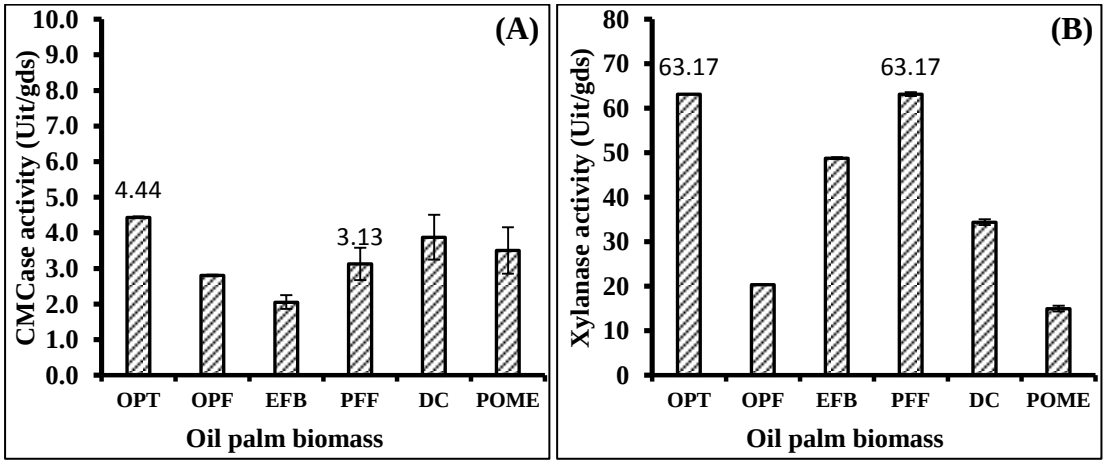

Figure 3.13 Comparison of CMCase (A) and xylanase (B) activity using oil palm trunk residues (OPTr), oil palm fronds residues (OPFr), empty fruit bunches (EFB), decanter cake (DC), palm pressed fibers (PPF) as a carbon source under SSF and using palm oil mill effluent (POME) as a carbon source under SmF by the formulated *Trichoderma koningiopsis* TM3.

5. Characterization of the crude enzymes from the formulated inoculum cultivated under SSF

Solid-stage fermentation (SSF) of OPT residues by the formulated inoculum of *T. koningiopsi*s TM3 gave the CMCase and xylanase activities of 0.687 and 8.274 Unit/ml at 5 days cultivation at room temperature (30±2 °C). Characterization of the enzymes revealed the optimum temperature for CMCase and xylanase at 50 °C (Figure 3.14) while the optimum pH of each enzyme was different (Figure 3.15). CMCase was most active at pH 4.4 and 4.8 while xylanase was most active within the pH range of 4.8 to 5.6. Therefore, these optimal values were within the optimal incubation pH (pH 4.4 to 5.6) for CMCase and xylanase from the previous study of *Trichoderma koningiopsis* TM3.

For thermostability (Figure 3.16), crude CMCase retained more than 75% of their activities after 5 h incubation at 40 °C and less than 50% of activity at room temperature (30±2 °C), 50 and 60 °C. The xylanase exhibited lower thermal stability as it only preserved 75% of activities at temperature below 40 °C and lost 73% and 79% of its activity within 1 h of incubation at 50 °C and 60 °C, respectively, while CMCase only lost 21% and 31% of its activities under the same condition. Based on these results, increasing temperature could improve the enzymolysis efficiency that could be attributed to the increased catalytic activity of cellulase, accelerating the rate of enzyme-catalyzed reaction and thereby causing more cellulose to be converted into reducing sugar (Ran *et al*., 2012). However, further increasing the temperature to 60 °C led to inactive cellulase owing to the denaturation of protein structure (Daniel *et al*., 2010). Nevertheless, the optimal temperature for exoglucanase (1.95 U/mL) and endoglucanase activity (1.88 U/mL) of *Aspergillus niger* was between 40 and 50 °C and decreased above 65 °C (Gautam *et al*., 2011).

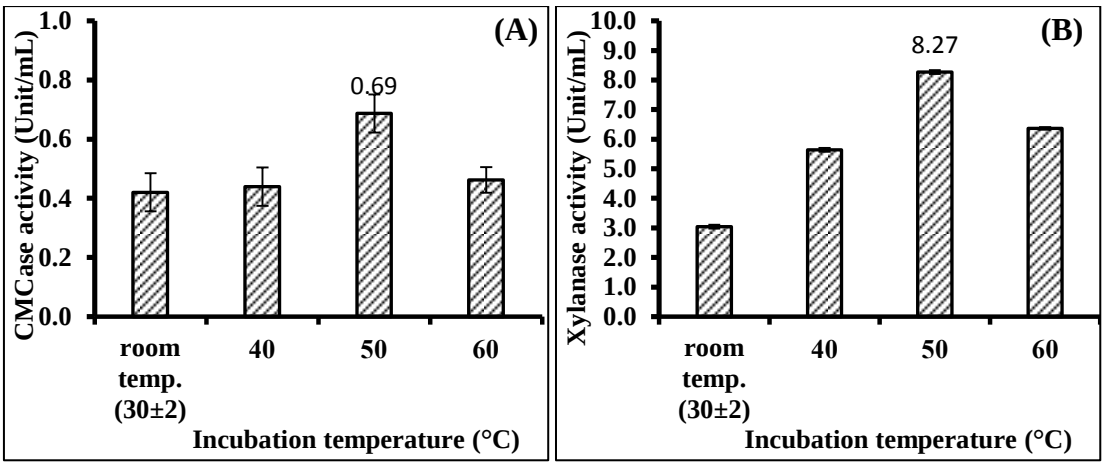

Figure 3.14 Effect of incubation temperature on CMCases (A) and xylanase (B) activity of *Trichoderma koningiopsis* TM3 in citrate buffer (incubation pH 4.8).

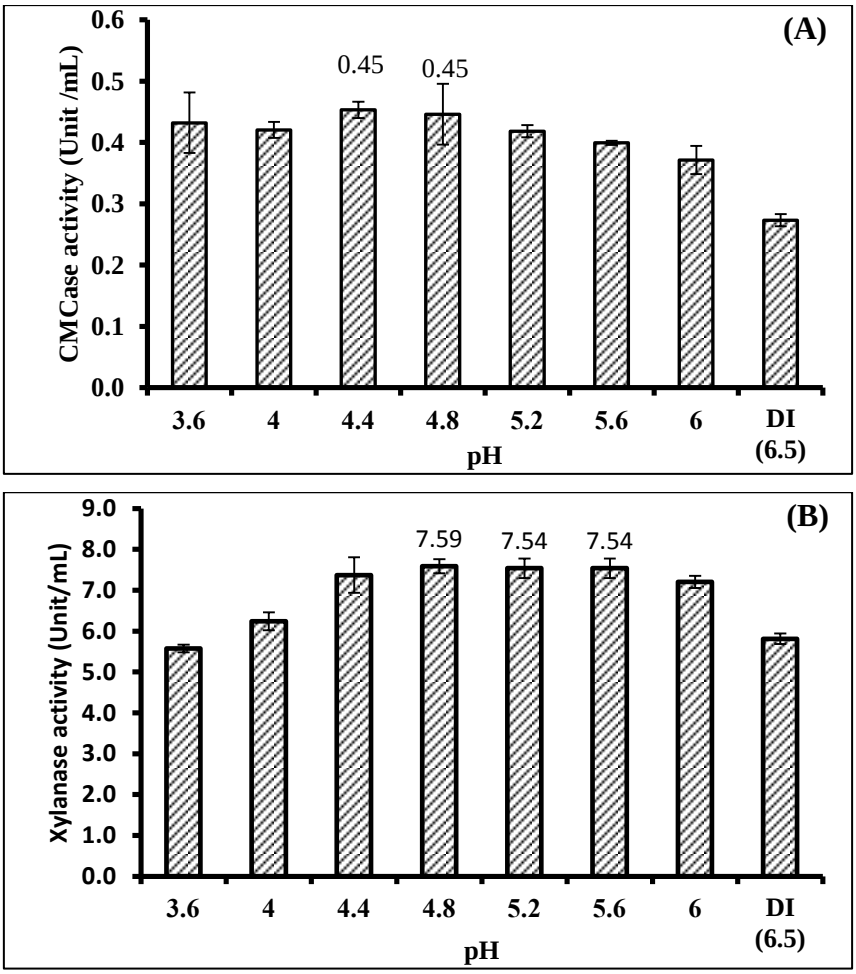

Figure 3.15 Effect of incubation pH on CMCases (A) and xylanase (B) activity of *Trichoderma koningiopsis* TM3 (incubation temperature at 50 °C for 30 and 15 min, respectively)

