Process Development for Carbon Dioxide Removal from Biogas and Lipid Production by Oleaginous Microalgae Cultivation

Sirasit Srinuanpan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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Candidate

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.

…….. Signature

(Mr. Sirasit Srinuanpan)

Candidate

ชื่อวิทยานิพนธ์ การพัฒนากระบวนการกำจัดก๊าซคาร์บอนไดออกไซด์ออกจากก๊าซชีวภาพและการผลิตไขมันโดยการเพาะเลี้ยงสาหร่ายไขมันสูง

ชื่อผู้แต่ง นาย ศิรสิทธิ์ ศรีนวลปาน

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ปีการศึกษา 2561

บทคัดย่อ

สาหร่ายสายพันธุ์โอเลจีเนียสมีอัตราการกำจัดก๊าซคาร์บอนไดออกไซด์สูงสามารถใช้ในการทำบริสุทธิ์ก๊าซชีวภาพได้อย่างมีประสิทธิภาพ สาหร่ายสายพันธุ์นี้ยังสามารถสะสมไขมันภายในเซลล์ได้มากกว่าร้อยละ 20 โดยน้ำหนักแห้ง ทำให้มีศักยภาพในการใช้แหล่งน้ำมันสำหรับการผลิตไบโอดีเซลได้อีกด้วย การศึกษานี้มีจุดมุ่งหมายเพื่อพัฒนากระบวนการเพาะเลี้ยงสาหร่ายสำหรับการทำบริสุทธิ์ก๊าซชีวภาพร่วมกับการผลิตไขมันอย่างมีประสิทธิภาพ ซึ่งจะรวมถึงการพัฒนากระบวนการเก็บเกี่ยวสาหร่าย การตรึงรูปสาหร่ายเพื่อนำกลับมาใช้ซ้ำ และความสามารถของสาหร่ายในการบำบัดน้ำทิ้งโรงงานอุตสาหกรรม โดยแบ่งการศึกษาออกเป็น 6 ตอน

ตอนที่ 1 การเพาะเลี้ยงสาหร่ายไขมันสูงหลายสายพันธุ์ด้วยก๊าซชีวภาพ เพื่อศึกษาความสามารถของสาหร่ายในการลดปริมาณก๊าซคาร์บอนไดออกไซด์และการเพิ่มปริมาณก๊าซมีเทนในก๊าซชีวภาพ ผลการทดลองพบว่าสาหร่ายไขมันสูงสามารถใช้ก๊าซคาร์บอนไดออกไซด์ที่อยู่ในก๊าซชีวภาพได้อย่างมีประสิทธิภาพ (>90%) และสามารถสะสมไขมันภายในเซลล์ได้สูงในช่วงร้อยละ 24-42 โดยสาหร่ายสายพันธุ์ *Scenedesmus* sp. เป็นสาหร่ายที่มีประสิทธิภาพสูงสุดในการกำจัดก๊าซคาร์บอนไดออกไซด์ จากการศึกษาสภาวะที่เหมาะสมของการเพาะเลี้ยงสาหร่ายเพื่อทำบริสุทธิ์ก๊าซชีวภาพและผลิตไขมัน พบว่าอัตราการพ่นก๊าซชีวภาพที่ 0.3 ปริมาตรอากาศต่อปริมาตรอาหารต่อชั่วโมง หัวเชื้อสาหร่ายเริ่มต้น 10^7 เซลล์ต่อมิลลิลิตร การเติมโพแทสเซียมไนเตรตเป็นแหล่งไนโตรเจน 0.8 กรัมต่อลิตร และการให้แสงที่ความเข้มแสง 5.5 กิโลลักซ์ เป็นสภาวะที่เหมาะสมที่ทำให้สาหร่ายสามารถกำจัดก๊าซคาร์บอนไดออกไซด์และทำให้ปริมาณก๊าซมีเทนเพิ่มขึ้นจากร้อยละ 60 เป็นมากกว่าร้อยละ 90 คิดเป็นอัตราการกำจัดก๊าซคาร์บอนไดออกไซด์สูงถึง 5.097 กรัมของก๊าซคาร์บอนไดออกไซด์ต่อลิตรต่อวัน และอัตราการผลิตไขมันเท่ากับ

88.57 มิลลิกรัมต่อลิตรต่อวัน นอกจากนี้ยังพบว่าการใช้กลยุทธ์เพิ่มอัตราการพ่นก๊าซชีวภาพแบบขั้นบันได สามารถส่งเสริมการเจริญของสาหร่ายและทำให้สาหร่ายมีชีวมวลสุดท้ายและปริมาณผลผลิตไขมันเพิ่มขึ้น 1.25 และ 1.79 เท่า ตามลำดับ เมื่อวิเคราะห์องค์ประกอบไขมันจากสาหร่ายพบว่าเป็นกรดไขมันอิ่มตัวที่มีความเสถียรต่อการเกิดออกซิเดชั่นและมีคุณภาพในการจุดติดไฟสูง

ตอนที่ 2 การเพิ่มประสิทธิภาพการทำบริสุทธิ์ก๊าซชีวภาพและการผลิตไขมันของสาหร่ายไขมันสูงสายพันธุ์ *Scenedesmus* sp. โดยการศึกษากลยุทธ์การเพิ่มระดับปัจจัยการเจริญแบบขั้นบันได ซึ่งมีปัจจัยการเจริญที่ศึกษาได้แก่ ความเข้มแสง ความเข้มข้นของไนโตรเจน และอัตราการพ่นก๊าซชีวภาพ พบว่ากลยุทธ์การเพิ่มอัตราการพ่นก๊าซชีวภาพแบบขั้นบันไดเหมาะสมกับการเจริญและการผลิตไขมันของสาหร่าย ในขณะที่กลยุทธ์การเพิ่มความเข้มแสงแบบขั้นบันไดเหมาะสมกับการกำจัดก๊าซคาร์บอนไดออกไซด์ และพบว่ากลยุทธ์การเพิ่มปัจจัยทั้งสามปัจจัยพร้อมกันแบบขั้นบันได ทำให้สาหร่ายมีการเจริญ การผลิตไขมัน และมีประสิทธิภาพในการกำจัดก๊าซคาร์บอนไดออกไซด์สูงสุด ทำให้ก๊าซชีวภาพที่ผ่านระบบการเพาะเลี้ยงสาหร่ายมีปริมาณก๊าซมีเทนเพิ่มขึ้นมากกว่าร้อยละ 98 คิดเป็นอัตราการกำจัดก๊าซคาร์บอนไดออกไซด์เท่ากับ 6.50 กรัมต่อวันต่อลิตรอาหาร ภายใต้กลยุทธ์ข้างต้นยังทำให้ได้ชีวมวลของสาหร่ายสูงถึง 4.40 กรัมต่อลิตร และมีปริมาณไขมันสะสมที่ร้อยละ 34.10 ไขมันสาหร่ายที่สกัดได้ประกอบด้วยกรดไขมันที่มีความยาวคาร์บอน 16 – 18 อะตอมในสัดส่วนมากกว่าร้อยละ 94

ตอนที่ 3 การหาสภาวะที่เหมาะสมของการทำบริสุทธิ์ก๊าซชีวภาพพร้อมกับการบำบัดน้ำทิ้งจากบ่อบำบัดไร้อากาศโรงงานสกัดน้ำมันปาล์มโดยการเพาะเลี้ยงสาหร่ายไขมันสูงสายพันธุ์ *Scenedesmus* sp. ตรึงรูปในเม็ดเจลอัลจิเนต ผลการทดลองพบว่าความเข้มข้นของหัวเชื้อสาหร่ายเริ่มต้นและอัตราส่วนปริมาตรเจลอัลจิเนตต่อปริมาตรอาหารที่เหมาะสมสำหรับการตรึงรูปสาหร่าย คือ 10^6 เซลล์ต่อมิลลิลิตรและร้อยละ 25 ตามลำดับ และพบว่าสภาวะที่เหมาะสมสำหรับการเพาะเลี้ยงสาหร่ายตรึงรูป คือ การใช้น้ำทิ้งที่อัตราส่วนน้ำต่อน้ำทิ้ง 4:1 และความเข้มแสง 9.5 กิโลลักซ์ ภายใต้สภาวะดังกล่าวสาหร่ายตรึงรูปสามารถกำจัดก๊าซคาร์บอนไดออกไซด์ได้ร้อยละ 88.46 และทำให้ปริมาณก๊าซมีเทนในก๊าซชีวภาพเพิ่มขึ้นมากกว่าร้อยละ 95 คิดเป็นอัตราการกำจัดก๊าซคาร์บอนไดออกไซด์เท่ากับ 4.63 กรัมต่อวันต่อลิตรอาหาร นอกจากนี้สาหร่ายตรึงรูปยังสามารถลดค่าซีโอดีในน้ำทิ้งได้มากกว่าร้อยละ 71 และสามารถลดปริมาณไนโตรเจนและปริมาณ

ฟอสฟอรัสได้ทั้งหมด โดยชีวมวลสาหร่ายสุดท้ายมีปริมาณ 2.98 กรัมต่อลิตร และมีปริมาณไขมันร้อยละ 35.92 นอกจากนี้ชีวมวลสาหร่ายยังมีปริมาณสารสีที่ประกอบด้วยคลอโรฟิลล์และแคโรทีนอยด์เท่ากับ 45.97 และ 26.06 มิลลิกรัมต่อกรัมชีวมวลสาหร่าย ตามลำดับ เมื่อวิเคราะห์องค์ประกอบไขมันที่ได้พบว่าประกอบด้วยกรดไขมันที่มีสัดส่วนของคาร์บอน 16 – 18 อะตอมมากกว่าร้อยละ 98

ตอนที่ 4 การศึกษาการเพิ่มปริมาณไขมันในสาหร่ายไขมันสูงโดยการจำกัดสารอาหาร และการหาสภาวะที่เหมาะสมในการเก็บเกี่ยวชีวมวลสาหร่าย โดยเปรียบเทียบการเพาะเลี้ยงสาหร่ายไขมันสูง 2 สายพันธุ์ที่แยกได้จากทะเลสาบสงขลาในประเทศไทย ได้แก่ สายพันธุ์*Micractinium reisseri* SIT04 และ *Scenedesmus obliquus* SIT06 ผลการทดลองพบว่าการจำกัดธาตุเหล็กและฟอสฟอรัสไม่มีผลต่อการเจริญของสาหร่ายอย่างมีนัยสำคัญ ในขณะที่การจำกัดไนโตรเจนทำให้สาหร่ายมีการเจริญลดลง แต่การจำกัดไนโตรเจนทำให้สาหร่ายมีปริมาณไขมันเพิ่มขึ้น 1.5-1.6 เท่า ซึ่งมากกว่าปริมาณไขมันที่เพิ่มขึ้นจากการจำกัดธาตุเหล็กและฟอสฟอรัส (1.2 เท่า) โดยสาหร่ายสายพันธุ์ *S. obliquus* SIT06 ให้การเจริญและสะสมไขมันได้สูงกว่า และพบว่าสาหร่ายมีการสะสมไขมันที่มีองค์ประกอบกรดไขมันอิ่มตัวสูงขึ้นภายใต้สภาวะการจำกัดไนโตรเจน สำหรับการเก็บเกี่ยวชีวมวลสาหร่ายเป็นการใช้สารช่วยตกตะกอนไคโตซานจากการหาสภาวะที่เหมาะสมโดยวิธีพื้นผิวตอบสนอง (Response Surface Methodology: RSM) พบว่าสามารถใช้ปริมาณไคโตซานเพียง 64 มิลลิกรัมต่อลิตร ในการตกตะกอนสาหร่ายได้มากกว่าร้อยละ 99 คิดเป็นค่าใช้จ่าย 0.098 บาทต่อกรัมสาหร่าย

ตอนที่ 5 เป็นการหาสภาวะที่เหมาะสมในการเพาะเลี้ยงสาหร่าย *S. obliquus* SIT06 แบบโฟโตออโตทรอฟิกด้วยวิธี RSM และศึกษาการเก็บเกี่ยวชีวมวลสาหร่ายด้วยวิธีสร้างเพเลตของเชื้อราชนิดสาย ผลการทดลองพบว่าสภาวะที่เหมาะสมในการเลี้ยงสาหร่าย คือความเข้มแสงที่ระดับ 87 ไมโครโมลโปรตอนต่อตารางเมตรต่อวินาที ความเข้มข้นของโซเดียมไนเตรตที่ใช้เป็นแหล่งไนโตรเจนเท่ากับ 1.1 กรัมต่อลิตร และค่าพีเอชเริ่มต้นเท่ากับ 8 โดยสาหร่ายสามารถผลิตชีวมวลสาหร่ายได้สูงสุดเท่ากับ 1.99 กรัมต่อลิตร และสาหร่ายมีการสะสมไขมันสูงถึงร้อยละ 40.86 สำหรับการเก็บเกี่ยวชีวมวลสาหร่าย ได้ศึกษาการเติมสปอร์ของเชื้อราชนิดสายลงไปในอาหารเลี้ยงสาหร่ายที่อยู่ในระยะ late-log phase พบว่าเชื้อราชนิดสาย *Cunninghamella*

echinulata TPU 4652 สามารถเก็บเกี่ยวชีวมวลสาหร่ายได้อย่างมีประสิทธิภาพ โดยให้ค่าประสิทธิภาพการเก็บเกี่ยวชีวมวลสูงสุดถึงร้อยละ 92.7 และชีวมวลรวมของเพเลตสาหร่าย-เชื้อรา (microalgae-fungi pellets) ที่เก็บเกี่ยวได้มีน้ำหนักเท่ากับ 4.45 กรัมต่อลิตร และมีปริมาณไขมันเท่ากับ 1.21 กรัมต่อลิตร ไขมันที่สกัดได้มีองค์ประกอบหลักเป็นกรดไขมันสายยาวประเภท C16:0, C18:0 และ C18:1 ที่มีคุณสมบัติทางเชื้อเพลิงสอดคล้องกับมาตรฐานไบโอดีเซลสากล แสดงให้เห็นว่ามีศักยภาพในการนำใช้เป็นแหล่งน้ำมันสำหรับผลิตไบโอดีเซล

ตอนที่ 6 เป็นการศึกษาวิธีการที่รวดเร็วในการเก็บเกี่ยวและตรึงรูปชีวมวลสาหร่ายไขมันสูงโดยใช้เพเลตเชื้อราชนิดสาย (pellet-forming filamentous fungi) พบว่าเชื้อราชนิดสายสายพันธุ์ *Trichoderma reesei* QM9414 มีความสามารถในการสร้างเพเลตได้ดีที่สุดภายใต้อัตราการเขย่าเท่ากับ 100 รอบต่อนาที จึงนำมาใช้ในการเก็บเกี่ยวและตรึงชีวมวลสาหร่ายไขมันสูงสายพันธุ์ *Scenedesmus* sp. การใช้อัตราส่วนของปริมาตรเพเลตเชื้อราต่อปริมาตรสาหร่ายที่ 1:2 ทำให้สามารถเก็บเกี่ยวชีวมวลสาหร่ายได้มากกว่าร้อยละ 94 ภายในระยะเวลา 10 นาที อัตราส่วนของเพเลตเชื้อราต่อปริมาณสาหร่ายที่ใช้มีผลต่อระยะเวลาในการเก็บเกี่ยวและปริมาณเซลล์สาหร่ายเริ่มต้นในตัวตรึง สาหร่ายตรึงรูปด้วยเชื้อราสามารถนำไปบำบัดน้ำทิ้งจากโรงงานอาหารทะเลได้อย่างมีประสิทธิภาพภายใต้สภาวะที่ไม่มีการฆ่าเชื้อ โดยสามารถลดค่าซีโอดี ปริมาณไนโตรเจน และปริมาณฟอสฟอรัสได้มากกว่าร้อยละ 74, 44 และ 93 ตามลำดับ ภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (scanning electron microscopy: SEM) แสดงให้เห็นว่าเซลล์สาหร่ายไม่เพียงถูกกักเก็บในเพเลตเชื้อรา แต่ยังถูกตรึงด้วยเอ็กโซพอลิแซ็กคาไรด์ที่อยู่บนเส้นใยของเชื้อรา ไขมันที่สกัดได้จากเพเลตสาหร่าย-เชื้อราประกอบด้วยกรดไขมันสายยาวซึ่งมีความยาวของคาร์บอนระหว่างช่วง 16 – 18 มากกว่าร้อยละ 83 ซึ่งเหมาะสมสำหรับใช้ในการผลิตไบโอดีเซล

การศึกษานี้แสดงให้เห็นถึงศักยภาพของสาหร่ายไขมันสูงในการทำบริสุทธิ์ก๊าซชีวภาพ และผลิตไขมันที่มีศักยภาพเป็นแหล่งน้ำมันสำหรับผลิตไบโอดีเซล รวมทั้งได้นำเสนอกลยุทธ์ที่มีประสิทธิภาพในการส่งเสริมการเจริญ การสะสมไขมัน และการกำจัดก๊าซคาร์บอนไดออกไซด์ของสาหร่าย ซึ่งเป็นการพัฒนากระบวนการและนวัตกรรมของการเก็บเกี่ยวสาหร่ายที่มีประสิทธิภาพ นอกจากนี้สาหร่ายไขมันสูงยังมีความสามารถในการบำบัดน้ำทิ้งโรงงาน

อุตสาหกรรมได้ โดยน้ำมันที่สกัดได้จากทั้งสาหร่ายและเพเลตสาหร่าย-เชื้อรามีองค์ประกอบที่คล้ายกับของน้ำมันพืชเช่น น้ำมันปาล์ม คาดว่าผลการวิจัยนี้จะเป็นประโยชน์ต่ออุตสาหกรรมก๊าซชีวภาพ และการพัฒนาอุตสาหกรรมการผลิตเชื้อเพลิงชีวภาพจากสาหร่าย

Thesis Title	Process development for carbon dioxide removal from biogas and lipid production by oleaginous microalgae cultivation
Author	Mr. Sirasit Srinuanpan
Major Program	Biotechnology
Academic Year	2018

ABSTRACT

Oleaginous microalgae has high CO_2 removal rate which could be used for biogas purification effectively. They also can accumulate high lipid content >20% dry basis and has potential to be utilized as biodiesel feedstocks. This study aimed to develop the efficient process of microalgae cultivation for biogas purification coupling with lipid production as well as the development of harvesting process, immobilization of microalgae for repeat-use and the ability of microalgae for phytoremediation of industrial wastewater. There are main six parts in this study.

Part I is the cultivation of several oleaginous microalgae using biogas and the evaluation of their ability to remove CO_2 and improve methane content in biogas. All oleaginous microalgae could effectively remove CO_2 in biogas (>90%) and accumulate lipid content in range the range of 24-42%. Among the species tested, *Scenedesmus* sp. was most effective in CO_2 removal. The optimal conditions for both biogas purification and lipid production were: gas flow rate of 0.3 L h^{-1} per L-medium, inoculum sized at 10^7 microalgal cells mL^{-1}, added with KNO_3 0.8 g L^{-1} as nitrogen source and illuminated at 5.5 klux light intensity. Under these conditions, methane content in biogas was increased from 60% up to >90% corresponding to high CO_2 removal rate of 5.097 g-CO_2 day^{-1} per 1 L-medium coupled with lipid productivity of 88.57 mg L^{-1} day^{-1}. In addition, with the strategy of stepwise-increasing gas flow rate the final biomass and lipid productivity were 1.25 and 1.79 folds increased. The microalgal lipids were composed of fatty acids with fuel properties of high oxidation stability and high ignition quality.