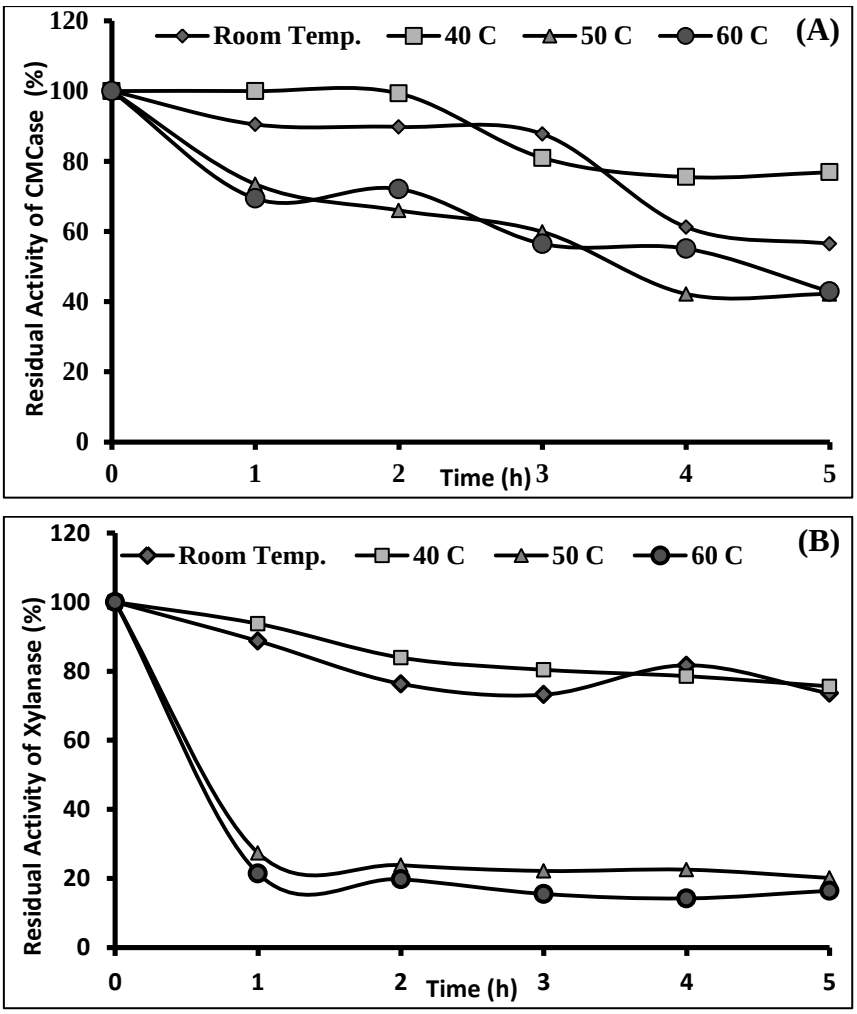

Figure 3.16 Thermostability of crude CMCase (A) and xylanase (B) activity of *Trichoderma koningiopsis* TM3

6. Application of the enzymes for production of sugars from OPTr and use for production of ethanol and acetic acid

6.1. Precipitation of the crude enzymes

The crude enzyme from the supernatant of formulated *Trichoderma koningiopsis* TM3 was concentrated by using acetone which was reported to be better than ammonium sulphate precipitation because of better recovery of activity (Adeleke *et al*., 2012). In addition, organic solvents with small dielectric constants, e.g. acetone and methanol, discourage the dispersion of protein molecules in the media. Thus, the solubility of proteins can be lowered and precipitation can be induced by lowering the effective dielectric constant of the media. This is commonly achieved by adding a water-soluble solvent such as acetone to an aqueous solution of protein. Acetone had

the advantage that it is relatively inexpensive and is available in a pure form with few contaminants that may inhibit or poison the enzyme. It is also frequently used in sterol extraction (Wang, 2009). The concentrate enzyme (Table 3.8) possessed CMCase and xylanase activities of 3.22 and 54.14 Unit/ml, respectively, with the yields of 60.04% and 68.74%, respectively.

Table 3.8 Summary of precipitation of the crude enzymes from supernatant of the formulated *Trichoderma koningiopsi* TM3

Purification Step	Total volume (ml)	Enzymes	Enzymes activity (Unit/ml)	Total activity (Units)	Yield (%)
Crude enzyme	200	CMCase	0.54	107.40	100
		Xylanase	7.88	1575.20	100
Acetone precipitation	20	CMCase	3.22	64.48	60.04
		Xylanase	54.14	1082.86	68.74

6.2. Effect of enzyme concentration on sugar production from OPT residues

The efficacy of crude enzymes (CMCase and xylanase) concentrations (0-40 Unit/g OPT) from *Trichoderma koningiopsis* TM3 in hydrolyzing the OPT residues was evaluated. The time course of enzymatic saccharification revealed the irrespective of the substrate and pretreatment used, the release of reducing sugar and glucose increased with increase in the saccharification time (Figure 3.17). Among various enzymes concentrations and reaction times tested, the optimum enzymes concentration was 25 Unit/g OPTr and hydrolyzed for 15 h. The maximum reducing sugars was 11.92 g/l with the yield of 0.48 w/v. Therefore, the enzymes from this strain exhibited higher saccharification efficiency on the OPTr than those from many brown rots fungi reported earlier (Table 3.9). The commercial enzyme from

Trichoderma reesei was found to release higher amount of glucose during enzymatic saccharification of cellulose substrates than those enzymes from brown rot fungi. The higher saccharification of cellulosic materials by the commercial enzymes could be because they contain different celluloses in pure form (90%, w/w purity) as they had been delignified (chlorite pretreated) (Gupta *et al*., 2011; Deswel *et al*., 2011). The crude enzymes from *Trichoderma koningiopsis* TM3 exhibited comparatively higher saccharification efficiency on the OPT residues than the enzymes from brown rots fungi reported earlier (Table 3.9). A release of only 32 mg sugar/g substrate from avicel after 43 h (Yoon and Kim, 2005) and only 3.53 mg sugar/g substrate released from enzymatically hydrolyzed *Pinus densiflora* (Lee *et al.*, 2008) were reported. This result showed that the OPTr was a better substrate than the other agricultural wastes (rice straw, wheat straw, etc.) as it gave the highest yield of reducing sugar without addition of the commercial enzyme (i.e. Novozyme). The sugar yield of this strain was almost 15 times higher than that of *Formitoplis palustris* acting on Avicel (32 mg/g after 43 h) (Yoon and Kim, 2005).

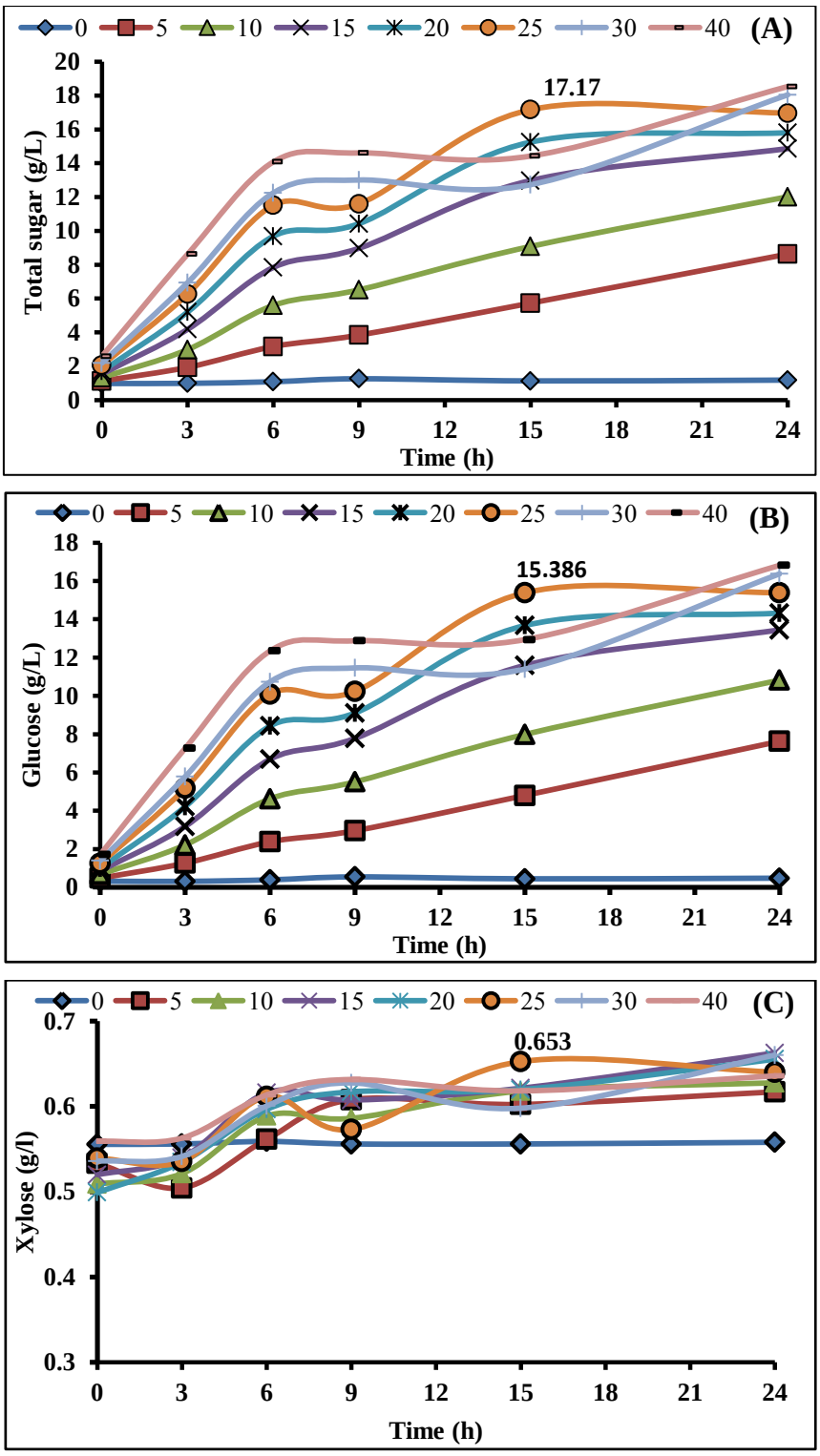

Figure 3.17 The efficacy of acetone precipitated enzymes concentrations (0-40 Unit/g OPTr) from *Trichoderma koningiopsis* TM3 in hydrolyzing the OPT residues; total sugar (A), glucose (B) and xylose (C)

(Initial enzyme concentration; CMCase 3.22 Unit/ml and xylanase 54.14 Unit/ml)

Table 3.9 Comparison of enzymes hydrolysis of different substrates by cellulase from different fungi and commercial preparations

Source of Enzymes	**Substrate**	**Reducing sugar***	**Reference**
Laetiporus sulphureus	*Pinus densiflora*	70.9	Lee *et al*. (2008)
Trichoderma reesei and Novozyme 188	Corn cob	826.2	Gupta *et al*. (2011)
Trichoderma reesei and Novozyme 188	Prosopis	838.9	Gupta *et al*. (2011)
Trichoderma reesei and Novozyme 188	Lantana	777.6	Gupta *et al.* (2011)
Fomitopsis pinicola	*Pinus densiflora*	3.53	Lee *et al*. (2008)
Fomitopsis palustris	Avicel	32	Yoon and Kim (2005)
Fomitopsis sp. RCK2010	Rice straw	157.2	Deswal *et al.* (2011)
Fomitopsis sp. RCK2010	Wheat straw	214.1	Deswal *et al*. (2011)
Trichoderma koningiopsis TM3	OPT residues	476.8	Present work

*mg/ g of substrate

6.3. Effect of yeast strain and co-culture on ethanol production from OPTr hydrolysate

Ethanol production from plant biomass has received considerable attention because of the expectation that bioethanol will alleviate demands for petroleum-based fuels (Sakihama *et al*., 2015). The hydrolysis of lignocellulosic biomass liberates sugars, primarily glucose and xylose, which are subsequently converted to ethanol by microbial fermentation (Husunuma and Kondo, 2012). *Saccharomyces cerevisiae* has been used in industrial bioethanol production due to its robustness and high ethanol productivity. Since xylose cannot be naturally fermented by *S. cerevisiae* (Hahn-Hagerdal *et al.*, 2001), *Candida shehatae* was employed as the strain that can utilize both glucose and xylose as substrates for ethanol fermentation (Yuvadetkun and Boonmee, 2016). In this study, the mono- and co-culture of *S. cerevisiae* TISTR5055, *C. shehatae* TISTR5843 were employed for ethanol fermentation from the OPT hydrolysate. Without addition of nutrients (polypeptone and yeast extract), the ethanol fermentation was almost complete after 36 h when the glucose was thoroughly consumed (Figure 3.18). Meanwhile, the minor sugar components in the hydrolysate medium, ie., cellobiose, xylose and arabinose with the initially concentration of 1.1, 0.99 and 0.56 g/l, respectively, were not detected (by HPLC) after 36 h. The amount of ethanol produced corresponded to 0.19, 0.03 and 0.18 g/g of the theroretical yield calculated based on consumption of glucose, cellobiose, xylose and arabionose by *S. cerevisiae* TISTR5055, *C. shehatae* TISTR5843 and co-culture, respectively. The ethanol production rate and yield were comparable with *S. cerevisiae* TISTR5055, *C. shehatae* TISTR5843 and co-culture (Table 3.10), indicating that *S. cerevisiae* TISTR5055 gave the highest ethanol production rate (0.053 g.l/h) and yield (0.19 g ethanol/g sugar used) and could supported for ethanol fermentation. However, this result is lower than the result from the ethanol production from oil palm empty fruit bunch via dilute-acid hydrolysis and fermentation by *Mucor indicus* (0.45 g/g) and *S. cerevisiae* (0.46 g/g) (Millati *et al*., 2011). The low ethanol yield in this study may be due to the lack of nutrients and this effect would be further investigated. It should be noted that both yeast strains exhibited the highest sugar assimilation on arabinose (75-77%) followed by cellobiose (61-75%), glucose (35-58%) and xylose (49-53%), respectively. Therefore,

improvement in the ethanol production from oil palm trunk hydrolysate could be obtained by supplementation of some nutrients that are essential for cell growth and metabolite production. The ethanol production from *S. cerevisiae* TISTR5055 in OPT hydrolysate, with addition of nutrients (yeast extract 3.0 g/l, malt extract 3.0 g/l and peptone 5.0 g/l) gave 2.64 folds the higher ethanol production (4.15 g/l) than without nutrients at 12 h cultivation (Fig. 3.19C and 3.19D). This result was the ethanol production using crude glycerol by *Kluyvera cryocrescens* that could produce high ethanol (about 11 g/l) when using yeast extract as supplement nutrient (Choi *et al*., 2011).

6.4. Acetic acid production from OPTr hydrolysate by two-stage fermentation and co-cultures (*S. cerevisiae* and *A. aceti*)

Acetic acid is one of the simplest organic carboxylic acid. This colourless weak acid is characterized by distinctive sour taste and pungent smell. Nowadays, this acid is considered as one of the key intermediate for many industries including: chemical, detergent, wood and food industries (Awad *et al.*, 2012). Production of acetic acid is carried out by chemical means using petrochemical feedstock or by the traditional approach of fermentative alcohol conversion using specific type of acetic acid bacteria. In this study, acetic acid was produced by two-stage fermentation (ethanol followed by acetic acid) and co-cultures fermentation using *S. cerevisiae* and *A. aceti* from the OPTr hydrolysate, with and without addition of YM nutrients. In two-stage process without addition of nutrients (Figure 3.19A), ethanol production by *S. cerevisiae* increased and rather stable during 12-24 h. After inoculation of *A. aceti* at 24 h, the ethanol production continued and reached the highest value of 2.87 g/L at 36 h. Simultaneous production of acetic acid was observed as *A. aceti* assimilated ethanol to produce acetic acid and reached the highest value (1.23 g/L) at 30 h after inoculation of *A.aceti* (Fig. 3.19A). With nutrients supplementation (Fig. 3.19B), ethanol production increased sharply and reached the maximum value (4.15 g/L) at 12 h fermentation. This resulted in 2.7 folds increase compared to without nutrients addition and 4.3 folds increase in production rate. This was due to the influence of some nutrients that were essential for cell growth and metabolite production (Choi *et al*., 2011). However, there was no production of acetic acid which may be due to the too high concentration of ethanol (about 2.8 g/L) that

inhibited the growth of acetic acid bacteria. For co-cultures fermentation, simultaneous production of ethanol and acetic acid was observed and reached the maximum ethanol concentration of 2.87 g/L at 18 h and 2.12 g/L at 24 h, respectively. The maximum acetic acid production and production rate were 1.7 fold and 4.0 fold higher than those from the two-stage fermentation. It was observed that addition of nutrients to the OPTr hydrolysate exhibited strong influence on ethanol production (4.01 g/L at 18 h cultivation) and acetic acid production (1.81 g/L at 36 h) (Fig. 3.19D). Therefore, co-culture fermentation of *S. cerevisiae* and *A. aceti* in the OPTr hydrolysate without nutrients supplementation could increase the ethanol production from mono-culture of *S. cerevisiae* TISTR5055 by 1.5 fold (from 1.91 g/L to 2.87 g/L) with 3 fold increase in ethanol production rate (productivity). Nutrient supplementation to the mono-culture could further enhance both the ethanol concentration (from 2.87 g/L at 18 h to 4.01 g/L at 18 h) and ethanol production rate (0.119 g/L/h to 0.223 g/L/h) by 1.4 fold. However, the nutrients supplementation had an adverse effect on acetic acid production as the acetic acid concentration decreased by1.17 fold (from 2.12 g/L at 24 h to 1.81 g/L at 36 h) and the production rate decreased by 1.76 fold (0.088 g/L/h to 0.050 g/L/h) (Table 3.10).