Part II is the further improvement of *Scenedesmus* sp. performance in biogas upgrading and lipid production by the strategies of step-wise increasing of growth factor levels. Three important growth factors for microalgae included light

intensity, nitrogen source and CO_2 flow rate. The stepwise-increasing of CO_2 flow rate was suitable for cell growth and lipid production while the stepwise increasing of light intensity was more suitable for CO_2 removal efficiency. Among the strategies attempted, the simultaneous stepwise-increasing of all three growth factors most effectively enhanced the performance of microalgae. Through this strategy, >96% of CO_2 was continuously removed from biogas and the CH_4 content in the purified biogas was >98%. This process also generated microalgal biomass at 4.40 g L^{-1} with a lipid content of 34.10%. The CO_2 removal rate by this process was as high as 6.50 g-CO_2 day^{-1} per 1 L microalgal culture. The microalgal lipids contained long chain fatty acids (C16-C18) >94% and their prospect fuel properties indicated their suitable use as biodiesel feedstocks.

Part III is the optimization of the conditions for simultaneous biogas purification and pretreatment of anaerobic digester effluent from palm oil mill by immobilized oleaginous microalga *Scenedesmus* sp. in alginate gel beads. The optimal culture conditions for immobilized microalga were: the use of initial cell concentration at 10^6 cells mL^{-1} and bead volume to medium volume ratio at 25% v/v. The optimal conditions for simultaneous biogas purification and pretreatment of secondary effluent were: the use of diluted effluent at 4:1 and light intensity at 9.5 klux. Through these conditions, 88.46% of CO_2 was removed from biogas and the methane content was increased more than 95%. The CO_2 removal rate was 4.63 g-CO_2 day^{-1} per 1 L-medium. After process operation, the immobilized microalgae effectively removed COD >71% and all of nitrogen and phosphorus. The final microalgal biomass obtained was 2.98 g L^{-1} with high lipid content of 35.92%. The pigments including chlorophylls and carotenoids in biomass were 45.97 and 26.06 mg g^{-1} biomass, respectively. Fatty acid compositions of microalgal lipids were C16-C18 (>98%).

Part IV aimed to increase the lipid content of oleaginous microalgae via nutrient starvations and optimize the cost-effective harvesting process. Two locally isolated oleaginous microalgae from Songkhla Lake in Thailand were identified as *Micractinium reisseri* SIT04 and *Scenedesmus obliquus* SIT06. Starvation of either ferrous or phosphorus did not significantly affect cell growth but the starvation of nitrogen did limit cell growth of both strains. However, the nitrogen

starvation stimulated lipid content of both strains by 1.5-1.6 folds which were higher than the lipid content increased by ferrous and phosphorus starvation (1.2 folds). *S. obliquus* SIT06 could grow and accumulated higher lipid content. The lipid accumulated during nitrogen starvation contained higher content of saturated fatty acids. The harvesting process through bioflocculation was optimized by Response Surface Methodology (RSM). The maximum flocculation efficiency greater than 99% was achieved using minimum dosage of chitosan at 64 mg L^{-1} which are fairly cost-effective at estimated chitosan around 0.098 Bath per gram microalgae biomass.

Part V aimed to optimize the photoautotrophic cultivation of *S. obliquus* SIT06 using RSM and to harvest microalgal cells by co-pelletization with filamentous fungi. The optimal conditions for photoautotrophic cultivation of *S. obliquus* SIT06 were: pH of 8.0, $NaNO_3$ as a nitrogen source at concentration of 1.1 g L^{-1}, and light intensity of 87 μmol proton m^{-2} s^{-1}. Under these conditions, the highest microalgal biomass obtained was 1.99 g L^{-1} with a high lipid content of 40.86%. To simplify harvesting process of microalgal cells, pellet-forming filamentous fungi were inoculated into the late log-phase of microalgae culture. Among the fungi tested, *Cunninghamella echinulata* TPU 4652 most effectively harvested the microalgal cells with the highest flocculation efficiency of 92.7%. Moreover, the biomass and lipids of microalgae-fungi pellets were as high as 4.45 and 1.21 g L^{-1}, respectively. The extracted lipids were mainly composed of C16:0, C18:0 and C18:1, and their estimated fuel properties meet with the international standards indicating their potential use as biodiesel feedstocks.

Part VI is the development of the rapid method for harvesting and immobilizing oleaginous microalgae using pellet-forming filamentous fungi. Among the fungi tested, *Trichoderma reesei* QM 9414 showed superior pellet forming ability under shaking speed at 100 rpm. Its pellets were used to harvest oleaginous microalga *Scenedesmus* sp. With increasing volume ratio of fungal pellets to microalgae culture up to 1:2, >94% of microalgal cells were rapidly harvested within 10 min. The ratio of fungal pellets could manipulate both harvesting time and initial concentration of microalgal cells in the pellets. The microalgae-fungal pellets were successfully used as immobilized cells for effective phytoremediation of secondary effluent from seafood processing plants under nonsterile condition. The chemical oxygen demand,

total nitrogen, and total phosphorus removal were >74%, >44%, and >93%, respectively. The scanning electron microscopy showed that the microalgal cells were not only entrapped in the pellets but also got attached to the fungal hyphae with sticky exopolysaccharides, possibly secreted by the fungi. The extracted lipids from the pellets were mainly composed of C16-C18 (>83%) with their suitability as biodiesel feedstocks.

This study has shown that oleaginous microalgae are the promising microorganisms that can be used not only for effective biogas purification but also production of lipids with high potential as biodiesel feedstocks. The effective strategies to increase microalgal growth, lipid content, CO_2 removal efficiency as well as the effective harvesting process and innovative immobilization of microalgae have been proposed. The oleaginous microalgae also show high ability for being used in phytoremediation of industrial wastewater. The extracted lipids from both microalgae and microalgae-fungal pellets have similar fatty acid compositions with those of plant oils. This study may contribute greatly to the biogas industry and the industrialized microalgae based biofuel production.

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Sirasit Srinuanpan

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LIST OF PAPERS

I. **Srinuanpan, S.**, Cheirsilp, B., Kitcha, W., Prasertsan, P. 2017. Strategies to improve methane content in biogas by cultivation of oleaginous microalgae and the evaluation of fuel properties of the microalgal lipids. Renew. Energy. 113: 1229-1241.

II. **Srinuanpan, S.**, Cheirsilp, B., Prasertsan, P. 2018. Effective biogas upgrading and production of biodiesel feedstocks by strategic cultivation of oleaginous microalgae. Energy. 148: 766-774.

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IV. **Srinuanpan, S**., Cheirsilp, B., Prasertsan, P., Kato, Y., Asano, Y. 2018. Strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks: nutrient starvations and cost-effective harvesting process. Renew. Energy. 113: 1229-1241.

V. **Srinuanpan, S.**, Cheirsilp, B., Prasertsan, P., Kato, Y., Asano, Y. 2018. Photoautotrophic cultivation of oleaginous microalgae and co-pelletization with filamentous fungi for cost-effective harvesting process and improved lipid yield. Aquacult. Int. 26: 1493-1509.

VI. **Srinuanpan, S.**, Chawpraknoi, A., Chantarit, S., Cheirsilp, B., Prasertsan, P. 2018. A rapid method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi and the application in phytoremediation of secondary effluent. Int. J. Phytoremediat. 20: 1017-1024.

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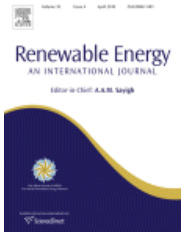

Title: Strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks: Nutrient starvations and cost-effective harvesting process

Author: Sirasit Srinuanpan,Benjamas Cheirsilp,Poonsuk Prasertsan,Yasuo Kato,Yasuhisa Asano

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1. Introduction

Fuel security, fossils oil price, and global warming are among the most important concerns of energy government in the world. Recently, there is an increasing need for exploration of sustainable energy resources. Biodiesel is one of renewable energy that has been produced from energy crop oils or animal fats. However, producing enough biodiesel from these feedstocks to satisfy existing demand would have a serious impact on food supplies. Among available alternative oil sources, oleaginous microalgae with a lipid content >20% have been receiving considerable attention because they can fix CO_2 into lipids and also produce high value products, such as chlorophylls and carotenoids (Jawaharraj *et al*., 2016). The lipid content in microalgae biomass is highly influenced by microalgae species, medium composition, and culture conditions especially nitrogen concentration, culture pH, gas flow rate and light intensity (Karpagam *et al*., 2015; Thawechai *et al*., 2016). In addition, many studies have reported the crucial conditions for high lipid accumulation in microalgae such as nitrogen limitation (Karpagam *et al*., 2014; Li *et al*., 2015) phosphorus and iron starvations (Yeesang and Cheirsilp, 2011; Fan *et al*., 2014). However, their effects on only lipid accumulation are not enough to evaluate their potential use as biodiesel feedstocks. The effects on the fatty acid composition and fuel properties as biodiesel should also be investigated.

Recently, the microalgae cultivation with feeding biogas has received considerable attention for improving CH_4 content in biogas. Biogas has been receiving increasingly interest as a renewable and sustainable source of energy. Crude biogas produced from anaerobic digestion of organic wastes is mainly composed of 50-80% methane (CH_4) and 30-45% carbon dioxide (CO_2) and also has trace components such as water, oxygen, and hydrogen sulfide depending on the feedstock (Kajolinna *et al*., 2015). The CH_4 content in biogas is the most important criterion, especially for the combustion process in engines and electricity production. Only biogas with CH_4 content >90% (v/v) meets requirement for being used as biofuel with high-efficiency combustion and high calorific value (Ryckebosch *et al*., 2011; Meier *et al*., 2015). The removal of CO_2 is an important key to increase CH_4 content in biogas. Despite physical/chemical absorption, water scrubbing, membrane separation,

and pressure-swing adsorption supporting a removal of CO_2 from biogas, these technologies require considerable large amount of energy, auxiliary materials, and chemicals. Moreover, they also generate wastes and wastewater that can pollute the environment (Nordberg *et al*., 2012; Yan *et al*., 2014). The biogas purification by microalgae through photosynthesis reaction has received considerable attention because the CO_2 fixation capability of microalgae is high and it also gives high biomass productivity (>0.1 g L^{-1} day^{-1}) and high-value products such as lipids and pigments. It could improve the quality of biogas because as CO_2 is removed, the CH_4 content increases. The CO_2 removal from biogas has been studied by using several species of microalgae i.e. *Scenedesmus* spp., *Nannochloropsis* spp., *Chlorella* spp. and *Neochloris* spp. The results showed that more than 90% of CO_2 could be removed from biogas while only 0.2-1% O_2 was detected in the gas phase (Yan and Zheng, 2013; Tongprawhan *et al*., 2014). It should be noted that the use of specific microalgae especially oleaginous microalgae strains that could produce high value products such as lipids as a feedstock for biodiesel, would be more attractive in order to offset the costs of this process. Thus these strategies could improve not only the quality of biogas but also the fixation of CO_2 into bioenergy by the oleaginous microalgae. Due to the high potential of this technique for environmental friendly and renewable energy production, the promising strategy to improve this technique should be developed to make it more viable at industrial scale.

Several researches have indicated that the microalgae could be one of the candidates for effluent treatment due to their capability of consuming the organic and inorganic component in effluent and generated biomass. The palm oil industry is one of the vital agro-industry in Thailand, which is the third largest palm oil producer in the world. In the process of crude palm oil (CPO) production from fresh fruit bunches, palm oil mill effluent (POME) was generated about 2.5 tons of effluent per ton of crude oil processed mainly from the process of sterilizing the fruits, extraction and clarification of oil (Pechsuth *et al*., 2001). POME is a thick brownish viscous liquid waste and characterized by high organic and inorganic contents that cause environmental concerns. POME normally is used as feedstock for biogas production through anaerobic digester. The effluent from anaerobic digester was then treated in a secondary treatment system as the effluent still contain high levels of carbon, nitrogen

and phosphorus. On the one hand, these compounds need to be removed in the tertiary treatment, and on the other hand they are suitable and cost-effective for microalgae cultivation (An *et al*., 2003; Cheirsilp *et al*., 2017; Xie *et al*., 2018). Previous reports have indicated that the chemical oxygen demand (COD), total nitrogen, and total phosphorus could effectively remove by microalgae (Ding *et al*., 2016; Cheirsilp *et al*., 2017). The microalgal biomass and lipid content were also obtained and also suitable use as biodiesel feedstocks. However, higher volume ratio of POME decreased the light penetration and resulted in lower biomass production (Ding *et al*., 2016). The ability of microalgae cultured in POME for growth and nutrient removal also depend on microalgae species. Therefore, to achieve the maximum biomass and nutrient removal, the suitable combination of POME concentrations and light intensity should be investigated.

In recent years, with the aim of simplifying recovery of microalgal biomass for biofuel production, the use of immobilized microalgae has gained increasingly attentions. This technique could increase the retention time of microalgae cells within bioreactors and promoted the metabolism of the microalgae. Gel entrapment method is the most common way to immobilize microalgae cells, in which natural polysaccharides such as agars, carrageenans and alginates are preferably used due to their low toxicity and high transparency (Lam and Lee, 2012). Alginate is commonly used for immobilization of microalgae and it also maintains the high viability of cells for extended periods of time (Ruiz-Marin *et al*., 2012). The use of immobilized microalgae in the beads could also reduce the amount of energy input for biomass recovery because the beads can be recovered by a simple separation method (e.g. sieving). However, some components in the wastewater such as higher phosphate and citrate likely affected the strength of the gel matrix and ultimately dissolved it (Jimenez-Pere *et al*., 2004). Recently, it has been reported that the re-calcification of alginate beads can resolve this problem (Lam and Lee, 2012; Castro-Ceseña and Sánchez-Saavedra, 2015). However, the possibility of using immobilized microalgae for removing CO_2 from industrial plants have been rarely reported. To develop the sustainable practices of immobilized microalgae, the immobilized microalgae beads should be attempted for simultaneous removing CO_2, treating effluent and contributing to microalgae based biofuel production.

In addition to cultivation conditions, the effective harvesting process of microalgae biomass should also be evaluated to be more sustainable in developing microalgae biofuel industry. The small size of microalgae cells and their low cell density make the harvesting process difficult and costly. The harvesting step is also important step whose cost would be as high as 20-30% of the total cost of biofuel production (Grima *et al*. 2003). Several methods including gravity sedimentation, filtration, centrifugation, coagulation flocculation, or combination of these have been implemented to harvest microalgal cells. Coagulation and flocculation are known as typical pre-concentration steps that can rapidly separate the microalgal biomass from the large amount of culture medium (Harith *et al*., 2009; Barros *et al*., 2015). The coagulations can be induced by pH adjustment and/or coagulants. The coagulants destabilize the colloidal system by neutralizing the forces of different origin that keep it stable. While the flocculents increase the sizes of flocs and agglomerate the suspended particles (Lopez-Maldonado *et al*., 2014). Recently, flocculations by biopolymer have been increasingly used due to their effectiveness and environmental friendly. The chitosan has been successfully used for bioflocculation of *Spirulina*, *Oscillatoria*, *Chlorella*, and *Synechocystis* spp. The optimal chitosan dosage required to maximize flocculation depended on the type of microalgae and their cell density (Divakaran and Pillai, 2002). Guar gum has also been used as bioflocculent in its both native and modified forms (Banerjee *et al*., 2014). The combined uses of pH for coagulation and biopolymer for flocculation have been performed (Harith *et al*., 2009). Therefore to minimize the dosage use of bioflocculent and maximize the harvesting efficiency, the important factors including the bioflocculent dosage, pH level and microalgal cell density for bioflocculation should be simultaneously optimized. It was expected that with these simultaneous optimizations the coagulation-flocculation processes could be maximized and lead to technical- and cost-effective process for harvesting of microalgal cells.

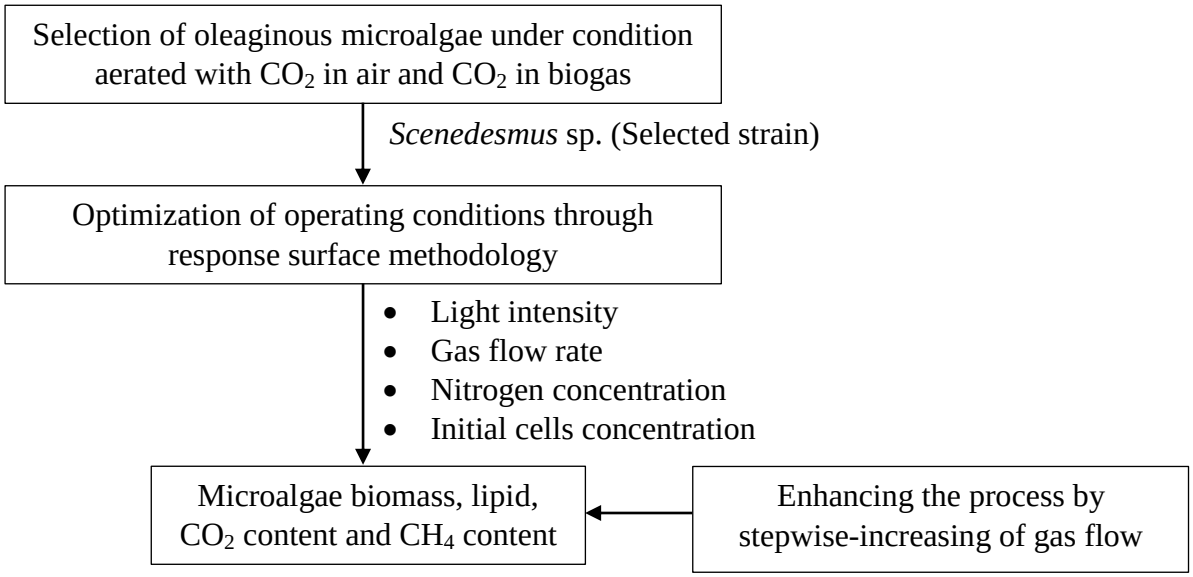
Recently, several researches have shown the possible use of pellet-forming filamentous fungi for harvesting of microalgal cells (Zhou *et al*. 2013; Talukder *et al*., 2014; Bhattacharya *et al*., 2017) and some of them attempted this method as immobilization technique (Wrede *et al*., 2014; Miranda *et al*., 2015). The filamentous fungi represent as attractive bioflocculating agents because of their self-

pelletization and possible entrapment of microalgae cells. Filamentous fungi can grow in different morphological forms including suspended mycelia, clumps, and pellets. The pellet-forming ability is influenced by culture conditions such as medium composition and viscosity, pH, inoculums, and shaking speed. The pellet form of the fungi is favorable since it can improve the culture rheology and make the repeated use of fungal cells possible and easily be harvested by a simple sieving method (Papagianni, 2004; Zhou *et al*., 2012; Zamalloa *et al*., 2017). Several filamentous fungi such as *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Isaria fumosorosea*, *Cunninghamella echinulata*, and *Mucor circinelloides*, have been reported for their capability of forming large pellets, which may provide an alternative cost effective way of harvesting microalgae biomass (Zhou *et al*., 2013; Wrede *et al*., 2014; Mackay *et al*., 2015). Generally, the fungal-assisted flocculation could be performed by two techniques. The first technique is the co-cultivation of microalgae with fungal pellets. This technique needs two steps of pelletization of fungal cells and co-cultivation with microalgae which required at least 72 and 48 h, respectively (Wrede *et al*., 2014; Miranda *et al*., 2015; Muradov *et a*l., 2015). The second technique is the co-pelletization in which the fungi grow and simultaneously flocculate microalgal cells in their pellets. This technique could reduce the operating steps and increase the overall productivity. Although several researchers have attempted this technique for harvesting of some microalgae (Zhou *et al*. 2012; Prajapati *et al*. 2014; Zamalloa *et al*. 2017), only one research group evaluated the lipid production of the microalgae-fungi pellets through autotrophic cultivation mode (Zhang and Hu, 2012). However, the lipid yield in their study was lower than 0.1 g L^{-1}. Therefore, the fast-growing microalgae with high lipid content as well as the filamentous fungi with the ability to effectively harvest these microalgae should be screened to improve the economics of microalgae based biofuel industry.