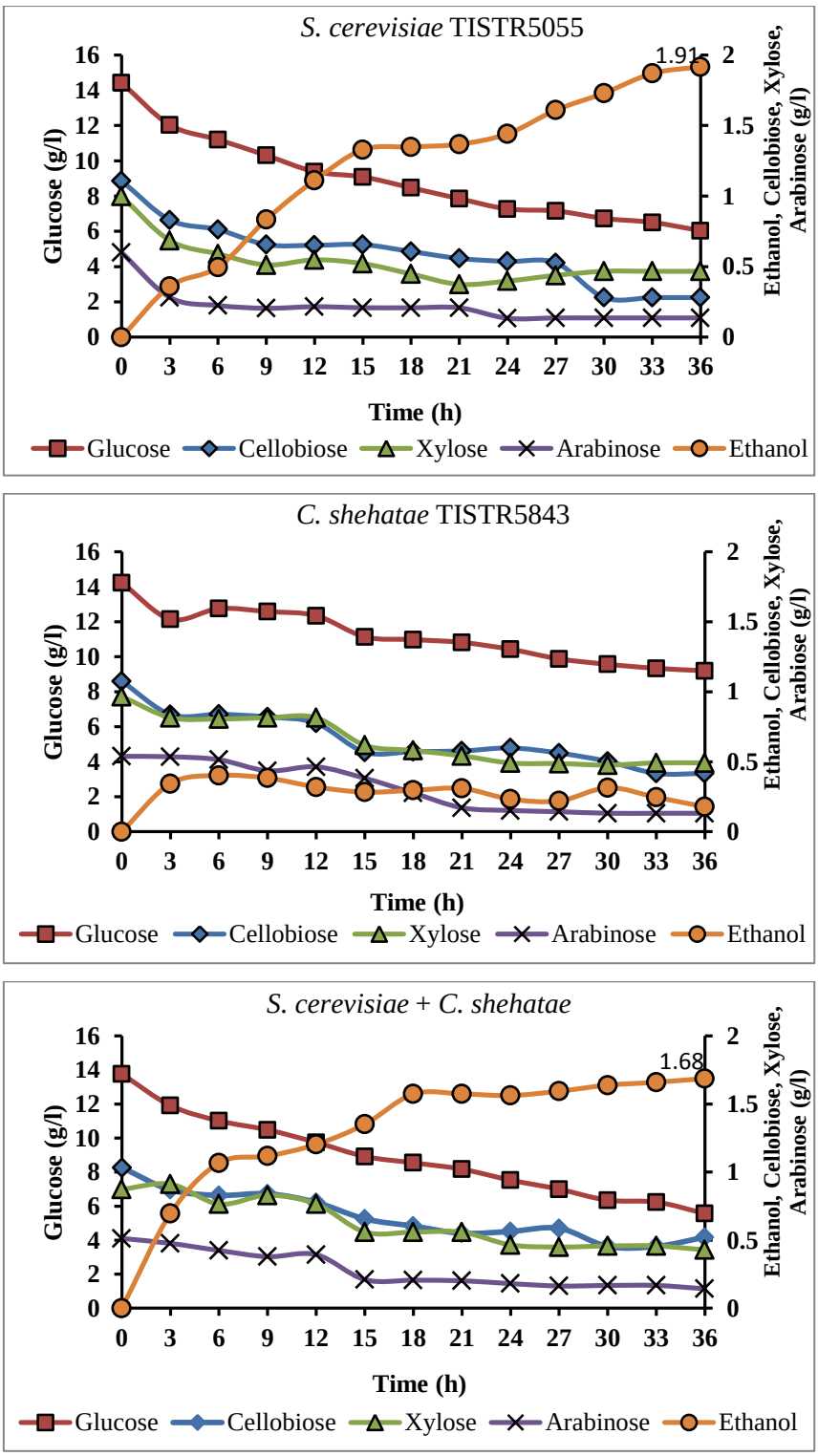

Figure 3.18 Time course of ethanol production from oil palm trunk hydrolysate, without addition of nutrients, by *Saccharomyces cerevisiae* TISTR5055, *Candida shehatae* TISTR5843 and co-culture in shake-flask culture at room temperature (30±2 °C) and 150 rpm shaking speed for 36 h, initial pH 4.9 without pH-control.

Table 3.10 Ethanol production by *Saccharomyces cerevisiae* TISTR5055, *Candida shehatae* TISTR5843 and co-culture using sugars from hydrolyzing the untreated OPT residues.

	Time (h)	**Cellobiose (g/l)**	**Glucose (g/l)**	**Xylose (g/l)**	**Arabinose (g/l)**	**Total sugars (g/l)**	**Ethanol (g/l)**	**Productivity (g/l.h)**	**Yield***
S. cerevisiae TISTR5055	0	1.108	14.425	0.998	0.599	17.150	0.000	0.053	0.187
	36	0.280	6.039	0.467	0.136	6.922	1.917		
C. shehatae TISTR5843	0	1.075	14.235	0.966	0.539	16.815	0.000	0.005	0.027
	36	0.416	9.201	0.492	0.132	10.241	0.178		
Co-culture	0	1.033	13.765	0.873	0.513	16.184	0.000	0.045	0.177
	36	0.522	5.566	0.428	0.143	6.659	1.687		

*g ethanol/g sugars used

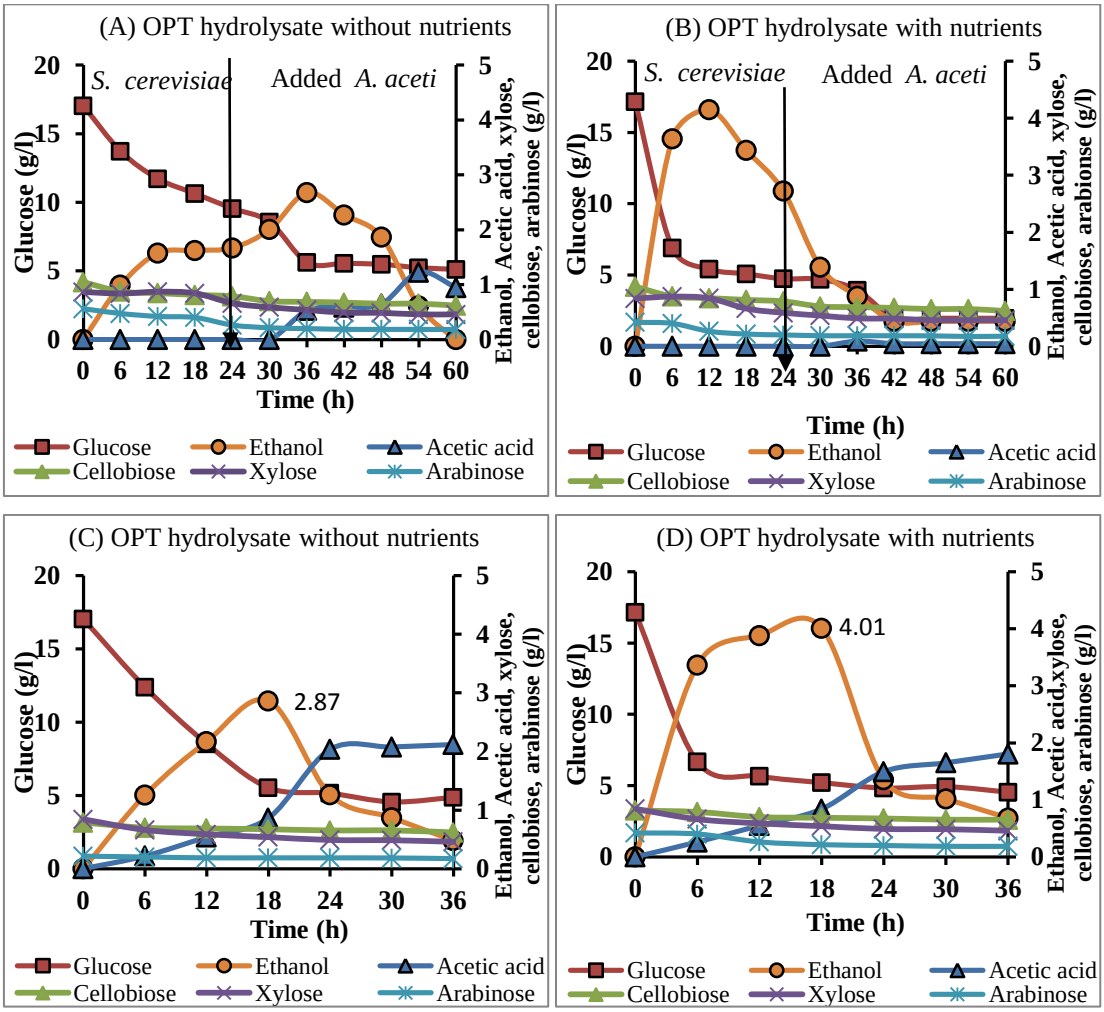

Figure 3.19 Time course of ethanol and acetic acid production by *Saccharomyces cerevisiae* TISTR5055 and *Acetobacter aceti* under two-stage (A, B) and co-culture (C, D) fermentation from oil palm trunk hydrolysate, without addition of nutrients (A, C) and with addition of nutrients (B, D) in shake-flask (150 rpm) culture at room temperature (30±2 °C)

Table 3.11 Summary of ethanol and acetic acid production by *Saccharomyces cerevisiae* TISTR5055 and *Acetobacter aceti* under two-stage and co-culture fermentation from oil palm trunk hydrolysate, with and without addition of nutrients in shake-flask culture at room temperature (30±2 °C) and 150 rpm shaking speed, initial pH 4.9 without pH-control.

Fermentation	**Nutrients**	**Highest Ethanol (g/l)**	**Highest Acetic acid (g/l)**	**Ethanol production rate (g/l.h)**	**Acetic acid production rate (g/l.h)**
Two-stage	with	4.15 (12 h)	-	0.346	-
	without	2.67 (36 h)	1.23 (36 h)	0.074	0.034
Co-culture	with	4.01 (18 h)	1.81 (36 h)	0.223	0.050
	without	2.87 (18 h)	2.12 (24 h)	0.119	0.088

7. Application of crude enzymes to increase biogas production from co-digestion of POME or POME hydrolysate with OPTr or OPTr hydrolysate

7.1. Characteristics of POME

Raw POME is a colloidal suspension containing 95–96% water, 0.6–0.7% oil and 4–5% total solids including 2–4% suspended solids. The suspended solids are mainly consist of debris from palm fruit mesocarp generated from three main sources, (1) sterilizer condensate, (2) sludge separator and (3) hydrocyclonewaste (Borja and Bnaks, 1994; Khalid and Wan Mustafa, 1992; Lam and Lee, 2011) where about 0.9, 1.5 and 0.1 m^3 of POME were generated waste for each tonne (1.13 m^3) of crude palm oil processed (Lam and Lee, 2011). Thus, it was estimated that in the year 2009, 43.8 million m^3 of POME was generated from Malaysian palm oil mills based on the total crude palm oil production of 17.56 million tonne (Malaysian Palm Oil Board, 2010). In fact, the palm oil industry was identified as one of the agricultural industry in Malaysia that generates the highest pollution load into rivers throughout the country (Wu *et al*., 2007).

Characteristics of POME used in this study were presented in Table 3.10. POME has a high total COD (53 g/l), showing a great potential for biogas production. POME has a lower pH value (4.43) because of the organic acids produced in the fermentation process, and its value fall within the range of pH about 4-5 (Lam and Lee, 2011). The volatile solids (VS) are high (43.98 g/l), indicating that the POME is rich in organics. POME includes dissolved constituents such as a high concentration of proteins, carbohydrates, nitrogenous compounds, lipids and minerals, which may be converted into useful materials using microbial processes (Singh *et al*., 2010). The rich organics suggested POME's potential as sustainable feedstock for crude enzymes for degradation and biogas production.

7.2. The efficiency of crude enzymes for saccharification of POME and OPTr

The efficacy of crude enzymes (CMCase and xylanase) (0-15 Unit/g TVS) from *Trichoderma koningiopsis* TM3 in hydrolyzing sterilized POME was evaluated at 40 and 50 °C incubation under constant agitation (150 rpm). Enzymatic saccharification revealed the irrespective of the substrate and pretreatment used, the

increase of sugars (glucose, xylose, cellobiose and arabinose) with the increase in the saccharification time and the dose of enzymes (Figure 3.20). The similar relationship between dose of enzymes and the level of sugars was observed in hydrolysate of POME. The maximum sugars concentrations were obtained from enzymatic hydrolysis using 15 Unit/g TVS at 50 °C for 18 h incubation. Under this condition, the POME hydrolysate contained mainly glucose (16.69 g/l) followed by xylose (4.39 g/l), cellobiose (3.49 g/l) and arabinose (2.35 g/l) with the yield of 0.3521 g/g TVS. In addition, the sugars concentration in non-sterilized hydrolysates of POME to those of sterilized POME was similar (Table 3.13) under the same condition.

For the efficacy of crude enzymes (CMCase and xylanase) (0-15 Unit/g OPT) from *Trichoderma koningiopsis* TM3 in hydrolyzing OPT residues was evaluated at 40 and 50 °C incubation under constant agitation (150 rpm). The maximum sugars concentrations were obtained from enzymatic hydrolysis using 15 Unit/g OPT at 50 °C for 18 h incubation. Under this condition, the OPT hydrolysate contained mainly glucose (19.41 g/l) followed by cellobiose (2.18 g/l), xylose (1.09 g/l), and arabinose (0.60 g/l) (Figure 3.21).

Table 3.12 Characteristics of raw palm oil mill effluent (POME) used in the experiment

Parameters	Unit	POME
pH	pH	4.43
Total COD	g/l	53.00
Total solids (TS)	g/l	51.86
Volatile solids (VS)	g/l	43.98
Suspended solids (SS)	g/l	17.89
Volatile suspended solids (VSS)	g/l	40.49
Alkalinity	mg.$CaCO_3$/l	10.00
Cellobiose	g/l	2.18
Glucose	g/l	4.50
Xylose	g/l	3.45
Arabionose	g/l	1.73
Acetic acid	g/l	4.76
Cellulose	%*	11.00
Hemicellulose	%*	7.00
Lignin	%*	7.87

*% dry basis

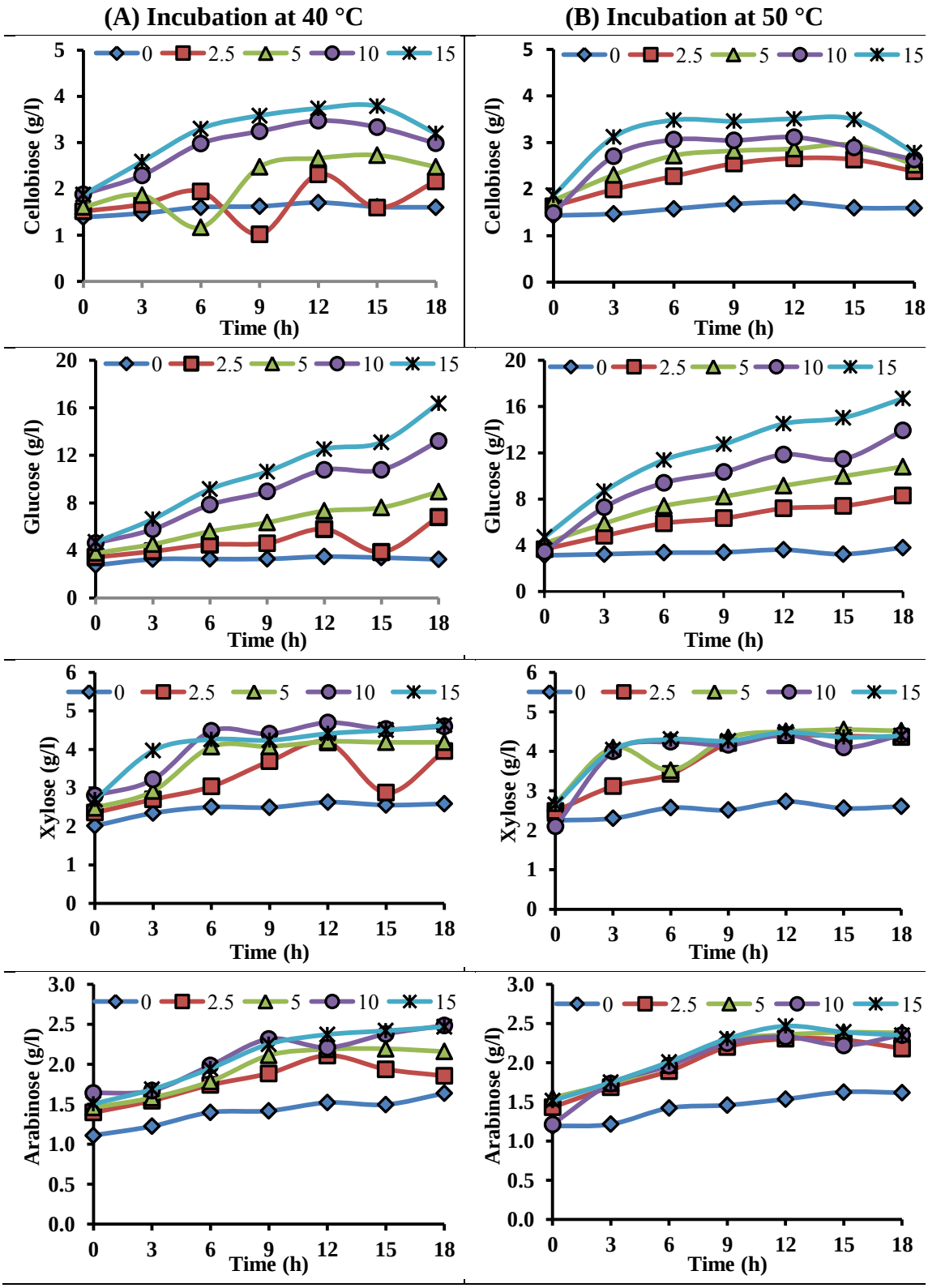

Figure 3.20 Enzymatic hydrolysis (0-15 Unit/g TVS) profile of the palm oil mill effluent (POME) at 40 °C (A) and 50 °C (B) for 18 h.

Table 3.13 Characteristics of palm oil mill effluent (POME) and oil palm trunk (OPT) residues after enzymatic hydrolysis at 50 °C for 18 h incubation.

Tested samples	Cellobiose (g/l)	Glucose (g/l)	Xylose (g/l)	Arabinose (g/l)	Acetic acid (g/l)
Non sterilized POME	1.9225	3.9276	2.7885	1.8529	2.4563
Non sterilized POME +Enzymes*	2.4327	8.1937	3.8912	1.9532	2.2785
Sugars increased	0.5102	4.2661	1.1027	0.1003	
Yield (g sugars/g TVS)			0.1360		
Sterilized POME	1.8606	4.9366	2.9128	1.7846	2.5683
Sterilized POME + Enzymes*	2.5770	9.0522	4.2728	2.0354	2.3922
Sugars increased	0.7164	4.1156	1.3600	0.2508	
Yield (g sugars/g TVS)			0.1465		
OPT residues	0.0538	0.1581	0.7512	0.2346	-
OPT residues + Enzymes**	2.1797	19.4106	1.0962	0.5988	-
Sugars increased	2.17259	19.2525	0.3450	0.3642	
Yield (g sugars/g OPT)			0.8854		