2. Objectives

1. To improve the methane content in biogas by cultivation of oleaginous microalgae and to evaluate the fuel properties of microalgae lipid (Paper I).
2. To improve the methane content in biogas and production of biodiesel feedstocks by stepwise-increasing of growth factors for microalgae cultivation (Paper II).
3. To optimize the condition for simultaneous phytoremediation of secondary effluent, biogas purification and production of biodiesel feedstocks by immobilized oleaginous microalgae (Paper III)
4. To increase the potential use of oleaginous microalgae as biodiesel feedstocks under nutrient-starved condition and to study the cost-effective process for the harvesting of microalgal biomass by coagulation-flocculation processes (Paper IV).
5. To improve the lipid yield of oleaginous microalgae cultivation under photoautotrophic mode and to study the cost-effective harvesting process by co-pelletization with filamentous fungi (Paper V).
6. To develop a rapid method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi and to study the possibility of immobilized cell for phytoremediation of secondary effluent and production of biodiesel feedstocks (Paper VI).

PAPER I



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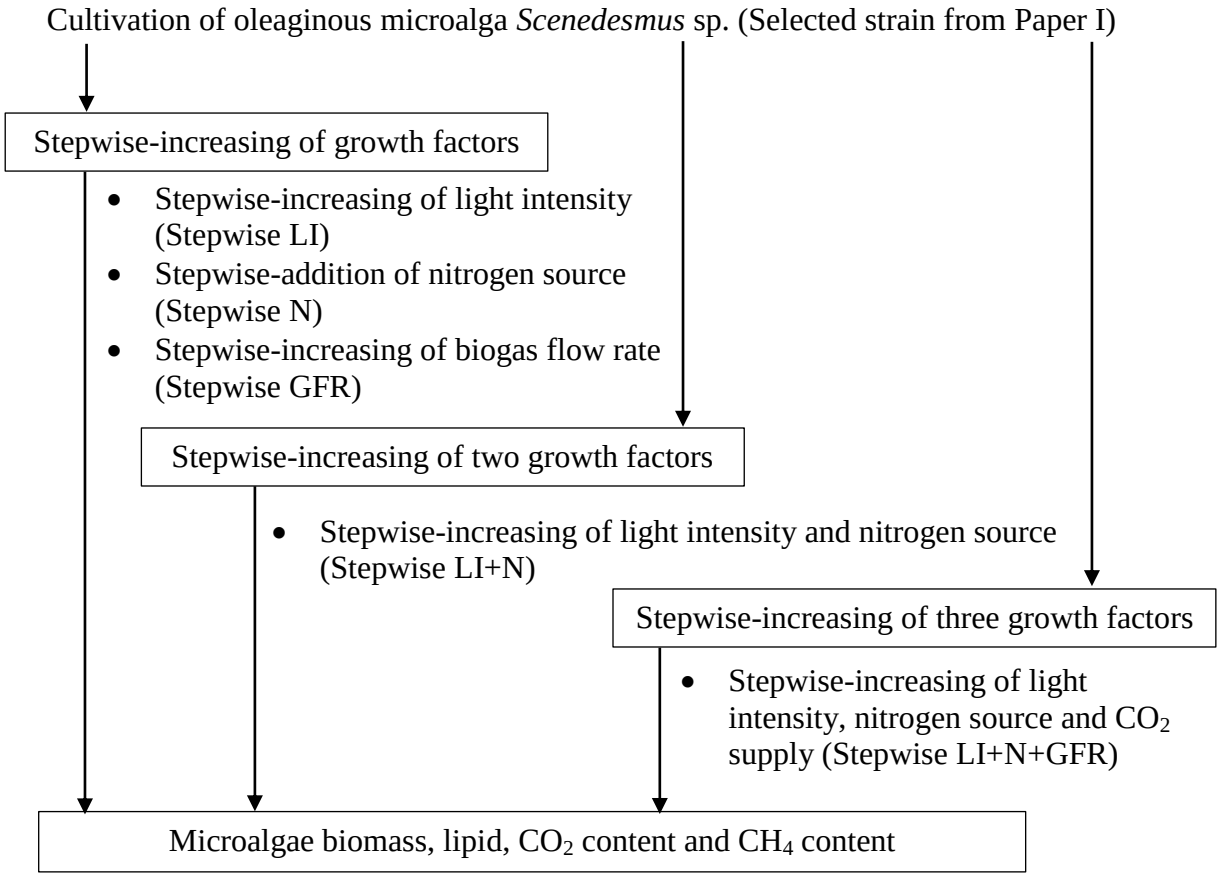
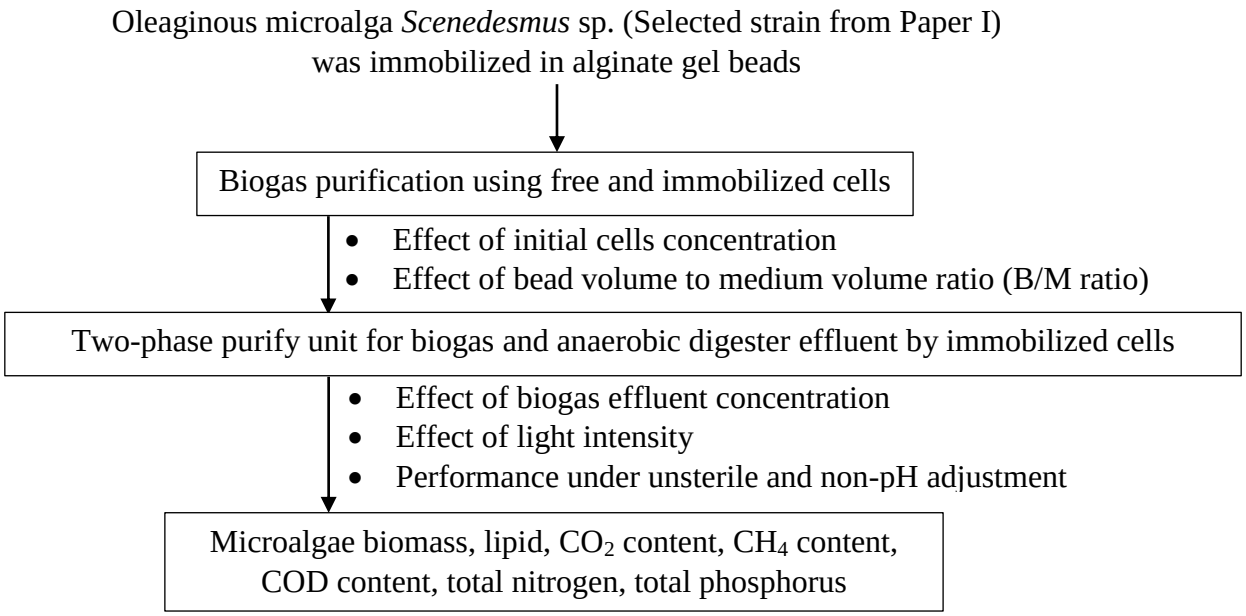


Figure 1 Experiment diagram of this study.

PAPER III



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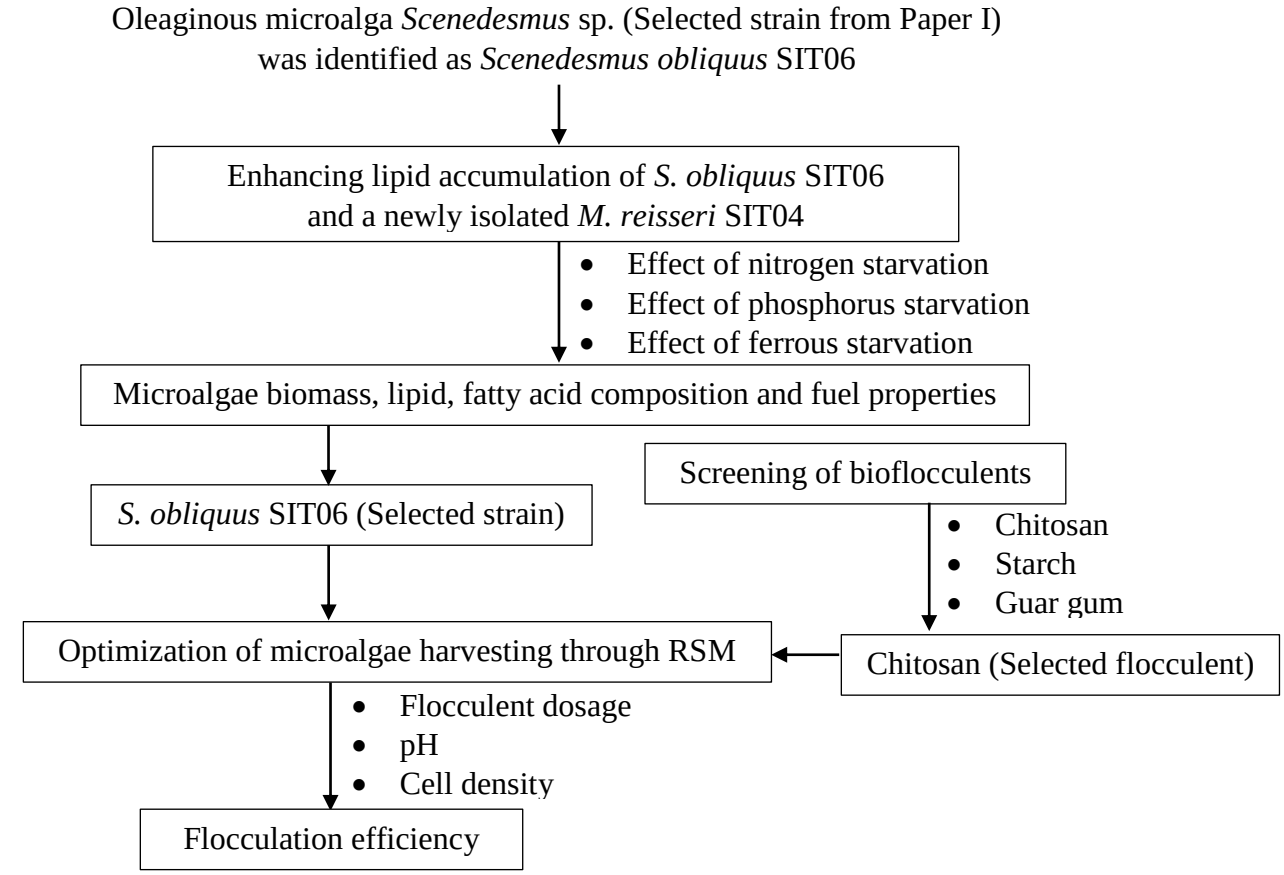
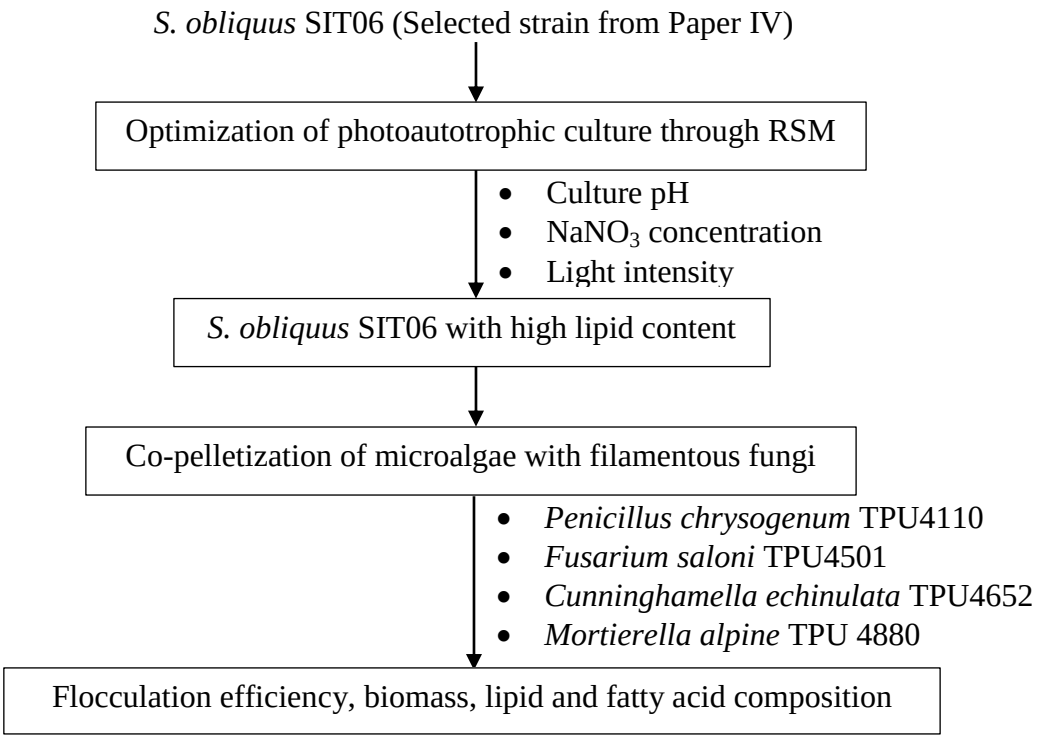


Figure 1 Experiment diagram of this study (cont.).

PAPER V



PAPER VI

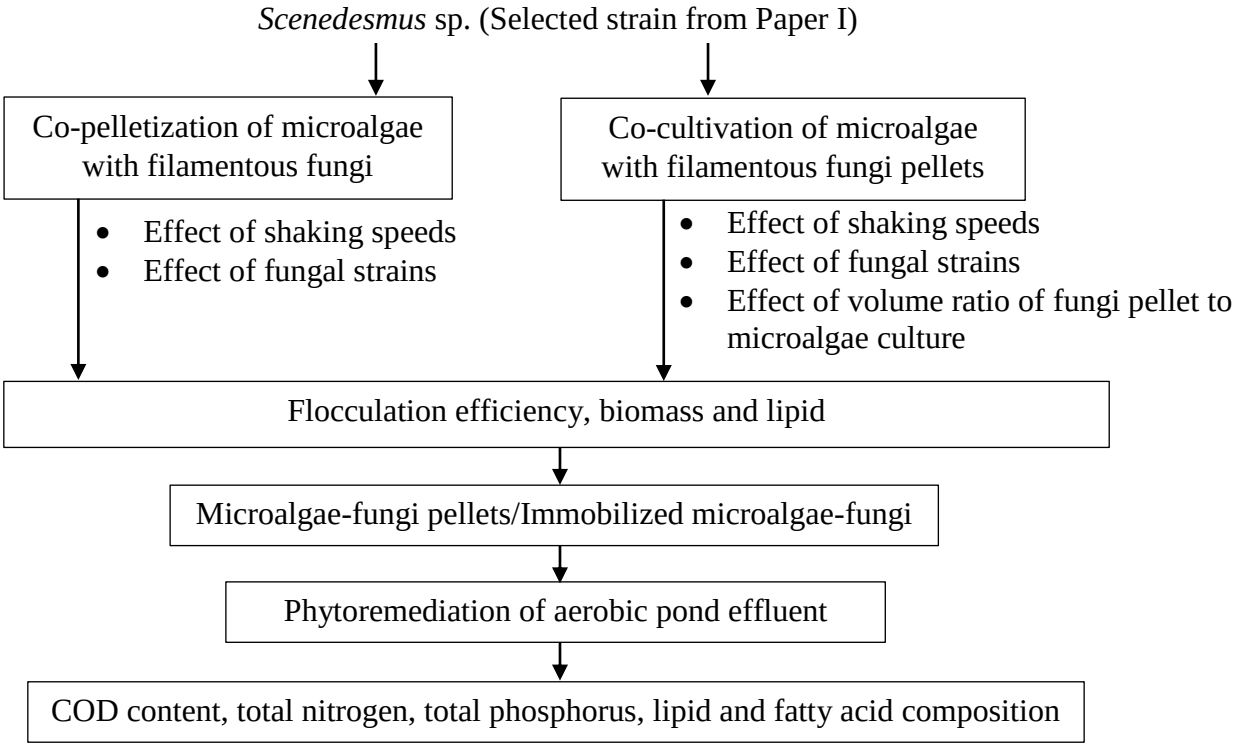


Figure 1 Experiment diagram of this study (cont.).

3. Literature reviews

3.1 Potential use of microalgae for biogas upgrading and production of biodiesel feedstocks

Biogas produced from anaerobic digestion of organic wastes is considered as a renewable and sustainable bioenergy. Crude biogas is mainly composed of methane (CH_4) at 50–80% and carbon dioxide (CO_2) at 35-40%. It also has trace amount of water, oxygen, and hydrogen sulfide (H_2S) depending on the composition of the organic wastes. The CH_4 content in biogas is most important for its combustion in engines and electricity production. Biogas with CH_4 content >90% (v/v) would give high-efficiency combustion and high calorific value. There are several physical/chemical methods to remove CO_2 and increase CH_4 content in the biogas. However, most of these technologies consume large amount of energy, auxiliary materials, and chemicals. They also generate wastes and wastewater. Therefore, the biological removal of CO_2 through microalgae cultivation has been attempted to improve the CH_4 content in biogas. Due to the high CO_2 fixation capability of microalgae, >75% of CO_2 in biogas could be removed. Moreover, through this strategy microalgae also give high biomass productivity and high-value products (Srinuanpan *et al*., 2017). The use of specific microalgae with high lipid content, namely oleaginous microalgae for removing CO_2 from biogas not only can purify the biogas but also produce biodiesel feedstocks. This integrated process is very attractive to offset the production costs of microalgae-based biofuels. Moreover, the concepts for cultivations of microalgae and its co-cultivation with other microorganisms for simultaneously removing CO_2 from biogas, phytoremediation of industrial wastewater and low-cost production of microalgae based biofuel are innovative and may contribute greatly to the sustainable and environmental friendly production of renewable energy.

Several microalgae are highly tolerant to high CO_2 and have high content of lipids which their fatty acid composition similar to those of plant oils. Many microalgae have been studied for CO_2 removal from biogas, such as *Chlorella* spp. (Kao *et al.*, 2012a; Kao *et al*., 2012b; Yan and Zheng, 2013; Zhao *et al*., 2013; Tongprawhan *et al*., 2014; Wang *et al*., 2015), *Leptolyngbya* spp. (Choix *et al*., 2017a), *Scenedesmus* spp. (Ouyang *et al*., 2015; Wang *et al*., 2015; Choix *et al*., 2017;

Srinuanpan *et al*., 2017; Srinuanpan *et al*., 2018), *Nannochloropsis* sp. (Meier *et al*. 2015;), *Selenastrum* spp. (Wang *et al*., 2015; Ouyang *et al*., 2015) and *Anabaena* spp. (Wang *et al*., 2015). According to these reports, the efficiency of CO_2 removal from biogas by microalgae could be as high as >90% and the CH_4 content in biogas after removing CO_2 increased more than 90% which meets requirement for being used as biofuel (Table 1). However, only few oleaginous microalgae have been attempted and evaluated for their ability to accumulate high lipid content during biogas upgrading process. *Chlorella* spp. (Tongprawhan *et al*., 2014) and *Scenedesmus* spp. (Choix *et al*., 2017b; Srinuanpan *et al*., 2017; Srinuanpan *et al*., 2018) have been examined for biogas upgrading and accumulation of lipids. Most of them could purify biogas and accumulate lipids > 20% of their biomass. More than 90% of fatty acids in the microalgal lipids are palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) which are suitable as biodiesel feedstocks.