* CMCase 15 Unit/g TVS and **CMCase 15 Unit/g OPT

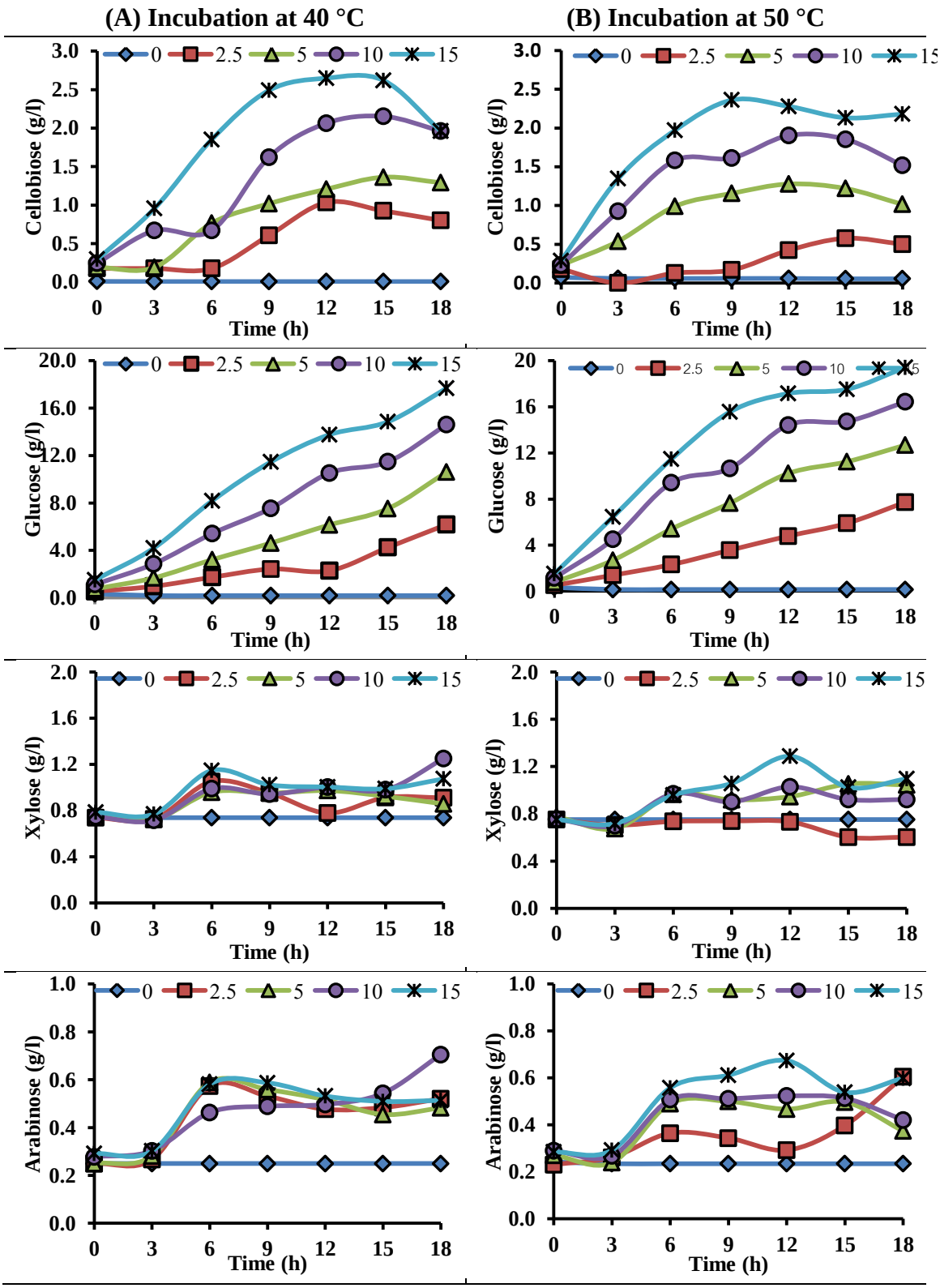

Figure 3.21 Enzymatic hydrolysis (0-15 Unit/g OPT) profile of oil palm trunk (OPT) residues at 40 °C (A) and 50 °C (B) for 18 h.

7.3. Biogas production of POME or POME hydrolysate with and without OPTr or OPTr hydrolysate

Cumulative methane production from co-digestion of POME or POME hydrolysate with OPTr or OPTr hydrolysate under mesophilic condition is shown in Figure 3.22. Methane production from POME (A) was relatively higher and earlier than that from the OPTr (C). The methane production yield per amount of organic waste (TVS) of POME (A) and POME hydrolysate (B) at the initial organic loading of 22.54 and 17.88 g/l were found to be 1,078 and 1,243 ml CH_4/g VS-added, respectively (Figure 3.23). The methane yield increased in the POME hydrolysate than in the raw POME due to its higher substrate concentration. This indicated that high organic content had potential to inhibit the process when overloaded. POME was a concentrate substrate with high content of lipid (8.4 g/l) and low pH (4.3) which could potentially inhibit or overload the process and resulting in the decrease in biodegradability (Fang *et al*., 2011).

The methane yield of OPTr and OPTr hydrolysate at initial organic loading of 15.22 and 15.48 g VS/l was 1,402 and 1,350 ml CH_4/g VS-added, respectively (Figure 3.23). Low methane yield was observed in OPTr hydrolysate, which indicated that they had potential to inhibit the process. Co-digestion of POME with OPTr, POME with OPTr hydrolysate, POME hydrolysate with OPTr and POME hydrolysate with OPTr hydrolysate at mixing ratios of POME/OPTr of 1:1 was conducted. Methane production increased in all the mixtures. The best result of methane yield was achieved from co-digestion of POME hydrolysate with OPTr (1,340 ml CH_4/g VS-added) (Figure 3.23). The methane yield from the POME and OPTr was separately digestion in each mixing, the increasing of methane production achieved by co-digestion was attributed to the increase of biodegradability of OPTr by co-digestion or synergetic methane potential. From large amount of POME and OPTr, co-digestion of POME hydrolysate with OPT residues could be more economic benefit. Mixing ratios of POME hydrolysate/OPTr is needed for further improving biodegradability of OPTr and methane production.

At the end of the 36 days digestion, COD removal from co-digestion of POME with OPTr was 30-56% (Table 3.14) which was lower than the COD removal from co-digestion of POME hydrolysate with OPTr (56%).

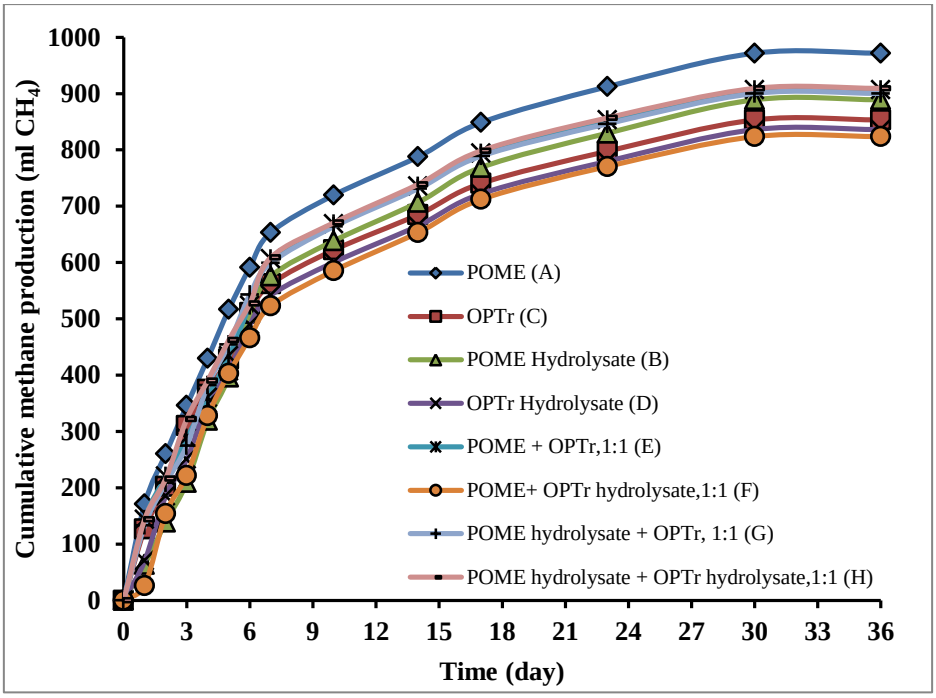

Figure 3.22 Cumulative methane production during 36 days batch fermentation at 37 °C of palm oil mill effluent (POME), POME hydrolysate, oil palm trunk residues (OPTr), OPTr hydrolysate, co-digestion of POME with OPTr and OPTr hydrolysate, and co-digestion of POME hydrolysate with OPTr and OPTr hydrolysate.

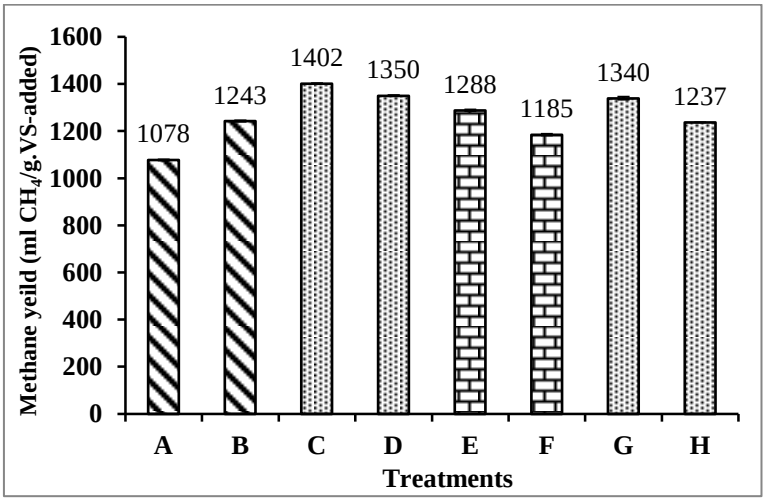

Figure 3.23. Methane yield from biogas production of palm oil mill effluent (POME) (A), POME hydrolysate (B), oil palm trunk residues (OPTr) (C), OPTr hydrolysate (D), co-digestion of POME with OPTr (E), co-digestion of POME with OPrT hydrolysate (F), co-digestion of POME hydrolysate with OPTr (G) and co-digestion of POME hydrolysate with OPTr hydrolysate (H) at 37 °C for 36 day.

Table 3.14 Summary of application of lignocellulolytic enzymes for methane production from co-digestion of palm oil mill effluent (POME) or POME hydrolysate with oil palm trunk residues (OPTr) or OPTr hydrolysate in batch fermentation at 37 °C for 36 day.