3.2 Factors affecting biogas upgrading and production of biodiesel feedstocks

3.2.1 Biogas composition

The cultivation of microalgae using CO_2 from biogas as a carbon source could be limited by the variation in concentration of CO_2, CH_4 and H_2S in biogas. Among these three components, H_2S is the most toxic compound to the microalgal cells. Depending on the organic wastes used in anaerobic digester, the concentration of H_2S in biogas varied from 0.005 to 2% (González-Sánchez and Posten, 2017). When the CO_2 was fed into the culture medium, the CO_2 will dissolve and could be formed in the type of soluble CO_2 or HCO_3^-. The CO_2 conversion rate could depend on the culture pH (Wang *et al*., 2012) The CO_2 fixation can be increased while maintaining an alkaline pH, because this will accelerate the absorption of CO_2 and HCO_3^- (Amaro *et al*., 2010).

Several studies have evaluated the effect of CO_2 concentration on the microalgae growth (Tang *et al*., 2011; Thaweechai *et al*., 2016; Zhao *et al*., 2015). Some microalgae exhibit excellent tolerance ability to high CO_2 concentration. The genus *Chlorella* and *Scenedesmus*, growing in the wide range of high CO_2 concentration, are considered as suitable strains for studying the possibility of CO_2 removal from biogas (Tongprawhan *et al*., 2014; Srinuanpan *et al*., 2017). At a low

concentration of CO_2, the growth of microalgae may be limited whereas a high CO_2 concentration may cause a reduction in pH and inhibit the growth of microalgae and finally reduce the CO_2 fixation ability. Wang et al. (2017) found that the microalgal biomass of *Chlorella vulgaris* increased with increasing CO_2 concentration from 25% to 55%. The appropriate concentration of CO_2 did promote the photosynthetic efficiency of the microalgae to reproduce with in a shorter time. Yan *et al*. (2014) suggested that CO_2 in biogas above 55% was harmful to the microalgal cells and inhibited the growth of *Chlorella* sp.

The effect of CH_4 content in biogas is one of important factors for the performance of microalgae to grow and remove CO_2 from biogas. Meier *et al*. (2015) have reported that 50-100% of CH_4 content in biogas did not affect the growth of *Nannochloropsis gaditana*, while Kao *et al*. (2012a) claimed that *Chlorella* sp. MM-2 grew less when the CH_4 content in biogas increased from 20% up to 80%. Yan *et al*. (2014) have reported that the acceptable range of CH_4 content in biogas for cultivation of *Chlorella* sp. was 45–55% (v/v). Srinuanpan *et al*. (2017) have evaluated the growth of two microalgae marine *Chlorella* sp. and *Scenedesmus* sp. using 40% CO_2 in biogas. Interestingly, both microalgae were able to grow similarly when using 40% CO_2 in air. They explained that CH_4 in biogas may be insoluble in culture medium at ambient condition and then did not affect the performance of the microalgae. It could be concluded that both tolerance to high CO_2 and high CH_4 content in biogas are great important characteristics of microalgae for being used in biogas upgrading.

In addition to CO_2 and CH_4 content in biogas, H_2S in biogas greatly influences microalgae growth and their CO_2 fixation ability. Only few researchers reported the effect of H_2S in biogas on the performance of microalgae. *Chlorella* sp. presented high biomass productivities and showed its robustness during its cultivation using biogas containing considerable amount of H_2S up to 150 ppm (Kao *et al*., 2012). The microalgae seemed to convert H_2S to sulfate in order to reduce the toxicity and the sulfate form could be easily assimilated by *Chlorella* sp. as a growth limiting substrate (Mera *et al*., 2016). But this ability is strongly pH dependent and low pH is more suitable for better H_2S uptake rate. Other types of sulfur i.e. thiosulfate, tetrathionate, thiocyanate and elemental sulfur are slightly less favorable for the

microalgae assimilation (González-Sánchez and Posten, 2017). At alkaline pH, the sulfides H_2S (liquid form) and hydrosulfide (HS^-) could be easily oxidized through biochemical reaction. González-Sánchez and Posten (2017) have proposed the kinetic growth of green microalga *Chlorella* sp. as a function of the H_2S concentration during its application in biogas upgrading process. They found that the H_2S >200 ppm could inhibit the cell growth of *Chlorella* sp. Sulfate came mainly from chemical reactions and it was later assimilated by the microalgae.

Microalgal lipids mostly accumulated in triglyceride form and can be converted into biodiesel either by chemical or enzymatic reactions. The fatty acid compositions of microalgal lipids significantly affect the properties of the biodiesel. Only few researches focused on the effect of CO_2 concentration on lipid accumulation and composition of microalgae. The effect of CO_2 concentration on lipid production of microalga *Chaetoceros muelleri* was studied by varying CO_2 concentration at 0.03%-30% (Wang *et al*., 2014). It seemed that the CO_2 concentration >10% could induce the accumulation of lipids in the microalgae. The microalgae could accumulate lipids as high as 43.40% of their dry biomass. The high CO_2 concentration also induced the synthesis of unsaturated fatty acids rather than saturated fatty acids. Tong *et al*. (2010) also found that the high CO_2 concentration did increase the content of unsaturated fatty acids, especially the polyunsaturated fatty acids, in the microalgal lipids. Similar trend was also found in the lipid accumulation by *Tetraselmis suecica* (Kassim and Meng, 2017). Only Tongprawhan *et al*. (2014) and Srinuanpan *et al*. (2017; 2018) reported the effect of biogas on the fatty acid compositions of the oleaginous microalgae. They found that the biogas with 40% CO_2 could stimulate the production of unsaturated fatty acids such oleic acid (C18:1) and linolenic acid (C18:3) in the microalgae. The unsaturated, especially polyunsaturated, fatty acids are favorable for the production of biodiesel with low melting points.

3.2.2 Biogas flow rate

Biogas flow rate is the key factor to maximize CO_2 removal from biogas and lipid production by the microalgae. Appropriate flow rate of biogas could help in good mixing, supply CO_2 needed for microalgae growth, and stripping O_2 from the culture medium preventing supersaturation of oxygen in the medium (>35

mg/L dissolved oxygen) (Yan and Zheng, 2013). As a result, increasing biogas flow rate can lead to an increase in microalgal biomass productivity and CO_2 removal efficiency. However, in case the supply of CO_2 is much higher than the ability of the microalgae to consume, the culture pH would become acidic. The acidic pH negatively affects the solubility and availability of nutrients, enzyme activity, and transport of substrate across plasma membrane and electron transport in respiration and photosynthesis (Tang *et al*., 2011). Therefore, too high biogas flow rate decreased the performance of the microalgae (Srinuanpan *et al*., 2017; 2018). Another possible reason for this phenomenon has been described that as the biogas flow rate increases, the size of gas bubble also increases and the specific surface area per gas volume decreased. Kao *et al*. (2012a) also reported that the CO_2 removal from biogas by microalgae decreased with high gas flow rate due to the decrease in retention time of large bubbles in the medium. In addition, the high gas flow rate also increased the evaporation rate of the culture medium (Su *et al*., 2016).

Srinuanpan *et al*. (2017) suggested that with a low biogas flow rate the microalgae could effectively purified biogas but the biogas would be treated at a low rate and resulted in low productivity of the process. The high biogas flow rate means that high amount of biogas could be treated at a high rate. As the percent of CO_2 removal is important for purifying and CO_2 removal rate is important for the productivity of the process, the strategy to improve both targets was developed by Srinuanpan *et al*. (2018). They suggested the stepwise-increasing of gas flow rate for enhancing both percent CO_2 removal and CO_2 removal rate. Through this strategy, the biogas was supplied as a low rate which is suitable for low amount of microalgal cells. As the microalgae grew and their biomass increased, the biogas flow rate was increased to supply more CO_2. This strategy enhanced the overall process productivity.

Interestingly, the gas flow rate also affects the lipid content and lipid composition of the microalgae. Widjaja *et al*. (2009) reported that the higher CO_2 flow rate used the higher lipid content obtained. Similarly Binnal and Babu (2017) also found that the lipid content of microalga *Chlorella protothecoides* increased when the CO_2 flow rate increased. It is possibly due to the high CO2 flow rate could constantly supply high carbon to nitrogen ratio which has been proven to play an

important role in elevating the lipid content in microalgal cells (Su *et al*., 2017). Carvalho and Malcata (2005) have reported that the concentration of palmitoleic acid in the flagellate *Pavlova lutheri* increased with increasing gas flow rate, while EPA (20:5) concentrations decreased. High gas flow rate also enhanced the production of polyunsaturated fatty acids (Tang *et al*., 2011).

3.2.3 Light energy

3.2.3.1 Light intensity and photoperiods

Light is an important energy source for the photosynthesis by the microalgae. When the light energy is insufficient, the microalgae growth is restricted. The biomass productivity is low and the microalgae even consumed the storage energy (carbohydrates/lipids) during photorespiration (Zhao *et al*., 2013). Therefore, during low light intensity period an increase in photoperiod (i.e., longer lighting time) could support the better microalgal growth (Xue *et al*., 2011; Yan and Zheng, 2013). On the other hand, with above light saturation or high intensity of light the microalgae could not grow, the microalgae are unable to absorb light efficiently because the higher light intensity caused the destroyed light adapter i.e. photosystem I and II (Yan and Zheng, 2013; Jeong *et al*., 2013). Thus, the decrease in the light/dark regime (i.e., shorter lighting time) could avoid this phenomenon and reduce the photoinhibition effect (Xue *et al*., 2011; Yan and Zheng, 2013).

The effect of light intensity on CO_2 removal from biogas by microalga *Chlorella* sp. has been studied by Zhao *et al*. (2013). They found that the light intensity at 1200 and 1600 μmol m^{-2} s^{-1} with light–dark cycle 12 h:12 h were the optimal conditions to achieve >90% of methane. While Yan and Zheng (2013) found that the moderate light intensity at 350 μmol m^{-2} s^{-1} with middle photoperiod 14 h light:10 h dark was optimum for biogas upgrading by *Chlorella* sp. The optimal light intensity for removing CO_2 from biogas by *Scenedesmus obliquus* and *Selenastrum bibraianum* have been reported to be 150–170 μmol m^{-2} s^{-1} with a 12 h light–dark cycle (Ouyang *et al*., 2015). It should be noted that the optimal light intensity and photoperiods was controversial with different microalgal strains. In fact, the optimal light intensity would change upon the change in density of the microalgal cells. During an initial stage of cultivation, as the microalgae cell density is low the light

intensity should not exceed the saturation level to the microalgal cells otherwise photoinhibition may occur and damage the microalgal cells. In addition, using low light intensity during the initial stage of cultivation could decrease power consumption. On the contrary, as microalgae grew and high density of microalgal cells causes mutual cell shading. Therefore, the relationship between light intensity and cell density are essential at the end of the culture time to avoid the effects of insufficient lighting (Pilon *et al*., 2011; Yan *et al*., 2013; Das *et al*., 2011). Therefore, it is suggested that a constant light intensity is not suitable for technical and economic microalgae cultivation because not only the photoinhibition would occur during initial stage but also light energy would be wasted. Several researchers suggested that the optimal light intensity for supporting the microalgae growth should be increasing intensity which depends on cell density during cultivation to avoid light limitation and photoinhibition when the microalgae was cultured in constant light intensity. The optimal light intensity supply strategy proposed by Yan *et al*. (2013) is as follows: Step 1, 0-48 h with 800 μmol m^{-2} s^{-1}; Step 2, 48-96 h with 1200 μmol m^{-2} s^{-1}; and Step 3, 96-144 h with 1600 μmol m^{-2} s^{-1}. Srinuanpan *et al*. (2018) suggested the stepwise-increasing of light intensity during the biogas upgrading by the microalgae as follows: increasing the light intensity at a step of 6.8 μmol m^{-2} s^{-1} every 48 h based on the growth curve of the microalgae. This strategy successfully enhanced the microalgae growth and their ability to remove CO_2 from biogas.

Although several reports suggested that high light intensity could enhance the microalgae growth, it reduced lipid content of the microalgae. Cherisilp and Torpee (2012) reported that a low light intensity most effectively increased lipid accumulation. Since the high light intensities enhanced the growth, the microalgae might use synthesized energy for cell division rather accumulate it in lipid form (Yeesang and Cheirsilp, 2011; Gerorge *et al*., 2014). However, many researchers reported different trends of results. Liu et al. (2012), Difusa *et al*. (2014) and He *et al*. (2015) have shown that a high light irradiance was favored for more lipid and hydrocarbon contents rather than more biomass. They explained that under above light saturation the microalgae photosynthesis decreased; overall anabolic reaction flux was harshly limited, more photosynthetic flow of carbon and energy from carbohydrate and protein turning into the lipids synthesis. They also found that the

neutral lipid in microalgae was significant promoted under high light intensity, and the fatty acid compositions were good for the production of biodiesel. It could be concluded that the optimal light intensity for supporting the microalgae growth and lipid production are largely different and also differ with microalgae strains.

3.2.3.2 Light wavelength

Light wavelength is essential parameters for photosynthesis in the microalgae and CO_2 fixation. However, sunlight/natural light for supporting the growth of microalgae is not consistent due to uncertainty light intensity on rainy days and sunny days and changing the weather which may cause light limitation and photoinhibition. Culturing microalgae with artificial light could solve this problem. Yan *et al*. (2016) reported that using light-emitting diode (LED) is one of light source that it could save operation cost of microalgae cultivation than using ordinary fluorescent lamps because LEDs light contain the absorption bands of pigments i.e. chlorophyll and carotenoid or comprise only a combination of the growth efficient and inefficient light spectra.

Generally, the light wavelength in range of 400-700 nm was absorbed by microalgae for photosynthesis through chlorophylls (450-475 nm for chlorophyll a and 630-675 nm for chlorophyll b) and carotenoids (400-550 nm) (Kim *et al*., 2013). Several studies reported red light (600–700 nm) and blue light (400–500 nm) stimulate the growth of microalgae, CO_2 removal from biogas and lipid content of the microalgae. Yan *et al*. (2016a) studied the effects of LED light wavelengths (White, Red, Blue and Yellow) on biogas upgrading and biomass production by microalga *Chlorella* sp. They found that the optimum light wavelength for supporting microalgae growth and removing CO_2 from biogas was red light wavelength. Ho *et al*. (2011) reported that red light wavelength could enhance the photosystem II relative photosystem I. The chlorophyll in microalgae cell could also efficiently absorb the red light wavelength rather than the others (Zhao *et al*., 2013). But, the shorter wavelengths i.e., blue light has a high probability of striking the light-harvesting complex at the maximum power. Consequently, blue light wavelength could be highly inhibitor for CO_2 removal from biogas through microalgae photosynthesis and then cause photo-inhibition (Zhao *et al*., 2013). On the contrary, Zhao *et al*. (2013) and

Yan *et al*. (2016a) suggested that using red, yellow, and white light as a longer wavelength or combination of red and other light wavelength could avoid light saturation and photo-inhibition.

However, several researches indicated the controversial results. Kim *et al*. (2013), Yan and Zheng (2013), Yan *et al*. (2016b) found that sufficient red and blue light should be provided for adequate photosynthesis of microalgae. Providing red and blue light wavelengths must be selectively sufficient since the microalgae *Chlorella* sp. and *Scenedesmus* sp. could utilize artificial light energy for photosynthesis through chlorophyll a and chlorophyll b by absorbing light wavelength of 450–475 nm and 630–675 nm, respectively. They also reported that using white light wavelength which is providing light wavelength of 400-700 nm contributed to a higher microalgae growth rate than that of using light wavelength of red or blue. While combining rad and blue light wavelength gave the high microalgae growth rate than with a single light wavelength, irrespective of the combining ratio. The photosynthetic efficiency of microalgae was increased by simultaneously providing both red and blue light wavelengths, which satisfy the wavelength ranges needed for microalgal photosynthesis. Therefore, the mixed light wavelength of red and blue could enhance the CO_2 removal from biogas by microalgae. Although the mixed light wavelength of red and blue is suitable for microalgae biogas upgrading, the suitable mixed light wavelength should be evaluated depending on selected microalgae species.

The lipid composition of the microalgae is also affected by light wavelength (Teo *et al*., 2011). It has been reported that light induce triacylglycerides synthesis, formation of particularly chloroplast membranes and growth (Wahidin *et al*., 2013). The cellular fatty acid content of microalga *Nannochloropsis* sp. was highest when exposed to green LED, volumetric FAME productivities were highest for blue LED (Das *et al*., 2011). Ra *et al*. (2016) reported that blue and green LEDs influence microalgae growth and lipid production of microalga *Nannochloropsis* sp., respectively. It was possible that increasing of fatty acid and lipids could be due to compensatory increase in the amount of light adapter in chloroplasts i.e. thylakoid under low ability of light adsorption. Green light wavelength could stimulus the plant response and one of these responses is that the more thylakoid membranes in the

grana stack of plant leaves was observed than those in sunlight-exposed leaves (Zhang *et al*., 2011; Hulberg *et al*., 2014). However, these reports were controversial with those of Teo *et al*. (2014) who studied that two microalgae strains (*Tetraselmis* sp. and *Nannochloropsis* sp.) was cultivated under different light wavelength. They reports that the highest growth rate and lipid content were found in blue light supply. They explained that both ribulose bisphosphate carboxylase/oxygenase (Rubisco) and carbonic anhydrase enzymes affect the utilization of carbon dioxide in microalgae cell. Both enzymes are principally under the blue light wavelength. Moreover, the higher triglycerides accumulation was observed under the blue LED light feeding strategy.

Interestingly, the mixed light wavelength has been used to enhance the lipid accumulation in the microalgae. Ra *et al*. (2016) studied a two-stage cultivation strategy to improve the lipid production of microalgae by using LEDs light as a light source. After first stage with the blue LED (465 nm), the green LED light (520 nm) was exported for the second stage culture. They found that green LED light wavelength could significantly induce the microalgae cell into stress condition and then reached the maximum lipid accumulation and highest lipid content compared with one-stage of cultivation with blue light. While Kim *et al*. (2014) found that innovative process with wavelength shift (red light and then blue light or blue light and the red light) increased biomass and biodiesel yield.

3.2.4 Nitrogen source and its concentration

Nitrogen source is important growth factors for microalgae as it involves in the synthesis of nucleic acids, phospholipids and proteins. It has been reported that increasing nitrogen concentration could lead to the increase in biomass (Arumugam *et al*., 2013). Although increasing nitrogen concentration at initial could increase the ability of the microalgae, when their levels are above the optimal level the inhibition occurs (Li *et al*., 2008; Arumugam *et al*., 2013). Arumugam *et al*. (2013) reported that KNO_3 performed better than $NaNO_3$, Urea, $CaNO_3$, NH_4NO_3 and NH_4Cl for biomass production of *Scenedesmus*. This is because nitrogen and potassium in KNO_3 both are important nutrients for microalgae. In addition, they also suggested that the nitrogen concentration beyond 10 mM or 1.01 g L^{-1} could

inhibit the microalgae growth and led to the low biomass concentration. The optimum nitrogen concentration for the growth of *Scenedesmus* in their study was 5–10 mM (0.5–1.0 g L^{-1}). Therefore the KNO_3 as nitrogen source could enhance the microalgae growth and CO_2 removal. The used of KNO_3 as a nitrogen source and effect of its concentration on biogas upgrading by the microalgae have been reported (Tongprawhan *et al*., 2014; Srinuanpan *et al*., 2017). It was found that the CO_2 removal and lipid productivity of two microalgae *Chlorella* sp. and *Scenedesmus* sp. increased with increasing KNO_3 concentration. The optimal KNO_3 concentration for CO_2 removal from biogas was 0.62-0.8 g/L.

As the excess nitrogen concentration and nitrogen insufficiency during microalgae cultivation may limit the microalgae growth and result in lower CO_2 removal efficiency, the stepwise-addition of nitrogen source has been proposed by Srinuanpan *et al*. (2018). In their study, 0.2 g/L of KNO_3 was fed every 48 h and this strategy positively affected microalgae growth and CO_2 removal efficiency. However, the lipid production was slightly lower than that of the nitrogen-limited condition. It is well known that nitrogen limitation is the most effective and commonly used strategy for improving lipid accumulation in microalgae (Cheirsilp and Torrpee, 2012). During microalgae growth, starch would be first be synthesized to reserve energy, then lipid would be produced as a long-term storage mechanism in case of prolonged nitrogen deficiency (Siaut *et al*., 2011). Although a nitrogen starvation strategy is very effective in increasing lipid content in microalgae, the nitrogen deficiency often led to a significant decrease in the microalgae growth, thereby causing the negative effect on lipid productivity and CO_2 removal from biogas. Therefore, the engineering approaches should be conducted to optimize the cultivation time for CO_2 removal from biogas and the microalgae growth period (nitrogen-sufficient condition) and lipid accumulation period (nitrogen-deficient condition) to ensure high CO_2 removal efficiency and lipid productivity.

3.3 Combining biogas upgrading, production of biodiesel feedstock and wastewater treatment

The cultivation of microalgae by using commercial medium as nutrient sources is still not practical in the industrial level. Therefore, many

researchers in this field have paid increasing attention on the alternative use of industrial wastewater as good nutrient sources for microalgae cultivation. Most industrial wastewaters contain high organic/inorganic carbon, high nitrogen, high phosphorus which could be consumed by the microalgae (Cheirsilp *et al*., 2017). Recently, a number of studies have reported the simultaneous biogas upgrading and nutrients removal by microalgae. Table 2 summarizes previous researches on the cultivation of microalgae in different sources and concentrations of wastewater to simultaneously treat the wastewater and remove CO_2 from biogas. Cultivating *Chlorella* sp. in domestic digestate effluent produces a methane (CH_4) content (v/v) >90%, while removing about >80% chemical oxygen demand (COD), >70 % total nitrogen (TN), and >70% total phosphorus (TP) from wastewater (Yan and Zheng 2013; Wang *et al*., 2015; Zhao *et al*., 2013; Zhao *et al*., 2015; Ouyang *et al*., 2015). Yan *et al*. (2016), Xu *et al*. (2015), Prandini *et al*. (2016) reported that the *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, *Scenedesmus* spp. can achieve the methane content >70% when it grew in the swine wastewater. Meanwhile, COD, TN and TP were effectively removed >60%, >60% and >70%, respectively. Serejo *et al*. (2015) also found that the microalgae could upgrade >90% of methane when it grew in the vinasse digestate.

Not all types of wastewater could promote the microalgae growth and enhanced the biogas upgrading ability. The wastewater with high concentration of some compounds that possibly inhibit the microalgal growth should be pretreated or diluted before use. Xu *et al*. (2015) suggested that the Chemical Oxygen Demand below 1,600 mg/L was suitable for culturing the microalgae to upgrade biogas. Concentrated effluent has poor light penetration through the culture medium that inhibits growth. Higher nutrients concentrations and lower light penetration contributed to lower microalgae growth rates and longer duration of lag time and then resulted in lower CO_2 removal efficiency. Ding *et al*. (2016) reported that the excessive nutrient content in undiluted wastewater can be toxic to microalgae and affect their growth.

Although many researches focused on combination of two processes, biogas upgrading with wastewater treatment by the microalgae (Yan and Zheng 2013; Wang *et al*., 2015; Zhao *et al*., 2013; Zhao *et al*., 2015; Ouyang *et al*., 2015; Yan *et*

al., 2016, Xu *et al*., 2015; Posadas *et al*., 2017; Toledo-Cervantes *et al*., 2017; Rodero *et al*., 2018; Marin *et al*., 2018a; Marin *et al*., 2018b; MarianaFranco-Morgado *et al*., 2018; Cao *et al*., 2017), the combination of three processes, biogas upgrading with wastewater treatment and production of biodiesel feedstocks has been reported only by Khan *et al*. (2018). They studied the feasibility of combining microalgae cultivation for biogas upgrading and production of biodiesel feedstock using wastewater. The digestate was used as the culture medium for microalgae growth, while raw biogas is also supplied for upgrading through the removal of CO_2 and lipid production by microalga *Chlorella minutissima*. The microalgae enriched the biogas with methane up to 81.6%. The total lipid content based on dry biomass was 26%. The produced lipids have high potential for biodiesel production due to the similar fatty acid methyl ester profile as other oil bearing crops. The idea of integrating three processes of biogas upgrading, wastewater treatment and lipid production by oleaginous microalga would promote the industrialization of microalgae-based biofuels. This strategy possesses potential to significantly reduce the biodiesel production costs, remediate wastewater and upgrade biogas.

3.4 Recent concepts of biogas upgrading, phytoremediation of wastewater and production of biodiesel feedstocks

Co-cultivated microalgae-fungi systems and co-cultivated microalgae-activated sludge/bacterial systems have been proposed and their wide applications have been reported. Table 3 compares the performance of co-cultured microalgae-fungi, co-cultured microalgae-activated sludge and co-cultured microalgae-bacteria for biogas upgrading and phytoremediation of wastewater. For the co-cultured microalgae-fungi system, Cao *et al*. (2017) compared the different microalgae *Chlorella vulgaris*-fungi strains including *Pleurotus geesteranus*, *Ganoderma lucidum*, and *Pleurotus ostreatu* on the biogas upgrading and nutrient removal. They found that the nutrient and CO_2 were efficiently removed in fungi *Ganoderma lucidum*-microalgae *Chlorella vulgaris*. The CO2 removal efficiency was 75.61% and the methane content was increased up to 90% with red-blue light/dark ratio of 14 h:10 h. The removal efficiencies of COD, total nitrogen (TN) and total phosphorus (TP) were 78.09%, 86.24%, 86.74% and 75.61%, respectively. Several reports also found

that *G. lucidum* is a suitable fungal strain that could rapidly grow in swine wastewaters and also gave the high pollutants removal efficiency (Table 3). Afterwards, the obtained strains were used for co-cultivation with the microalgae. The COD, TN, TP and CO_2 were effectively removed. The CH_4 content was higher than 90% which meets the biogas standard for used as fuel. Zhang *et al*. (2017) reported that when the co-cultured microalgae and fungi were cultured in the effluent, the removal efficiency of the pollutants in biogas effluent and CO_2 in biogas were increased. In the process, they prepared the initial culture by combined microalgae with fungi pellet. Generally, the fungal-assisted flocculation could be performed by two techniques. The first technique is the co-cultivation of microalgae with fungal pellets. This technique needs two steps of pelletization of fungal cells and co-cultivation with microalgae which required at least 72 and 48 h, respectively (Wrede *et al*. 2014; Miranda *et al*. 2015; Muradov *et al*. 2015; Srinuanpan *et al*., 2018b). The second technique is the co-pelletization in which the fungi grow and simultaneously flocculate microalgal cells in their pellets. This technique could reduce the operating steps and increase the overall productivity (Srinuanpan *et al*., 2018b).

In the system of wastewater treatment, the activated sludge was generated as a byproduct which wildly used as removing contaminant due to their absorbability and high surface area. In addition, they could bind with microalgae which are called immobilized microalgae-activated sludge (Fytili and Zabaniotou, 2008). The co-cultured microalgae with activated sludge gave the high removal efficiency of pollutant in biogas effluent and CO_2 in biogas by adsorbability (Zhang *et al*., 2017). In the co-cultured microalgae-activated sludge system, the microalgae got attached to the activated sludge. In this consortia system, a symbiosis between microalgae and the indigenous bacteria from the activated sludge would occur. As the microalgae could generate oxygen through their photosynthesis, this could satisfy the oxygen requirement by the indigenous bacteria while the indigenous bacteria from the activated sludge could produce CO_2 and serve as carbon source for microalgae growth (Srinuanpan *et al*., 2018b). The SEM images of the microalgae–sludge pellets are showed in the report of Anbalagan *et al*. (2017). Sun *et al*. (2016) studied the removing CO_2 from biogas by three microalgal strains (*Chlorella vulgaris*, *Scenedesmus obliquus*, and *Neochloris oleoabundans*) mixed with activated sludge

using biogas slurry as nutrient medium. They found that activated sludge supported a superior biogas slurry treatment performance and the mixed culture containing *Scenedesmus obliquus* achieved the best combined effects. More than 75% of CO_2 was removed from biogas and the CH_4 content was increased up to 90%. The nutrient removal including COD, TN and TP was more than 50%. The performance of co-cultured microalgae-fungi system, co-cultured microalgae-activated sludge system and mono-culture microalgae system for biogas upgrading and nutrient removal in wastewater were compared in the reports of Zhang *et al*. (2017), Wang *et al*. (2017) and Guo *et al*. (2018). They found that the order of both biogas upgrading and nutrient removal is ranked the same, that is microalgae–fungi > microalgae–activated sludge > microalgae.

The potential of microalgae-bacterial was also confirmed in many reports (Table 3). They suggested that the system of microalgae-bacterial cultivation through open pond connected with external absorption column decreased the O_2 concentration below standard levels and also captured CO_2 and H_2S from biogas simultaneously. The liquid to biogas flow (L/G) ratio is one of the key operational parameters determining the biogas composition after purification on this system. They found that the optimum L/G ration was 0.5 which allowed obtaining a biogas complying with most fuel standard (0.4% CO_2, 0.03% O_2, 2.4%N_2, and 97.2% CH_4).

The production of biodiesel feedstock which is lipid from microalgae-activated sludge biomass cultured in wastewater and fed with biogas was evaluated in the reports of Serejo *et al*. (2014), Sun *et al*. (2016), Franco-Morgado *et al*. (2018) and Toledo-Cervantes *et al*. (2018). They found that the lipid content in microalgae-activated sludge was in ranged of 10-40% of their biomass. However, the lipid production in the microalgae-fungi biomass cultured in wastewater fed with biogas had no reported. In the report of Srinuanpan *et al*. (2018b) who found that the lipid content in microalga-fungi biomass cultured in secondary effluent fed with air was found to be 15.83%. The extracted lipids were mainly composed of C16–C18 (>83%) with their suitability as biodiesel feedstocks. Therefore, the novel technology including microalgae-fungi system, microalgae-activated sludge and microalgae-bacteria are promoting combining biogas upgrading, the production of biodiesel

feedstock and wastewater treatment. These strategies may contribute greatly to the industrialized microalgae based biofuel production and biorefinery concept.

3.5 Biological flocculation by microorganism

Microalgae harvesting via biological methods which used microorganism as flocculants is an alternative way to separate the microalgae biomass from liquid phase. As this method requires low energy inputs and no addition of chemicals, it is therefore suitable as a new insight for immobilization of microalgal cells. Recently, co-culturing of microalgae with filamentous fungi is considered to be plausible microalgal harvesting method (Srinuanpan *et al*., 2018c). Zhou *et al*. (2013) studies the harvesting of microalgae *C. vulgaris* by using pellet forming *Aspergillus oryzae* under heterotrophic mode. The factors including spore density, glucose concentration and pH range were optimized to achieve the maximum cell recovery and the optimal condition were 1.2×10^4 fungal spores/mL, 20 g L^{-1} glucose, and 4.0–5.0 pH. After operation, more than 63% of microalgae cells were harvested in heterotrophic mode whereas 10 g L^{-1} glucose was high enough to recover 100% autotrophically grown algal cells. Srinuanpan *et al*. (2018c) reported that the microalgae were harvested within 10 min by oleaginous fungal *Trichoderma reesei*. Using oleaginous fungal *Trichoderma reesei* pellets could not only harvest the microalgae but also enhance the lipid yields as biodiesel feedstock. Other fungi used to entrap algal cells are *Cunninghamella echinulata*, *Rhizopus oryzae*, *Isaria fumosorosea Penicillium expa*nsum and *Mucor circinelloides* (Gultom and Hu, 2013; Chen *et al*., 2018).

Generally, the fungal-assisted flocculation could be performed by two techniques. The first technique is the co-cultivation of microalgae with fungal pellets. This technique is called fungal pellet-assisted microalgae harvesting. It needs two steps of pelletization of fungal cells prior to co-cultivation with microalgae which required at least 72 h and 48 h in the first and second step, respectively (Wrede *et al.*, 2014; Miranda *et al.*, 2015; Muradov *et al.*, 2015). The second technique for fungal-assisted flocculation is the co-pelletization. With this technique which is call spore-assisted microalgae harvesting, the fungi can grow and simultaneously flocculate microalgal cells in their pellets. This technique could reduce the operating step and

increase the overall productivity. Chen *et al*. (2018) compared that the performance between fungal pellet- and spore-assisted microalgae harvesting methods for algae bioflocculation. They found that the harvesting efficiency did not different that more than 98% microalgae cells can be harvested by both methods. Fungal pellet-assisted microalgae harvesting method has a great advantage over spore-assisted microalgae harvesting method in terms of process time and mass of microalgae biomass harvested. Interestingly, acidic pH and fungi: algae ratio are a key factor in fungal pellet-assisted microalgae harvesting, but not in spore-assisted microalgae harvesting. The suitable fungi: microalgae ratio was 1:2. High temperature significantly affected the harvesting efficiency of spore-assisted microalgae by accelerating the formation of fungal pellets (Chen *et al*., 2018; Srinuanpan *et al*., 2018c). The researchers concluded that the energy consumption in fungal pellet-assisted harvesting was much less than that in spore-assisted harvesting. This was because the fungal pellet-assisted harvesting was conducted in the dark and the harvest time was much shorter than that required in spore-assisted harvesting. Muradov *et al*. (2015) and Srinuanpan *et al*. (2018c) indicated that the fungi may potentially neutralize the negative charges on the microalgal surface and enable the attachment of microalgal cells to the fungal cell wall. Another possibility would be because the fungal cells can secrete exopolysaccharides during interaction with other microorganisms and the microalgal cells may get attached with the sticky exopolysaccharides secreted by the fungi (Selbmann *et al*., 2003; Miranda *et al*. 2015; Srinuanpan *et al*., 2018c).

Recently, the innovative biological flocculations of microalgae using flocculant from bacteria and also the bacterial cells have been proposed (Ndikubwimana *et al*., 2016). The flocculation efficiency of marine microalgae *Chlorella vulgaris* and freshwater microalgae *C. protothecoides* using commercial γ-PGA bioflocculant produced by *Bacillus subtilis* was as high as 95% (Zheng *et al*., 2012). Several novel flocculant-producing bacteria isolated from various wastewaters have been reported and optimized for their bioflocculant production with high yields (Peng *et al*., 2014; Liu *et al*., 2015; Guo *et al*., 2018). To reduce the production cost, various industrial wastes such as phenol-containing wastewater, swine wastewater, palm oil mill effluent etc., have been used as low-cost media for the bacterial growth and bioflocculant production (Peng *et al*., 2014;

Chaisorn *et al*., 2016; Chen *et al*., 2016). Most of them contained enough nutrition for bacterial growth but their imbalanced nutrients are likely to limit the bacteria growth and bioflocculant production (Adebami and Adebayo-Tayo, 2013, Ramsden *et al*., 2010; Guo *et al*., 2018).

Interestingly, co-cultivation of microalgae with flocculant-producing bacteria could reduce the time for bacteria cultivation. Both microorganisms can produce exopolysaccharides that are indistinguishable from each other. Furthermore, these polymers may be involved in cell-to-cell interaction without any negative response such as cell lysis or cell stress (Lee *et al*., 2009; Ummylama *et al*., 2017). Ummylama *et al*. (2017) reported that some bacteria strains from the genera *Flavobacterium*, *Terrimonas* and *Sphingobacterium* could effectively use for combining bacteria with microalgae to harvest the microalgae *C. vulgaris*. Ji *et al*. (2018) reported that the bacteria cells almost were get attached with the surface of microalgae cells of *C. vulgaris* as a result of co-cultivation of microalgae *C. vulgaris* with *Bacillus licheniformis* at ratio of 1:3 in synthetic wastewater. Adjusting pH (base addition), the adhesion of *Bacillus licheniformis* to *Chlorella vulgaris* surface in synthetic wastewater was strengthened and the mass and energy exchanges between algae and bacteria were also strengthened (Liang *et al*., 2015). Also, adding of bacterial broth to the microalgal culture showed great flocculation efficiency of 83% within 24 h, indicating that both bacterial cells and bacterial extracellular metabolites play an important role in the process of flocculation (Liang *et al*., 2015; Lee *et al*., 2013).

Cellulose producing bacteria is one of bacteria which could use for microalgae harvesting. Co-cultivation of microalgae with cellulose producing bacteria did not only harvest the microalgae biomass but also obtain the living cellulose composite. Das *et al*. (2016) studies the possibility of microalgae harvesting by co-culture with bacterial cellulose and the possible symbiotic relationships between two microorganisms. They demonstrate the microalgae *Chlamydomonas reinhardtii* cells could grow and also was immobilized in the bacterial cellulose produced by acetobacter *Acetobacter aceti*. Both microorganism integrate into a symbiotic consortium and get embedded in the produced cellulose composite. During co-cultivation, the acetobacter cells could use sugars as carbon source to produce cellulose and acetic acid as major

byproducts. The produced cellulose was concurrently assimilated by the microalgae and the produced oxygen by the microalgae was used by the bacteria in their environment. It should be concluded that the acetobacter could not only produce cellulose at the air–water interface but also around the photosynthetic microalgae which get integrated in the cellulose gel. The proposed novel approach for immobilizing and harvesting the microalgae using the symbiosis with acetobacter is not only economical but also environmental friendly without the use of synthetic binding agents and in turn increase their production efficiency.

Moreover, co-cultivation of microalgae with activated sludge which called activated microalgae granules or activated microalgae floc shows an alternative system of microalgae-bacteria cultivation. This strategy is presented to save the cost of microalgae harvesting processes. Tiron *et al*. (2017) used the activated sludge granule to harvest the microalgae in aerobic wastewater by co-cultivation of *Chlorella* with activated sludge. They found that more than 99% of microalgae cell was harvested only by fast sedimentation of the granules. These strategies could not only harvest the microalgae biomass but also phytoremediation of wastewater (Wagner *et al*., 2016). These results suggest a potential application of co-culture system for effective nutrient removal from wastewater and simultaneous microalgae harvesting. This performance highlighted the viability of the granular activated microalgae system for sustaining a microalgae harvesting procedure with neither cost nor energy inputs.