Treatment	CH_4 (%)	Cumulative CH_4 (ml)	CH_4 Yield*	pH		TVS (g/l)		TVS removal (%)	COD (g/l)		COD removal (%)
				Initial	Final	Initial	Final		Initial	Final	
POME	69.17	971.78	1078	7.04	7.36	22.54	13.04	42.15	35.15	20.78	40.88
POME hydrolysate	68.97	889.01	1243	7.18	7.38	17.88	9.30	47.98	30.45	14.33	52.94
OPTr	63.25	853.24	1402	7.08	7.31	15.22	9.56	37.18	24.9	13.65	45.20
OPTr hydrolysate	64.71	836.08	1350	7.08	7.31	15.48	8.38	45.86	22.77	15.75	30.84
POME with OPTr	67.02	906.81	1288	7.05	7.44	17.60	9.64	45.23	26.35	13.30	49.52
POME with OPTr hydrolysate	66.34	823.63	1185	7.01	7.32	17.38	8.74	49.71	30.60	13.27	56.61
POME hydrolysate with OPTr	65.81	900.26	1340	7.05	7.37	16.80	8.92	46.90	25.05	13.65	45.49
POME hydrolysate with OPTr hydrolysate	65.52	909.22	1237	7.04	7.39	18.38	9.18	50.05	25.95	14.38	44.56

* ml CH_4/g VS-added

7.4. Microbial community profile from batch reactor operated for methane production

A sequence based survey of bacterial diversity of 9 sludges samples from batch reactors operated for methane production at 37 °C was conducted. Using the results of the DGGE analysis, we identified microbes that were the partial gene sequences, confirming the differences in the bacterial and archaeal communities between the co-digestion of POME or POME hydrolysate with OPTr or OPTr hydrolysate for biogas production (Figure 3.24, 3.25). DGGE profiling of bacterial community (Figure 3.24) realed that at the first day fermentation, there were no substantial differences in dominant bacterial species in all experiments, although *Clostridium* sp. and *Pseudomonas* sp. was constituted the major groups in these communities. *Clostridium* sp. is known to convert organic matters into VFAs (Feng *et al*., 2015), and *Pseudomonas* sp. is known to involved in hydrocarbon degradation (Shukor *et al.*, 2009; Gopinath *et al*., 2015). At 18 days fermentation, *Acinetobacter* sp. was more abundant than *Clostridium* sp. and *Pseudomonas* sp. in all experiments. *Acinetobacter amitratus* could produce cellulase enzyme (Ekperigin, 2007) and *Acinetobacter* sp. MU1_03 were able to remove hydrogen sulfide (Potivichayanon *et al*., 2006). In addition, *Acinetobacter lwoffii* could denitrify the sludge material into small molecules, forming NH_3-N. It could also degrade lignin (Ku *et al*., 2000; Gao *et al*., 2012). In this study, TVS removal in all experiments was 37-50% at steady-state. It is reasonable to suggest that an increased decomposition of organic matter occurs in reactor, based on the observed shifts in microbial populations.

In archaea community (Figure 3.25), *Methanosarcina* sp. was found to be the dominant in all experiments on the first day fermentation and remained in the reactor until 18 days fermentation. These archaea were dominant and played an important role in methane production (Karakashev *et al*. 2005; Mamamin *et al*., 2015) as they could utilize diverse substrates in extreme environments (Galagan *et al*., 2002). In addition, *Methanosarcina* species were reported to be dominant at high acetate concentration; the results were consistent with the high acetate concentration in POME that fed to methane reactors. Others dominant archaea bands were related to *Metanospirillum* sp. and *Methanoculleus* species, which were responsible for hydrogenotrophic methanogenesis (Shin *et al*., 2010). Based on the results of this

study, it can be interpreted that co-digestion not only influence specific bacterial communities, but also facilitate to increase the microbial populations. Furthermore, as a result of increase in the bacterial population, co-digestion is more efficient in the elimination of organic matters and the conversion of VFAs (56.61% COD removal), where *Methanosarcina* sp. (dominant in all experiments) rapidly converted the increased portion of VFAs into CH_4. Thereafter, methanogenesis by *Methanosarcina* sp. (using various substrates), and methanogenesis by *Metanospirillum* sp. (using H_2 and CO_2) occur simultaneously, resulting in the increase of methane production in all experiments. The methanogenic performance on the treated POME using crude enzyme and co-digestion was as high as that from untreated, suggesting that treated POME using crude enzyme and co-digestion could be efficiently coupled with a subsequent step for methanogenic process.

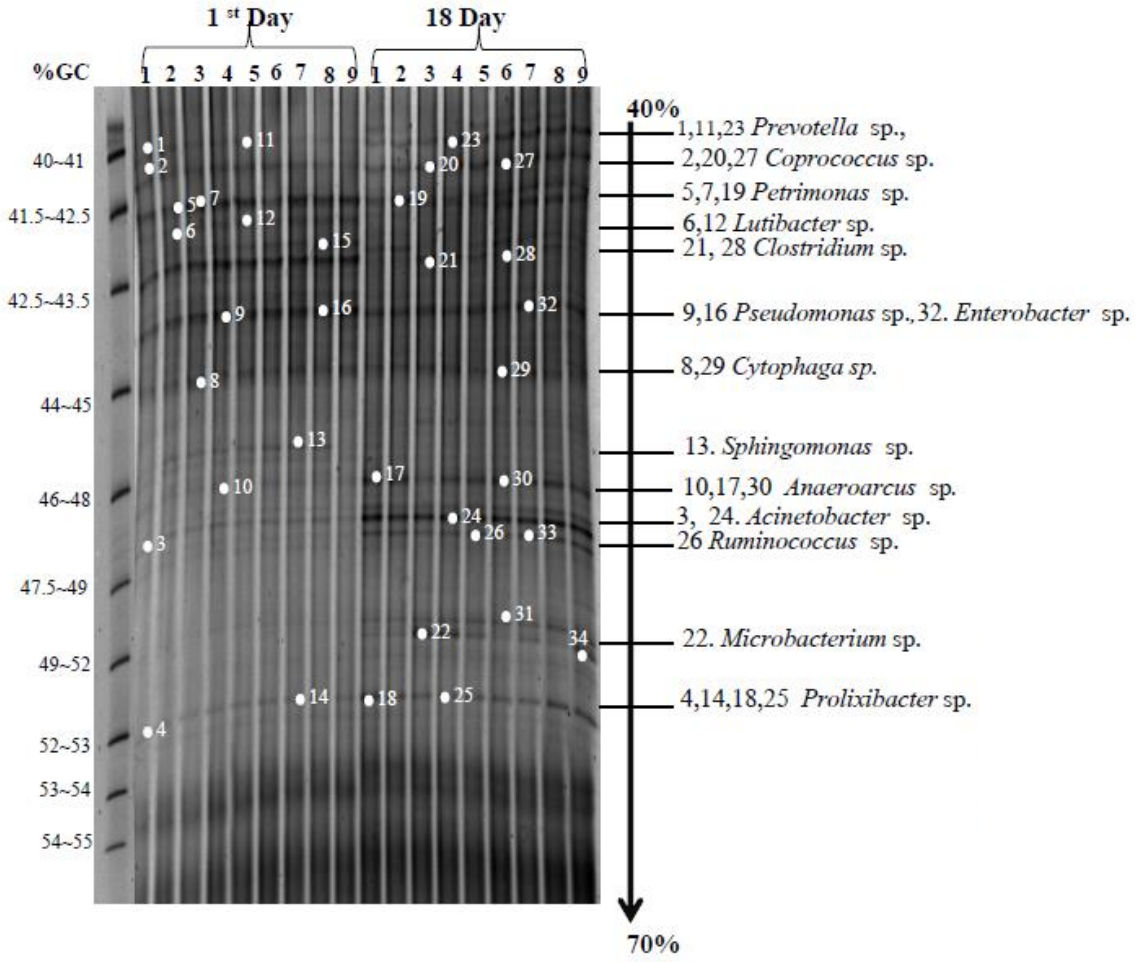

Figure 3.24 DGGE profile of bacterial community in sludge from batch reactor operated for methane production of palm oil mill effluent (POME) (1), POME hydrolysate (2), oil palm trunk residues (OPTr) (3), OPTr hydrolysate (4), co-digestion of POME with OPTr (5), co-digestion of POME with OPTr hydrolysate (6), co-digestion of POME hydrolysate with OPTr (7), co-digestion of POME hydrolysate with OPTr hydrolysate (8) and inoculum with DI water (control) (9).