Table 1 Performance of using microalgae for biogas upgrading and production of lipid as a biodiesel feedstock

Microalgae strain	Biogas composition	CH_4 content after removing CO_2 (%v/v)	Lipid production	References
Mutant strain of *Chlorella* sp.	Desulfurized biogas containing 70 % CH_4, 20% CO_2 and 8% N_2	84-87	-*	Kao *et al*. (2012a)
Mutant strain of *Chlorella* sp.	Desulfurized biogas containing 69% CH_4, 20% CO_2 and <50 ppm H_2S	85-90	-	Kao *et al*. (2012b)
Chlorella sp.	Crude biogas containing 70.65% CH_4, 26.14% CO_2, 0.23% O_2, 3.11% H_2O and <0.005% H_2S	92.16	-	Yan and Zheng (2013)
Chlorella sp.	Crude biogas containing 67.35% CH_4, 28.41% CO_2, 0.73% O_2, 3.48% H_2O and <0.005% H_2S	92.74	-	Zhao *et al*. (2013)
Chlorella sp.	Synthetic biogas containing 50% CH_4 and 50%CO_2	94.7	94.7 mg/L/day[b]	Tongprawhan *et al*. (2014)
Chlorella sp.	Crude biogas containing 61.38% CH_4, 32.57% CO_2, 0.54% O_2, 5.52% H_2O and <0.005% H_2S	84.21	-	Wang *et al*. (2015)

Table 1 continued

Microalgae strain	Biogas composition	CH_4 content after removing CO_2 (%v/v)	Lipid production	References
Leptolyngbya sp.	Synthetic biogas containing 25% CO_2 and 75% CH_4	99.56	-	Choix *et al*. (2017a)
Scenedesmus obliquus	Crude biogas containing 61.75% CH_4, 32.28% CO_2, 0.31% O_2, 2.68% H_2O and <0.005% H_2S	94.41	-	Ouyang *et al*. (2015)
Scenedesmus obliquus	Crude biogas containing 61.38% CH_4, 32.57% CO_2, 0.54% O_2, 5.52% H_2O and <0.005% H_2S	84.28	-	Wang *et al*. (2015)
Scenedesmus obliquus	Synthetic biogas containing 25% CO_2 and 75% CH_4	96.5	40%[a]	Choix *et al*. (2017b)
Scenedesmus sp.	Synthetic biogas containing 40% CO_2 and 60% CH_4	>90	1.65 g/L with 43.77%[a]	Srinuanpan *et al*. (2017)
Scenedesmus sp.	Synthetic biogas containing 40% CO_2 and 60% CH_4	>98	1.50 g/L with 34.1%[a]	Srinuanpan *et a*l. (2018a)
Nannochloropsis gaditana	Synthetic biogas containing 28% CO_2 and 72% CH_4	98.6	-	Meier *et al*. (2015)

Table 1 continued

Microalgae strain	Biogas composition	CH_4 content after removing CO_2 (%v/v)	Lipid production	References
Selenastrum capricornutum	Crude biogas containing 61.38% CH_4, 32.57% CO_2, 0.54% O_2, 5.52% H_2O and <0.005% H_2S	78	-	Wang *et al*.(2015)
Selenastrum bibraianum	Crude biogas containing 61.75% CH_4, 32.28% CO_2, 0.31% O_2, 2.68% H_2O and <0.005% H_2S	79	-	Ouyang *et al*. (2015)
Anabaena spiroides	Crude biogas containing 61.38% CH_4, 32.57% CO_2, 0.54% O_2, 5.52% H_2O and <0.005% H_2S	81	-	Wang *et al*. (2015)

[*] Not available

[a] Lipid productivity (mg/g/gay)

[b] Lipid content (%w/w of dry biomass)

Table 2 Performance of microalgae cultured in wastewater for biogas upgrading and production of biodiesel feedstock

Microalgae strain	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Chlorella sp.	Crude biogas containing 67.35% CH_4, 28.41% CO_2, 0.73% O_2, 3.48% H_2O and <0.005% H_2S	Biogas effluent - pH =6.77 - COD =986.05 mg/L - TN =357.41 mg/L - TP =37.24 mg/L	92.16	88.74	83.94	80.43	-*	Yan and Zheng (2013)
Chlorella sp.	Crude biogas containing 61.38% CH_4, 32.57% CO_2, 0.54% O_2, 5.52% H_2O and <0.005% H_2S	Biogas effluent - pH =6.99 - COD =789.13 mg/L - TN =189.15 mg/L - TP =15.53 mg/L	84.21	64.76	55.67	53.84	-	Wang *et al*. (2015)

Table 2 continued

Microalgae strain	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Chlorella sp.	Crude biogas containing 67.35% CH_4, 28.41% CO_2, 0.73% O_2, 3.48% H_2O and <0.005% H_2S	Biogas effluent - pH =6.50 - COD =1,203.61 mg/L - TN =492.02 mg/L - TP =53.28 mg/L	92.74	85.35	77.98	73.03	-	Zhao *et al*. (2013)
Chlorella vulgaris	Crude biogas containing 67.32% CH_4, 34.45% CO_2, 0.62% O_2, 3.66% H_2O and <0.005% H_2S	Biogas effluent - pH =6.84 - COD =1,013.87 mg/L - TN =308.75 mg/L - TP =9.93 mg/L	80.04	59.27	51.32	63.22	-	Zhao *et al*. (2015)

Table 2 continued

Microalgae strain	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Scenedesmus obliquus	Crude biogas containing 67.32% CH_4, 34.45% CO_2, 0.62% O_2, 3.66% H_2O and <0.005% H_2S	Biogas effluent - pH =6.84 - COD =1,013.87 mg/L - TN =308.75 mg/L - TP =9.93 mg/L	82.64	63.12	53.04	59.14	-	Zhao *et al*. (2015)
Nannochloropsis oleoabundans	Crude biogas containing 67.32% CH_4, 34.45% CO_2, 0.62% O_2, 3.66% H_2O and <0.005% H_2S	Biogas effluent - pH =6.84 - COD =1,013.87 mg/L - TN =308.75 mg/L - TP =9.93 mg/L	80.06	61.03	51.74	54.33	-	Zhao *et al*. (2015)

Table 2 continued

Microalgae strain	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Scenedesmus obliquus	Crude biogas containing 61.75% CH_4, 32.28% CO_2, 0.31% O_2, 2.68% H_2O and <0.005% H_2S	Biogas effluent - pH =7.14 - COD =1,294.32 mg/L - TN =419.49 mg/L - TP =14.84 mg/L	94.41	93.03	84.12	86.76	-	Ouyang *et al*. (2015)
Chlorella pyrenoidosa	Crude biogas containing 63.84% CH_4, 31.02% CO_2, 0.56% O_2, 3.59% H_2O and <0.005% H_2S	Swine biogas effluent - pH =6.79 - COD =962.41 mg/L - TN =360.28 mg/L - TP =31.46 mg/L	92.87	92.67	80.87	79.33	-	Yan *et al*. (2016)

Table 2 continued

Microalgae strain	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Scenedesmus obliquus	Crude biogas containing 58.67% CH_4, 37.54% CO_2, 0.79% O_2, 3.01% H_2O and <0.005% H_2S	Diluted swine biogas effluent - pH =6.43 - COD =1600 mg/L - TN =60 mg/L - TP =65 mg/L	88.25	75.29	62.54	88.79	-	Xu *et al*. (2015)
Chlorella sp.	Synthetic biogas containing 70% CH_4, 29.5% CO_2 and 0.5% H_2S	Real wastewaters - pH =6.43 - COD =592 mg/L - TN =580 mg/L - TP =34 mg/L	94	70	80	85	-	Posadas *et al*. (2017)

Table 2 continued

Microalgae strain	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Chlorella minutissima	Synthetic biogas containing 70% CH_4, 29.5% CO_2 and 0.5% H_2S	Biogas effluent - COD =1,745 mg/L - TN =1815 mg/L - TP =48 mg/L	96	85	85	100	-	Toledo-Cervantes *et al*. (2017)
Chlorella vulgaris	Synthetic biogas containing 70% CH_4, 29.5% CO_2 and 0.5% H_2S	Vinasse Wastewater - pH =7.84 - COD =306 mg/L - TN =71 mg/L - TP =3.3 mg/L	98.9	51	37	86	<10%	Serejo *et al*. (2015)

Table 2 continued

Microalgae strain	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Scenedesmus spp.	Raw swine biogas containing 70.7% CH_4, 26.1% CO_2, 0.23% O_2, H2S ≅1550 ppm	Swine biogas effluent - pH =7.9 - N-NH_3 =120 mg/L - P-PO_4^{3-} =90 mg/L	70	-	83	78	-	Prandini *et al*. (2016)
Chlorella minutissima	Biogas containing 63.53% CH_4 and 36.8% CO_2	Real wastewaters (the nutrient concentration did not show)	86.4	-	-	-	26%	Khan *et al*. (2018)

*Not available

Table 3 A novel technology of microalgae cultivation in wastewater for biogas upgrading and production of biodiesel feedstock

Co-culture technology	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Co-cultivation of microalgae with fungi								
Microalgae *Chlorella vulgaris* – fungi *Ganoderma lucidum*	Crude biogas containing 64.58% CH_4, 31.72% CO_2, 0.54% O_2, 3.15% H_2O and <0.005% H_2S	Biogas effluent - pH =6.95 - COD =1,213 mg/L - TN =239 mg/L - TP =13 mg/L	90	74	76	77	-*	Zhang *et al*. (2017)
Microalgae *Chlorella vulgaris* – fungi *Ganoderma lucidum*	Synthetic biogas containing 62.38% CH_4 and 31.19% CO_2	Biogas effluent - pH =6.97 - COD =1,496 mg/L - TN =278 mg/L - TP =28 mg/L	92	70	76	78	-	Cao *et al*. (2017)

Table 3 continued

Co-culture technology	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Co-cultivation of microalgae with fungi								
Microalgae *Chlorella vulgaris* – fungi *Ganoderma lucidum*	Synthetic biogas containing 64.92% CH_4 and 35.08% CO_2	Biogas effluent - pH =7.15 - COD =1,041 mg/L - TN =288 mg/L - TP =12 mg/L	93	86	86	86	-	Wang *et al*. (2017)
Microalgae *Chlorella vulgaris* – fungi *Ganoderma lucidum*	Synthetic biogas containing 48.87% CH_4 and 55.13% CO_2	Biogas effluent - pH =6.91 - COD =998 mg/L - TN =202 mg/L - TP =21 mg/L	92	68	62	64	-	Zhou *et al*. (2018)

Table 3 continued

Co-culture technology	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Co-cultivation of microalgae with fungi								
Microalgae *Chlorella vulgaris* – fungi *Ganoderma lucidum*	Crude biogas containing 64.59% CH_4, 33.79% CO_2, 0.38% O_2, 1.23% H_2O and <0.01% H_2S	Biogas effluent - pH =6.97 - COD =1,496 mg/L - TN =278 mg/L - TP =28 mg/L	86	83	81	84	-	Guo *et al*. (2018)
Co-cultivation of microalgae with activated sludge/bacteria								
Microalgae *Scenedesmus obliquus* – activated sludge	Synthetic biogas containing 54.5% CH_4, 45% CO_2 and 0.5% H_2S	Biogas effluent - pH =7.43 - COD =999 mg/L - TN =257 mg/L - TP =14 mg/L	93	65	63	74	≈10%	Sun *et al*. (2016)

Table 3 continued

Co-culture technology	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Co-cultivation of microalgae with activated sludge/bacteria								
Microalgae *Chlorella vulgaris* – activated sludge	Crude biogas containing 64.58% CH_4, 31.72% CO_2, 0.54% O_2, 3.15% H_2O and <0.005% H_2S	Biogas effluent - pH =6.95 - COD =1,213 mg/L - TN =239 mg/L - TP =13 mg/L	93	72	75	74	-	Zhang *et al*. (2017)
Microalgae *Chlorella vulgaris* – activated sludge	Synthetic biogas containing 64.92% CH_4 and 35.08% CO_2	Biogas effluent - pH =7.15 - COD =1,041 mg/L - TN =288 mg/L - TP =12 mg/L	88	75	74	74	-	Wang *et al*. (2017)

Table 3 continued

Co-culture technology	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Co-cultivation of microalgae with activated sludge/bacteria								
Microalgae *Scenedesmus* – activated sludge	Synthetic biogas containing 70% CH_4, 29.5% CO_2 and 0.5% H_2S	Domestic wastewater - pH =7.15 - TOC =176 mg/L - TN =106 mg/L - TP =33 mg/L	95	90	81	64	-	Garcia *et al*. (2017)
Microalgae *Chlorella vulgaris* – activated sludge	Crude biogas containing 64.59% CH_4, 33.79% CO_2, 0.38% O_2, 1.23% H_2O and <0.01% H_2S	Biogas effluent - pH =6.97 - COD =1,496 mg/L - TN =278 mg/L - TP =28 mg/L	83	81	83	83	-	Guo *et al*. (2018)

Table 3 continued

Co-culture technology	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Co-cultivation of microalgae with activated sludge/bacteria								
Microalgae *Picochlorum sp. and Halospirulina sp.* – bacteria	Synthetic biogas containing 69.5% CH_4, 30% CO_2 and 0.5% H_2S	Mineral salt medium - pH =9.3	>96	-	52-55	12-29	-	Morgado *et al.* (2018)
Unknow microalgae species – bacteria	Synthetic biogas containing 70% CH_4, 29.5% CO_2 and 0.5% H_2S	Domestic wastewater - pH =7.15 - IC =1500 mg/L - TN =1719 mg/L	76-99	-	-	-	-	Rodero *et al.* (2018)
Unknow microalgae species – bacteria	Synthetic biogas containing 70% CH_4, 29.5% CO_2 and 0.5% H_2S	Domestic wastewater - pH =9 - IC =1663 mg/L - TN =336 mg/L -TP =25 mg/L	97.9	-	-	-	-	Marin *et al.* (2018a)

Table 3 continued

Co-culture technology	Biogas composition	Nutrient concentration	CH_4 content after removing CO_2 (%v/v)	Nutrient removal efficiency			Lipid production	References
				COD removal (%)	TN removal (%)	TP removal (%)		
Co-cultivation of microalgae with activated sludge/bacteria								
Unknow microalgae species – bacteria	Synthetic biogas containing 70% CH_4, 29.5% CO_2 and 0.5% H_2S	Domestic wastewater - pH =9 - TOC =16-523 mg/L - IC =450-600 mg/L - TN =374-718 mg/L -TP =26-135 mg/L	99.6	-	-	-	-	Marin *et al.* (2018b)

* Not available

4. Results and Discussions

4.1 Strategies to improve methane content in biogas by cultivation of oleaginous microalgae and the evaluation of fuel properties of the microalgal lipids (Paper I)

This study aimed to evaluate various species of oleaginous microalgae for CO_2 removal from biogas and simultaneously producing lipid. The growth and lipid production of five oleaginous microalgae including marine *Chlorella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., *Botryococcus* sp. and freshwater *Chlorella* sp. were studied in the modified Chu13 medium under condition aerated with 40% CO_2 in air (imitating CO_2 content in biogas). Among the five strains tested, marine *Chlorella* sp. and *Scenedesmus* sp. grew fastest with the maximum specific growth rates of 0.34 day^{-1} and 0.33 day^{-1}, respectively, followed by freshwater *Chlorella* sp. and *Nannochloropsis* sp. The lipid content of these four strains were in the range of 17-28%. Although *Botryococcus* sp. accumulated lipid as high as 42.06% of its dry biomass, it had a long lag time for adaptation with 40% CO_2, resulting in a low growth rate and reached a low lipid productivity. The increase in initial cell concentration may increase the tolerance against high levels of CO_2 and reduce the time for adaptation (Lee *et al*., 2002). It has been reported that the lipid content of *Chlorella* sp. and *Scenedesmus* sp. could be increased up to >55% under optimized conditions (Liu *et al*., 2008; Mata *et al*., 2010).

When comparing the biomass obtained, marine *Chlorella* sp. gave the highest biomass of 0.87 g L^{-1} followed by *Scenedesmus* sp. (0.81 g L^{-1}) while those of other strains were in the range of 0.3-0.6 g L^{-1}. The maximum biomass concentrations of marine *Chlorella* sp. and *Scenedesmus* sp. were higher than those previously reported marine *Chlorella* sp. (0.6 g L^{-1}) and *Scenedesmus* sp. (0.4 g L^{-1}) (*Tongprawhan* et al., 2014) while were comparable to those of *Scenedesmus obliquus* (0.82 g L^{-1}) (Tang *et al*., 2010). The lipid productivity of marine *Chlorella* sp. and *Scenedesmus* sp. (23-26 mg L^{-1} day^{-1}) were higher than those of other three strains. Yoo *et al*. (2010) have compared the cultivation of three microalgae including *Botryococcus braunii*, *Scenedesmus* sp. and *Chlorella vulgaris* under condition aerated with 10% CO_2 at 0.3 air volume per medium volume per min (vvm). They

found that *Scenedesmus* sp. gave the highest lipid productivity of 20.65 mg L^{-1} day^{-1} followed by *C. vulgaris* (6.91 mg L^{-1} day^{-1}). Several reports also confirmed that these two species were able to grow under high CO_2 content of 10-50% (Tang *et al*., 2011; Morais and Costa, 2007).

The tolerance to high CO_2 content and high CH_4 content in biogas are great important characteristics of oleaginous microalgae for being used in CO_2 removal from biogas. As marine *Chlorella* sp. and *Scenedesmus* sp. gave comparative high growth rate and high lipid productivity under condition aerated with 40% CO_2 in air, they were selected for the experiments using synthetic biogas. The synthetic biogas was prepared by mixing with CH_4 at a ratio of 40:60 v/v. It was found that the biomass of marine *Chlorella* sp. was slightly higher than that of *Scenedesmus* sp. when using biogas. But their lipid productivities were not significantly different. It should be noted that the performance of marine *Chlorella* sp. and *Scenedesmus* sp. using biogas were similar to those using 40% CO_2 in air. These results indicated that the CH_4 content in biogas at 60% had no negative effect on the performance of these two microalgae. Several researchers have also attempted to use microalgae for CO_2 removal from biogas (Meier *et al*., 2015; Yan *et al*., 2014). Only Tongprawhan *et al*. (2014) attempted to use oleaginous *Chlorella* spp. for this purpose. However, the lipid production in their studies (94.7 mg L^{-1}) was much lower than the lipid productions by marine *Chlorella* sp. and *Scenedesmus* sp. in this study (300-320 mg L^{-1}). During 7 days of cultivation, CH_4 content in the outlet biogas increased higher than 98%. The percent CO_2 removal were 95% and 98% for marine *Chlorella* sp. and *Scenedesmus* sp., respectively. The percent CO_2 removal in this study are comparable to the results of Yan and Zheng (2013) and Yan *et al*. (2014) who used *Chlorella* sp. for biologically removing CO_2 from biogas. Comparing between two strains, the CO_2 fixation rate by *Scenedesmus* sp. (2.55 g CO_2 day^{-1} L^{-1}) were slightly higher than those by marine *Chlorella* sp. (2.47 g CO_2 day^{-1} L^{-1}). Thus, *Scenedesmus* sp. was selected as the most suitable strain for CO_2 removal from biogas coupled with lipid production.

Response surface methodology was used to optimize operating conditions for CO_2 removal from biogas and lipid production by the CO_2 removal from biogas and lipid production by the selected *Scenedesmus* sp. The independent

variables for the process were gas flow rate (L h^{-1} per 1 L-microalgal culture), initial cell concentration (log cells mL^{-1}), KNO_3 concentration (g L^{-1}) and light intensity (klux). The experimental results were concerned with biomass (g L^{-1}), lipid productivity (mg L^{-1} day^{-1}), percent CO_2 removal from biogas (%) and CO_2 fixation rate (g-CO_2 day^{-1} L^{-1}) using the four-factor BBD experimental design. The conditions at the center point were a gas flow rate of 0.3 L h^{-1} L^{-1}, an initial cell concentration of 10^6 cells mL^{-1}, KNO_3 concentration of 0.5 g L^{-1} and a light intensity of 4.5 klux.

The optimum conditions for maximizing biomass were gas flow rate of 0.317 L h^{-1} per 1 L-microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.75 g L^{-1}, and light intensity of 5.09 klux. The maximum calculated value for microalgal biomass was 3.37 g L^{-1}. The initial cell concentration is a fundamental parameter that controlled the growth rate and fermentation time. The higher initial cell concentration would give a faster growth rate and hence higher biomass production (Tongprawha *et al*., 2014). Another major factor that affects the biomass is nitrogen source and its concentration. Arumugam *et al*. (2013) reported that KNO_3 performed better than $NaNO_3$, Urea, $CaNO_3$, NH_4NO_3 and NH_4Cl for biomass production of *Scenedesmus*. This was because nitrogen and potassium in KNO_3 are the two important nutrients for algal growth. In addition, they also suggested that the nitrogen concentration beyond 10 mM or 1.01 g L^{-1} exhibited poor microalgal biomass production, which may be due to the deleterious effect of nitrogen at high concentrations. The optimum nitrogen concentration for the growth of *Scenedesmus* in their study was 5-10 mM (0.5-1.0 g L^{-1}).

The lipid productivity significantly depended on the initial cell concentration. When using low initial cell concentration the lipid productivity was limited, while using high initial cell concentration the lipid productivity was as high as 85.30-98.00 mg L^{-1} day^{-1}. In addition, the optimum conditions for maximizing lipid productivity were: slightly high gas flow rate of 0.413 L h^{-1} per 1 L microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.61 g L^{-1}, and light intensity of 5.43 klux. The maximum response value for lipid productivity was estimated to be 100.61 mg L^{-1} day^{-1}. Compared with the optimal conditions for biomass, higher gas flow rate with lower KNO_3, namely higher carbon to nitrogen ratio were more suitable for lipid production. Nitrogen deficiency severely affects

protein synthesis and reduces photosynthetic rates which result in metabolic flux towards lipid biosynthesis (Sing *et al*., 2015). Yeh and Chang (2011) reported that the nitrogen limitation is the key factor influencing the lipid accumulation of *Chlorella vulgaris*. Low nitrogen concentration could increase the lipid content of the microalgae. However, there is a tradeoff between biomass and lipid content. The microalgae could not grow well under nitrogen limitation and resulted in low biomass. Therefore to increase overall lipid productivity along with nitrogen stress, a two-step cultivation in which the condition for cell growth (low carbon to nitrogen ratio) is applied in the first step and the nitrogen stress (high carbon to nitrogen ratio) is applied in the second step, should be considered (Sing *et al*., 2015; Yeh and Chang, 2011; Praveenkumar *et al*., 2012).

The optimum conditions for maximizing the percent CO_2 removal were gas flow rate of 0.15 L h^{-1} per 1 L microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.62 g L^{-1}, and light intensity of 5.28 klux. The maximum response value for percent CO_2 removal was estimated to be 100%. The response surface of percent CO_2 removal indicated that percent CO_2 removal increased with decreasing gas flow rate and increasing KNO_3 concentration. As the gas flow rate increases, the size of gas bubble also increases and this reduces its retention time in the liquid and also its specific surface area per gas volume. These could then be the main reasons for the decrease in percent CO_2 removal at high gas flow rate.

It should be noted that the gas flow rate also affects the rate of CO_2 fixation. The CO_2 fixation rate is defined as the gram CO_2 removed per day per 1 L-microalgal culture. The CO_2 fixation rate increased with increasing gas flow rate. The optimum conditions for maximizing CO_2 fixation rate were: relatively high gas flow rate of 0.45 L h^{-1} per 1 L-microalgal culture, initial cell concentration of $10^{6.07}$ cells mL^{-1}, KNO_3 concentration of 0.80 g L^{-1}, and high light intensity of 4.98 klux. The estimated maximum CO_2 fixation rate was as high as 5.54 g-CO_2 day^{-1} L^{-1}. However, with these conditions the biomass and lipid productivity were only 1.70 g L^{-1} and 69.39 mg L^{-1} day^{-1}, respectively. The percent CO_2 removal was 71.29%.

The numerical optimization of culture conditions was carried out using Design-Expert statistical software based on the initial experimental results. When the

target was set to maximize both percent CO_2 removal and lipid productivity, the compromised optimal conditions were: relatively low gas flow rate of 0.15 L h^{-1} per 1 L-microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.65 g L^{-1}, and light intensity of 5.28 klux. These conditions gave the maximum CO_2 removal of 99.33% and the maximum lipid productivity of 96.18 mg L^{-1} day^{-1}. The final microalgal biomass was 2.80 g L^{-1}. The methane content in the biogas under these conditions was increased from 60% up to 99.73%. However, these conditions gave relatively low CO_2 fixation rate of 2.59 g-CO_2 day^{-1} L^{-1}. The second target was then set to maximize CO_2 fixation rate and lipid productivity. The obtained optimal conditions were different. They were: higher gas flow rate of 0.3 L h^{-1} L^{-1}, higher KNO_3 concentration of 0.8 g L^{-1}, and slightly higher light intensity of 5.5 klux but with the same initial cell concentration of 10^7 cells mL^{-1}. With these conditions, the high CO_2 fixation rate (4.013 g-CO_2 day^{-1} L^{-1}) and acceptable methane content (90.73%) could be obtained. It should be noted that this methane content meets the standard (>90%) for efficient combustion with an increase in calorific value, decrease in relative density and increase in the Wobbe index. The adequacy of the model in predicting the response efficiently was verified by comparing the observed and predicted value of the responses. The results of the measured responses were in close agreement with predicates value, and the deviation was found to be insignificant ($P > 0.05$). The final microalgal biomass and lipid productivity were 3.12 g L^{-1} and 88.57 mg L^{-1} day^{-1}, respectively.

Increasing gas flow rate can increase the amount of CO_2 which is the inorganic carbon source for the microalgae and this can lead to a higher biomass productivity. However, if the CO_2 supply is much higher than the CO_2 consumption by microalgae, the culture pH will drop rapidly. This acidic pH would then have an adverse effect upon the solubility and availability of nutrients, enzyme activity, and transport of substrate across plasma membrane and electron transport in respiration and photosynthesis (Tang *et al*., 2011). Olaizola *et al*. (2004) reported that the efficiency of CO_2 capture by microalgae is directly dependent on gas flow rate. As the percent of CO_2 removal is important for purifying and CO_2 removal rate is important for the productivity of the process, the strategy to improve both targets should be performed. The stepwise-increasing of gas flow rate is one candidate for enhancing

both percent CO_2 removal and CO_2 removal rate. At initial, the gas flow rate should be provided as a low rate which is suitable for low amount of microalgal cells. As the microalgae grew, the gas flow rate should be increased to supply more CO_2. Followed this strategy, the gas flow rate was stepwise-increased by 0.03 L h^{-1} per 1 L-microalgal culture every 2 days (Stepwise GFR). The results were compared with the control that using a constant rate of 0.3 L h^{-1} per 1 L-microalgal culture. The optimum conditions from RSM were used in this experiment. The initial cell concentration was 10^7 microalgal cells mL^{-1}. The culture was added with KNO_3 0.8 g L^{-1} as nitrogen source and illuminated at light intensity of 5.5 klux. With the Stepwise GFR, the final microalgal biomass and lipid productivity were increased by 1.25 and 1.79 folds, respectively. These results showed that increasing gas flow rate during cultivation is the effective strategy to increase the performance of the microalgae. The Stepwise GFR also showed higher lipid production (1.65 g L^{-1}) and higher lipid content (42.51%) than those of the control (0.98 g L^{-1} and 34.15%, respectively). These results were consistent with the results studies by Widjaja *et al*. (2009) who reported the higher CO_2 flow rate used the higher lipid content obtained. The CO_2 removal rate by Stepwise GFR was increased up to 5.10 g-CO_2 day^{-1} L^{-1} with the acceptable percent CO_2 removal (>75%). After cultivation with the Stepwise GFR, the CH_4 content in the biogas increased from 60% up to 90.15%. Yan *et al*. (2014) reported that the CH_4 content in biogas more than 90% (or CO_2 removal >75%) could provide high efficiency combustion. It should be also noted that the culture pH was in the range of 6.8-7.5 which had no significant effect on the CO_2 removal rate.

The lipids extracted from oleaginous *Scenedesmus* sp. cultivated under the optimal conditions for both CO_2 removal efficiency and lipid production, were converted to fatty acid methyl ester (FAME). Under both optimal conditions for percent CO_2 removal and CO_2 fixation rate, the major fatty acid of microalgal lipid were C16-C18 including palmitic acid, C16:0; heptadecanoic acid, C17:0; stearic acid, C18:0; oleaic acid, C18:1; linoleic acid, C18:2 and linolenic acid, C18:3. The content of unsaturated fatty acids of the microalgal lipid in this study was >52. It seems that an increase in light intensity, KNO_3 concentration and gas flow rate did increase the amount of total unsaturated fatty acids, especially oleaic acid (C18:1) and linolenic acid (C18:3). While low gas flow rate was good for accumulation of

saturated fatty acids including palmitic acid (C16:0) and short chain fatty acids (C14:0, C15:0).

The biodiesel fuel properties namely, iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP) were calculated based on the fatty acid compositions. Under optimum condition for percent CO_2 removal, the IV was found to be 111.18 g I_2/100 g oil which meets the European standard (EN-14214) (<120 g I_2/100 g oil). The SV of biodiesel from both optimum condition were 207.24 and 205.95 mg KOH/g oil respectively. Under optimum conditions for percent CO_2 removal, the CN was 47.62. The minimum value of CN as per the international biofuel standards of EN-14214 and ASTM D675140 are 47. The DU of biodiesel from both optimum conditions were in the range of 101-105. The LCSF and CFPP were in the range of 6-8 and 2-8 °C, respectively. These results suggested that microalgal lipid produced under optimal conditions for percent CO_2 removal showed better fuel properties than those produced under optimum conditions for CO_2 fixation rate due to higher CN and lower SV.

The fatty acid compositions of lipid obtained from Stepwise GFR were determined. The fatty acids profiles are composed mostly of palmitic acid (C16:0), heptadecanoic acid (C17:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). More than 95% of fatty acids were covered with C16-C18 which is suitable for biodiesel production. Given the fatty acid profiles and its impact on the quality of produced biodiesel, it has been reported that palmitic acid and stearic acid are most favorable for biodiesel production (Talebi *et al*., 2013).The biodiesel fuel properties were calculated based on the fatty acid composition. The IV, SV, CN, DU, LCSF and CFPP were found to be 115.81 g I_2/100 g oil, 206.51 mg KOH/g oil, 46.96, 96.43, 6.84 °C, and 5.03 °C, respectively. It could be concluded that the fuel properties of microalgal lipid are in accordance with the international standards (EN-14214 and ASTM D675140).

From this paper, it could be concluded that i) CO_2 in biogas could be efficiently removed by oleaginous microalgae, ii) after process optimization, methane content in biogas could be increased >90%, iii) stepwise-increased gas flow rate enhanced CO_2 removal rate and lipid productivity, and iv) microalgal lipids-derived

biodiesel showed good fuel properties. The conclusion diagram for this paper is shown in Figure 2.

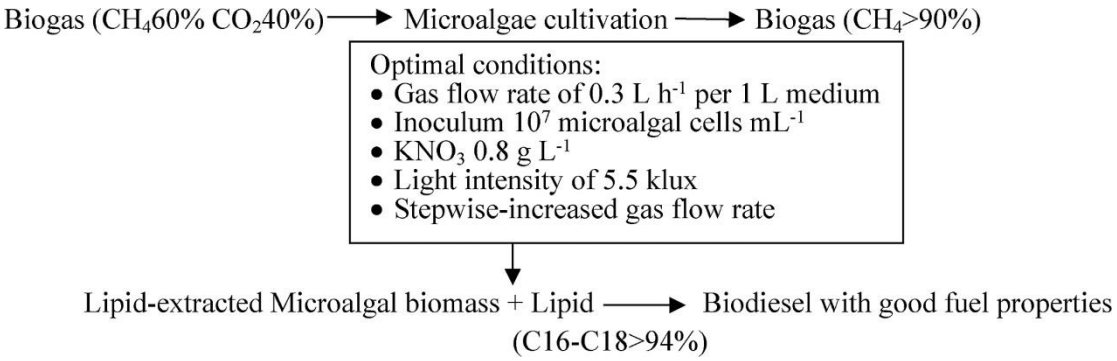


Figure 2 Conclusion diagram for Paper I.

4.2 Effective biogas upgrading and production of biodiesel feedstocks by strategic cultivation of oleaginous microalgae (Paper II)

In this study, oleaginous green microalga *Scenedesmus* sp. was used as potential microalgae to biologically remove CO_2 from biogas and simultaneously produce lipids. The biogas upgrading and lipid production by this microalga were enhanced by strategic cultivation. From the previous section, the optimum conditions for cultivation of oleaginous *Scenedesmus* sp. using synthetic biogas (CO_2:CH_4 40:60) were obtained. They were: light intensity at 5.5 klux, initial KNO_3 concentration at 0.8 g L^{-1}, biogas flow rate at 0.3 L h^{-1} per 1 L microalgae culture and initial cell concentration of 10^7 cells mL^{-1}. Several studies have reported that the supplementation of high levels of growth factors at initial negatively affected the performance of the microalgae (Srinuanpan *et al*., 2017; Yan and Zheng, 2013; Jeong *et al*., 2013; Li *et al*., 2008; Arumugam *et al*., 2013; Kao *et al*., 2012). Especially, light intensity is considered as the most important factor that affects both photosynthesis activity and the key enzymes involving in lipid biosynthesis process (Lv *et al*., 2010). The use of high light intensity at initial may induce the photoinhibition and negatively affect the cell growth and lipid production (Cheirsilp and Torpee, 2012; Thawechai *et al*., 2016; Srinuanpan *et al*., 2017). However, the more microalgae grow the higher levels of growth factors are needed. Therefore, these growth factors should be efficiently provided during the growth of microalgae. In this study, three important growth factors including light intensity, nitrogen source and

CO_2 supply were individually and simultaneously stepwise-increased in order to support the increased microalgae cells and their performance during cultivation.

The stepwise-increasing of light intensity was performed by increasing the light intensity at a step of 0.5 klux every 2 days based on the growth curve of the microalgae (Stepwise LI). The microalgae grew better with the significant increase of cell concentration. The final biomass and lipid production were increased up to 3.80 $g\,L^{-1}$ and 1.48 $g\,L^{-1}$, respectively which were 1.22 and 1.51 folds of the cultivation with constant light intensity at 5.5 klux. The specific growth rate and lipid productivity were increased up to 0.28 day^{-1} and 136.88 $mg\,L^{-1}\,day^{-1}$, respectively which were 1.40 and 1.61 folds of those with constant light intensity at 5.5 klux. This was likely due to the increase of photoautotrophic growth with an increased light intensity during cultivation. These results were consistent with the results of Cheirsilp and Torpee (2012), who found that the microalgae growth and lipid production of marine *Chlorella* sp. and *Nannochloropsis* sp. increased when applying stepwise-increasing of light intensity during cultivation. The lipid content of the microalgae in Stepwise LI was also increased from 34.15% up to 39.0%. As the microalgae growth was enhanced, the nitrogen concentration rapidly decreased. The low nitrogen concentration might lead to the higher ratio of carbon to nitrogen which could increase the lipid accumulation in the microalgae (Hu *et al*., 2008). The CO_2 removal and CH_4 content also increased when applying Stepwise LI. As 84.6% of CO_2 was removed from biogas, the CH_4 content in biogas was increased from 60% up to 93.6%. Wang *et al*. (2015) reported that the CO_2 removal efficiency more than 75% (or CH_4 content in biogas >90%) is required for high-efficiency combustion and high heating value. The CO_2 removal rate by Stepwise LI was also increased up to 4.37 g-CO_2 day^{-1} per 1 L microalgal culture. These results indicate that the stepwise-increasing of light intensity is a promising strategy to improve the microalgae growth and lipid production.

To avoid the limitation by nitrogen depletion, the stepwise-addition of nitrogen source (Stepwise N) was attempted. The nitrogen source was added at 0.2 $g\,L^{-1}$ every 2 days. Through this strategy, the nitrogen source could be efficiently supplied to support the cell growth. The Stepwise N showed higher cell concentration and microalgae biomass than the control. The microalgae biomass and specific growth

rate reached 3.36 g L^{-1} and 0.23 day^{-1}, respectively. Due to the improved cell growth, the CO_2 removal by Stepwise N was increased up to 79.24%. Consequently, the CH_4 content in biogas was increased up to 91.67%. The CO_2 removal rate was 4.12 g-CO_2 day^{-1} per 1 L microalgal culture. From these results, it could be concluded that the stepwise-addition of nitrogen source positively affected microalgae growth and CO_2 removal. However, the lipid production was slightly lower than that of the control. It has been reported that the excessive nitrogen source could stimulate cell division but decrease acetyl-CoA carboxylase (ACCase) activity in lipid biosynthesis pathway (Lv *et al*., 2010).Therefore, the strategy to balance between microalgae growth and lipid production should be performed.

In addition to light intensity and nitrogen concentration, CO_2 supply is also important factor for the microalgae growth and efficiency of CO_2 removal from biogas. It has been reported that the efficiency of CO_2 capture by microalgae is directly dependent on CO_2 gas flow rate and the culture pH (Olaizola *et al*., 2004). In the previous study (Srinuanpan *et al*., 2017), it was found that when using low biogas flow rate the highly purified biogas could be obtained but the productivity of the process was low, namely the biogas was treated at a low rate. The CO_2 removal rate is an important parameter for biogas purification process. High CO_2 removal rate means that high amount of biogas could be treated at a high rate. Moreover, it was expected that the high gas flow rate would also provide the excessive carbon source which could enhance the lipid accumulation by the microalgae. The stepwise-increasing of biogas flow rate (Stepwise GFR) was then attempted. The biogas flow rate was stepwise-increased by 0.03 L h^{-1} per 1 L microalgal culture every 2 days. The results showed that the Stepwise GFR was effective strategy to enhance not only cell growth (microalgal biomass of 3.77 g L^{-1}) but also lipid production (1.65 g L^{-1} with lipid content of 43.77%). Compared to the individual stepwise-increasing of growth factor, Stepwise GFR most enhanced the specific growth rate (0.29 day^{-1}) and lipid productivity (158.13 mg L^{-1} day^{-1}) of the microalgae which were 1.45 and 1.86 fold of those with constant gas flow rate at 0.3 L h^{-1} per 1 L microalgae culture.

Among the three growth factors tested, it could be concluded that the stepwise-increasing of light intensity could effectively increase both cell growth (microalgal biomass of 3.8 g L^{-1}) and lipid production (1.48 g L^{-1} with lipid content of

39%) while the stepwise-addition of nitrogen source enhanced mainly cell division (specific growth rate of 0.23 day^{-1}) and cell growth (microalgal biomass of 3.36 g L^{-1}) but not lipid production (1.00 g L^{-1} with lipid content of 29.81%). As the process productivity (CO_2 removal rate) depends mainly on gas flow rate, the most effective strategy to increase not only microalgae growth (microalgal biomass of 3.77 g L^{-1}) and lipid production (1.65 g L^{-1} with lipid content of 43.77%) but also the process productivity (CO_2 removal rate of 5.10 g-CO_2 day^{-1} per 1 L microalgal culture) is the stepwise-increasing of gas flow rate.