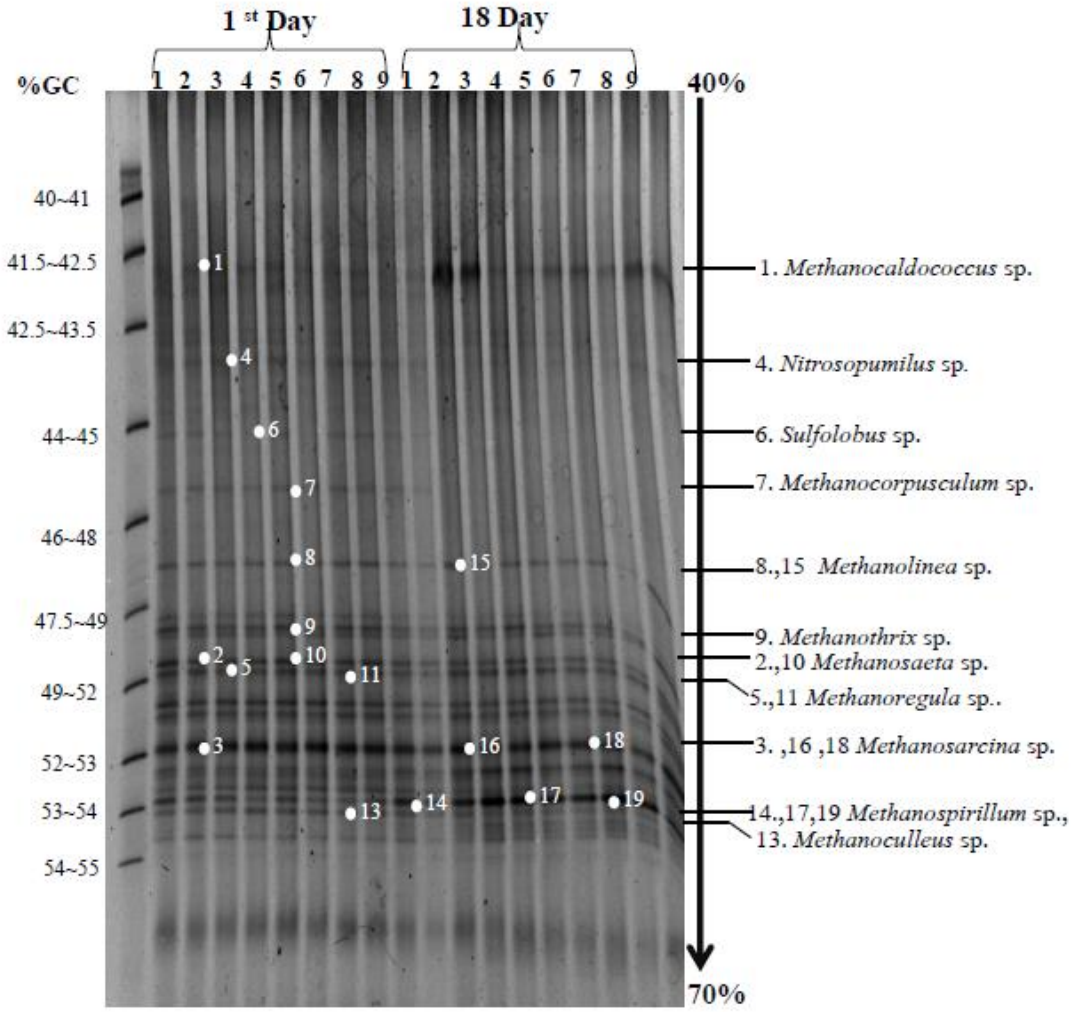

Figure 3.25 DGGE profile of archea community in sludge from batch reactor operated for methane production of palm oil mill effluent (POME) (1), POME hydrolysate (2), oil palm trunk residues (OPTr) (3), OPTr hydrolysate (4), co-digestion of POME with OPTr (5), co-digestion of POME with OPTr hydrolysate (6), co-digestion of POME hydrolysate with OPTr (7), co-digestion of POME hydrolysate with OPTr hydrolysate (8) and inoculum with DI water (control) (9).

CHAPTER 4

CONCLUSIONS AND SUGGESTIONS

4.1. Conclusions

4.1.1. The oil palm trunk (OPT) composted of 12.98-55.04% cellulose, 11.87-19.67% hemicelluloses and 1.30-13.00% lignin, depending on its length. The oil palm frond (OPF) contained 44.78% cellulose, 17.92% hemicelluloses and 12% lignin. Glucose was the dominant sugar in all parts of the sap from OPT and OPF. The OPF and bottom part of OPT contained a high level of glucose (38.18 and 30.20 g/kg, respectively).

4.1.2. Incubation temperature had an influence on enzyme production profile during natural fermentation of ground OPT and OPF. At room temperature incubation, the maximum CMCase (0.48 Unit/gds) was obtained at 15 days fermentation of OPT while the maximum xylanase activity (0.24 Unit/gds) was achieved at 6 days incubation of OPF. At 40 ˚C, the maximum CMCase and xylanase activity of 0.25 and 0.44 Unit/gds were achieved at 3 days incubation of OPF. There was no activity at 50 °C for both OPT and OPF.

4.1.3. Bacterial community developing on natural fermentation of OPT and OPF was comprised of acetic acid bacteria, lactic acid bacteria and starch-hydrolysing bacteria as the major groups in these communities. The acetic acid bacteria were *Gluconobacter mesenteroides, Gluconobacter oxydans* and *Ameyamaea chiangmaiensis*. The lactic acid bacteria were *Leuconostoc mesenteroides, Weissella confuse*. The starch hydrolytic bacteria, *Bacillus* sp., can be found. For yeasts and fungi community, *Kluyveromyces marxianus*, *Candida* sp., *Pichia kudriavzevii* and *Candida tropicalis* are considered common in natural fermentation. Fungi had low diversity in natural fermentation of OPT and OPF and only *Hexagonia hirta* and *Pycnoporus* sp were detected.

4.1.4. Eight out of the 20 fungal isolates could grow after 3 days incubation on oil palm biomass plates and encoded as the isolate TT1, TT2, TT3, TT4, TT5, TM1,

TM2 and TM3. Direct conversion of oil palm trunk residues (OPTr) and oil palm frond residues (OPFr) into enzymes by various isolated fungal strains were performed through solid-state fermentation (SSF) and submerged fermentation (SmF). Among 8 strains tested, the three isolates TT1, TM3 and TT2 produced the highest activity of CMCase, xylanase and FPase, respectively. They were identified as *Ceratocystis paradoxa*, *Trichoderma koningiopsis* and *Hypocrea nigicans*, respectively.

4.1.5. Comparison on enzymes production from the selected strains under SSF and SmF condition indicated that *C. paradoxa* TT1 produced the highest CMCase (18.16 U/g dry substrate (gds)) in SmF using OPTr as a carbon source and *T. koningiopsis* TM3 produced the highest xylanase and FPase (56.46 and 2.13 U/gds, respectively) in SSF using OPTr as a carbon source.

4.1.6. Formulation of *Ceratocystis paradoxa* TT1 and *Trichoderma koningiopsi*s TM3 in the package dried form and storage at room temperature and 4 °C for 6 months, the survival of the formulated *T. koningiopsis* TM3 remained at the same level (approximately 10^9 CFU per g dry weight). The formulated *T. koningiopsi*s TM3 and formulated *C. paradoxa* TT1 gave the highest efficiency of the inoculum for lignocellulolytic enzymes production in SSF and SmF, respectively. The formulated inoculum can utilize different oil palm biomass to produce lignocellulolytic enzymes. The maximal CMCase and xylanase obtained by the formulated *T. koningiopsi* TM3 using OPTr as a carbon source was 4.44 and 63.17 Unit/gds, respectively.

4.1.7. The optimum pH of the crude CMCase and xylanase was in the range of 4.8 to 5.6 while the optimum temperature was 50 °C. Thermal stability of the enzymes was only upto 40 °C for 5 h. In the acetone precipitation, the activity of CMCase and xylanase increased to 6 and 6.8 folds, respectively, with the recovery yields of 60 and 68%, respectively.

4.1.8. Application of enzymes for sugars production from OPTr and used for production of ethanol and acetic acid. The enzymes was used to hydrolyze OPTr at 50 °C for 24 h. The maximum sugars were obtained at the enzymes concentration of 25 Unit/g OPT at 15 h incubation. Ethanol production from the OPTr hydrolysate, the *S. cerevisiae* TISTR5055 gave the highest ethanol production rate (0.053 g/l.h) and yield

(0.187 g ethanol/g sugars used). Co-culture of *S.cerevisiae* TISTR5055 and *Acetobacter aceti* in OPTr hydrolysate without addition of nutrients increased the efficiency of acetic acid production in terms of acetic acid concentration, productivity and product yield.

4.1.9. The crude enzymes were applied for biogas production from palm oil mill effluent (POME) and OPTr. Enzymatic pretreatment of POME, causing the partial saccharification, positively affected the outcomes of anaerobic fermentation. The observed increase in the biogas yield (1,243 ml CH_4/g VS-added) and higher decrease in total organic content provided evidence that enzymatic pretreatment could intensify anaerobic organic biomass degradation processes in efficient and environmentally friendly manner. Co-digestion of treated POME by crude enzymes (CMCase 15 Unit/g TVS) with OPTr at mixing ratios of 1:1 on volume basis had high synergetic effect with the highest methane potential of 1,340 ml CH_4/g VS-added.

4.2. Suggestions

4.2.1. *T. koningiopsi* TM3 and *C. paradoxa* TT1 are newly isolated lignocellulosic enzyme producing fungi from oil palm trunk. They may be used for production of others enzymes, i.e., amylase, lipase for biodegradability in further studies.

4.2.2. Lignocellulolytic enzymes production rate from *T. koningiopsi* TM3 and *C. paradoxa* TT1 could be improved by optimization of medium composition and environmental condition (temperature, pH, etc.).

4.2.3. The enzymatic saccharification and enzymes recovery could be improved by immobilization of enzymes on granules.

4.2.4. Ethanol and acetic acid production could be improved by simultaneous saccharification and fermentation of oil palm biomass.

4.2.5. Optimum mixing ratios of POME hydrolysate/OPTr is needed for further improving biodegradability of OPTr and methane production.

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Lists of Publication and Proceedings

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Nutongkaew, T., Duangsuwan, W., Prasertsan, S. and Prasertsan, P. 2014. Physicochemical and biochemical changes during composting of different mixing ratios of biogas sludge with palm oil mill wastes and biogas effluent. Journal of Material Cycles and Waste Management. 16(1): 131-140.

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Nutongkaew, T., Noparat, P. and Parsertsan, P. Application of lignocellulolytic enzymes from the formulated inoculums for pretreatment of palm oil mill effluent and oil palm trunk residues prior to co-digestion for enhancing the efficiency of biogas production (Manuscript under preparation).

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Nutongkaew, T., Noparat, P. and Parsertsan, P. 2014. Bioconversion of oil palm biomass to lignocellulolytic enzymes by newly isolated fungi through solid-state and submerged fermentation. The 1st Joint Seminar between Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM) and Department of Industrial Biotechnology, Faculty of Agro-Industry On Friday 24th, 2014 Faculty of Agro-Industry (Oral presentation).

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