As the microalgae would require several growth factors to support their growth during cultivation, the simultaneous stepwise-increasing of light intensity together with nitrogen source (Stepwise LI + N) was then attempted. The cell concentration by this strategy (specific growth rate of 0.31 day^{-1}) was much higher than that of the control (specific growth rate of 0.20 day^{-1}) and that of the individual stepwise-increasing of growth factor (specific growth rate of 0.23-0.28 day^{-1}). This indicated that the effective cell division required both increasing light intensity and nitrogen source. Stepwise LI + N also showed higher final biomass (4.0 g L^{-1}) than that of the control (3.12 g L^{-1}) and the individual stepwise-increasing of growth factor (3.36-3.80 g L^{-1}). The specific growth rate was increased from 0.20 day^{-1} up to 0.31 day^{-1}. The CO_2 removal efficiency (88.47%) and CH_4 content (95.37%) were also higher than those of the control (76.91% CO_2 removal with 90.73% CH_4 content) and the individual stepwise-increasing of growth factor (75.45-84.06% CO_2 removal with 90.15-93.60% CH_4 content). However, the CO_2 removal rate (4.60 g-CO_2 day^{-1} per 1 L microalgal culture) by this strategy was lower than that by the Stepwise GFR (5.10 g-CO_2 day^{-1} per 1 L microalgal culture). This was because the overall biogas flow rate by Stepwise LI + N (0.30 L h^{-1} per 1 L microalgal culture) was lower than that of Stepwise GFR (0.39 L h^{-1} per 1 L microalgal culture).

To enhance the overall targets including microalgae growth, lipid production, CO_2 removal efficiency and CO_2 removal rate, all three growth factors including light intensity, nitrogen source and CO_2 supply were simultaneously stepwise-increased during microalgae cultivation (Simultaneous Stepwise LI + N + GFR). Through this strategy, the highest microalgae biomass of 4.4 g L^{-1} and the lipid production of 1.5 g L^{-1} were achieved. The specific growth rate

and lipid productivity were 0.36 day^{-1} and 139.38 $mg\,L^{-1}\,day^{-1}$, respectively. The CO_2 removal efficiency and CH_4 content were also highest at 96.26% and 98.5%, respectively. Interestingly, the CO_2 removal rate by this strategy was most increased up to 6.5 $g\text{-}CO_2\,day^{-1}$ per 1 L microalgal culture. As the simultaneous stepwise-increasing of three growth factors showed the best performance, it could then be concluded that light intensity, nitrogen source and CO_2 supply all are importance growth factors that have to be increasingly supplied along with the cell growth of microalgae.

The performance of all stepwise-increasing of growth factors are compared with that of the control. Considering the increase in the microalgae biomass, the improvement levels of microalgae growth could be concluded as follows: Simultaneous Stepwise LI + N + GFR > Simultaneous Stepwise LI + N > Stepwise GFR > Stepwise LI > Stepwise N. While the improvement levels of lipid production were as follows: Stepwise GFR > Simultaneous Stepwise LI + N + GFR > Stepwise LI > Simultaneous Stepwise LI + N > Stepwise N. For CO_2 removal efficiency, the Simultaneous Stepwise LI + N + GFR gave the highest CO_2 removal efficiency (96.26%) followed by Simultaneous Stepwise LI + N (88.47%), Stepwise LI (84.06%), Stepwise N (79.24%) and Stepwise GFR (75.45%). The improvement levels of CH_4 content in biogas after removing CO_2 were in accordance with those of CO_2 removal efficiency. The Simultaneous Stepwise LI + N + GFR also gave the highest CO_2 removal rate of 6.5 $g\text{-}CO_2\,day^{-1}$ per 1 L microalgal culture which was 1.63 folds of the control. Finally, these results suggested that the simultaneous stepwise-increasing of three growth factors was the most suitable strategy to simultaneously upgrade biogas and produce lipid by oleaginous *Scenedesmus* sp.

The fatty acid profiling of microalgal lipids from each strategy were characterized and used to estimate their fuel properties. The microalgal lipids were mostly composed of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). More than 94% of fatty acids were C16-C18. It should be noted that when the microalgae was cultivated through the stepwise-increasing of growth factors, the percentage of saturated fatty acids (SFA) increased while that of unsaturated fatty acids (USFA) decreased. Among individual stepwise-increasing of

growth factor, the SFA/USFA ratio of lipids obtained from Stepwise GFR was higher than those of other strategies. Interestingly, the SFA/USFA ratio of lipids from Simultaneous Stepwise LI + N + GFR was highest at 0.77.

The fuel properties of microalgal lipids including saponification number (SN), iodine number (IN), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP) were calculated based on their FAME profiles. The content of saturated and monounsaturated fatty acids (SFA and MUFA) could give high value of cetane number (CN) which denotes better combustion quality and easy start of the engine. The unsaturation of the oil determines iodine number (IN) indicating the oxidative stability of the fuel. The SN were found to be in the range of 205–208 mg KOH g^{-1} of oil. Consequently, the CN were found to be 42–48. It should be noted that the CN of microalgal lipids from Simultaneous Stepwise LI + N + GFR was ≥47, which meet the requirement for being used as biodiesel. The IN was found to be 111–135 g Iodine/100 g of oil. The microalgal lipids from Simultaneous Stepwise LI + N + GFR and Stepwise GFR meet with the standard value of IN (≤120 g Iodine/100 g of oil). The DU was found to be 96-112. The cold flow properties like LCSF and CFPP of microalgae were in range of 5-8 and 1-8 °C, respectively which were in accordance with the international standard revealing the good flow performance of biodiesel at low temperature. In conclusion, the microalgal lipids from Simultaneous Stepwise LI + N + GFR showed the best fuel properties which meet the biodiesel fuel specifications given by the regulatory international standard of ASTM D6751 and EN-14214. It should be noted that after the extraction of microalgal lipid the residual biomass could be used in various applications such as animal feed, fertilizer and even as feedstocks for further production of bioethanol and biogas.

From this paper, it could be concluded that i) oleaginous microalgae could purify biogas and simultaneously produce lipids, ii) stepwise-increasing of growth factors successfully enhanced microalgae performance, iii) simultaneous increasing of growth factors most enhanced biogas purification process, and iv) estimated fuel properties of microalgal lipids meet the international standards. The conclusion diagram for this paper is shown in Figure 3.

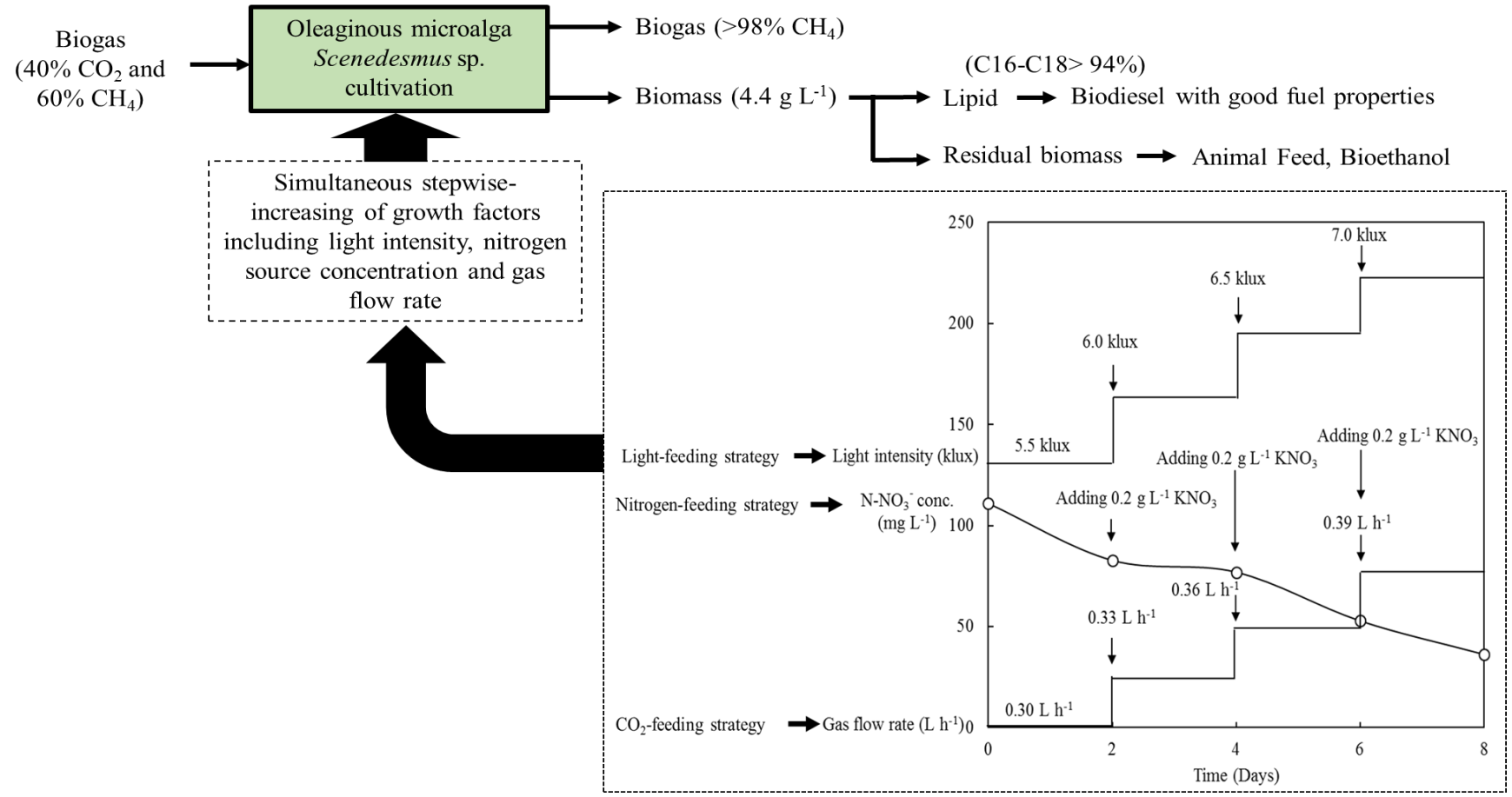


Figure 3 Conclusion diagram for Paper II.

4.3 Immobilized oleaginous microalgae as effective two-phase purify unit for biogas and palm oil mill effluent and production of biodiesel feedstock (Paper III)

This study aimed to evaluate the feasibility of using immobilized oleaginous microalgae *Scenedesnus* sp. for purification of biogas and phytoremediation of palm oil mill effluent in one step. The oleaginous microalga *Scenedesmus* sp. was immobilized in alginate gel beads in order to increase the performance of the microalgae and simplify the harvesting process. The results were compared with those using free cells.

The immobilized microalgae cells at low initial cell concentration of 10^6 cells mL^{-1} gave the biomass of 1.78 g L^{-1} which was close to that of free cells (1.89 g L^{-1}). For the production of lipids, immobilized microalgae cells accumulated lipid content of 33.93% which was also comparable to that of free cells. It was also found that the immobilized microalgae could grow without a long lag period after the beads were added in the medium when compared with the free cells, indicating that no significant mass transfer limitation occurred.

With the high initial cell concentration of 10^7 cells mL^{-1}, the free cells could grow and gave biomass increased up to 3.12 g L^{-1} with a lipid content of 34.15%. The immobilized cells grew similarly and gave the biomass of 2.99 g L^{-1} with lipid content of 32.83%. However, at day 8 the beads spit and the cells leaded out from the beads with 0.2 g L^{-1} of biomass detected in the medium on day 8. It might be due to overloading of biomass occurred in the beads with high cell density. Lam and Lee (2012) reported that the mass transfer of gas and nutrients is the key factor for cultivating immobilized microalgae. In addition, the results of this study was controversial with those of Cheirsilp *et al.* (2017), who found that a cell concentration of 10^7 cells mL^{-1} was suitable for the performance of oleaginous microalga *Nannochloropsis* sp. immobilized in alginate gel beads This might be due to the fact that the cells size of *Nannochloropsis* sp. (~3 µm) was much smaller than that of *Scenedesmus* sp. (>7.5 µm).

The immobilized microalgae with low initial cell concentration of 10^6 cells mL^{-1} contained chlorophylls and carotenoids at 16.54 and 9.67 mg g^{-1} biomass,

respectively which were comparable to those of the free cells (17.98 and 10.91 mg g^{-1} biomass, respectively). While a high initial cell concentration (10^7 cells mL^{-1}), the production of pigments including chlorophylls and carotenoids were lower. These results are in consistent with those reported by Abinandan and Shanthakumar (2016) who found that the chlorophyll content decreased when the inoculum size increased. The amount of chlorophylls is an indicator of the rate of photosynthesis of microalgae and also the availability of the light (Cheirsilp *et al*., 2017).

The percent CO_2 removal by free and immobilized microalgae at high initial cell concentration were higher than those using low initial cell concentration. The free and immobilized microalgae at high initial cell concentration (10^7 cells mL^{-1}) gave the maximum CO_2 removal of 75-77% and the CH_4 content was increased up to 90-91%. It should be noted that this methane content meets the standard (>90%) for efficient combustion with an increase in calorific value. CO_2 removal rate were as high as 3.9-4.0 g-CO_2 day^{-1} per 1 L-microalgal culture. Although the immobilized microalgae beads were broken, the CO_2 was also effectively removed due to both immobilized cells and leaked cells from the beads. At low cell concentration of 10^6 cell mL^{-1}, the percent CO_2 removal, CH_4 content and CO_2 removal rate by both free and immobilized microalgae were comparable to those at 10^7 cells mL^{-1}. More than 67-77% of CO_2 was removed from biogas and the CH_4 content was increased up to 86-91%. CO_2 removal rate were 3.5-4.0 g-CO_2 day^{-1} per 1 L-microalgal culture. It should be noted that the use of low initial cell concentration is more practical than the use of high initial cell concentration, initial cell concentration at 10^6 cells mL^{-1} was then chosen for further experiments.

The effect of bead volume on the growth of immobilized microalgae, lipid production and CO_2 removal efficiency were studied by varying the bead volume ratio of 25%, 50% and 75% medium volume. Among the three B/M ratio tested, the B/M ratio of 25% gave the maximum biomass of 2.00 g L^{-1} followed by the B/M ratio of 50% (1.78 g L^{-1}) and 75% (1.07 g L^{-1}), respectively. It should be noted that a lower B/M ratio gave higher microalgae biomass. The lipid productions by immobilized microalgae at low B/M ratio were also higher than those using high B/M ratio. The lipid production and lipid content were found to be 0.12-0.67 g L^{-1} and 18.98-33.93%, respectively. The lower B/M ratio caused less mass transfer limitation of nutrients and

gas. The contents of pigments decreased with increasing the B/M ratio. This could be due to the lower light penetration through the beads. In addition, the mass transfer limitation between liquid and solid phase may also alter the photosynthetic metabolism and reduce the production of pigments in microalgal cells. The lower B/M ratio achieved higher CH_4 content and lower CO_2 content, indicating the better biogas upgrading efficiency. At B/M ratio of 25%, the CO_2 removal (76%) and CH_4 content (90.37%) were highest as well as the maximum CO_2 removal rate of 3.95 g-CO_2 day^{-1} per 1 L-microalgal culture. Possibly, the decreased CO_2 removal efficiency might also be due to the coalescence of gas bubbles that the decrease in bubble retention time in the medium. The decrease in surface area per unit gas volume of the bubbles also reduced the CO_2 removal efficiency (Kao *et al*., 2012). The optimal B/M ratio of 25% was in consistent with the results reported by Cheirsilp *et al*. (2017). Therefore, the B/M ratio 25% was chosen for further experiment.

The immobilized microalgae were used in phytoremediation of anaerobic digester (AD) effluent. The chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) in the AD effluent were 9,500 mg L^{-1}, 2,005 mg L^{-1}, and 800 mg L^{-1}, respectively. However, the effluent was in dark brown colour with high turbidity. The AD effluent concentration was then diluted. Park *et al*. (2009) and Khalid *et al*. (2016) also suggested that POME should be diluted before used as nutrient medium. Dilution of the effluent could improve light penetration and increase the photosynthesis activity and biomass production. The AD effluent concentration at 20% (COD of 1900 mg L^{-1}, TN of 401 mg L^{-1}, and TP of 160 mg L^{-1}) gave the highest biomass production (2.19 g L^{-1}), followed by 40% AD effluent concentration (1.28 g L^{-1}). The AD effluent concentration >40% gave poor microalgae biomass. There are two possible reasons. One might be due to inhibition from high concentration of some compounds and another one might be the low light penetration. Both high concentration of inhibitory compounds and low light penetration resulted in low microalgae growth and long lag time. Generally, POME contains phenolic compounds such as caffeic acid, tannic acid, ferulic acid, 4-hydroxybenzoic acid, catechol, and 3-methylcatechol (Khongkhaem *et al*., 2016) which could be inhibitory to microalgae growth and photosynthesis reaction in microalgae cells (Hadiyanto and

Nur, 2012). Ding *et al*. (2016) also reported that the excessive nutrient content in undiluted wastewater could be toxic to microalgae and negatively affect their growth.

With 20% AD effluent concentration, the lipid production was highest at 0.61 g L^{-1} corresponding to the lipid content of 27.73% while the AD effluent concentration >20% gave the lower lipid content than 22%. Liu *et al*. (2012) found that the lipid yield and lipid content of *Scenedesmus* sp. was low due to the low light penetration. Through photosynthesis, large amounts of ATP and NADPH are produced as well as CO_2 is converted to glyceraldehyde-3-phosphate (G3P). Then, G3P is converted to pyruvate and thereafter to acetyl-CoA, via a series of catalytic reaction, initiates the lipid biosynthetic pathway (He *et al*., 2015). Therefore, increasing light intensity could lead to significant increase in photosynthesis reaction and subsequent lipid synthesis. In addition, high carbon and nitrogen concentration may also inhibit the activity of key enzyme (ACCase: Acetyl CoA Carboxylase enzyme) in lipid synthesis pathway (Minhas *et al*., 2016).

Using 20% AD effluent concentration also gave higher chlorophyll and carotenoid contents than those using effluent concentration. It was possible that high effluent concentration had low light penetration and resulted in low photosynthesis activity. Kamyab *et al*. (2016) reported that the reduction of pigment content is directly related to the reduction of photosynthesis activity. Moreover, in the effluent the microalgae might also grow with heterotrophic regime using organic carbon without light together with photosynthetic regime which is so called mixotrophic regime. García *et al*. (2005) reported that the cellular photosynthetic components depended on the duration of time that cells remained in light and dark zones.

Over time courses, the CO_2 content decreased, whereas the CH_4 content increased. Results indicated that lower effluent concentration achieved better biogas upgrading efficiency. The higher effluent concentration used, the lower biogas upgrading efficiency obtained. Low light penetration and high concentration of inhibitory compounds may inhibit the carboxylating activity and restrain the oxygenating activity of Rubisco, resulted in decreased performance of microalgae cells and lower CO_2 removal process (Yan *et al*., 2016).The highest CH_4 content obtained was 90.75% when using 20% AD effluent concentration. The higher effluent used, the lower biogas upgrading efficiency obtained. Considering CO_2 removal rate,

it was found that at 20% AD effluent concentration gave the highest values at 4.0 g-CO_2 day^{-1} per 1 L-microalgal culture. Thus, it could be concluded that the use of AD effluent concentration at 20% was suitable for biogas upgrading by immobilized oleaginous microalgae.

The DO value in this research slightly increased during the experimental period, which was found to be in the range of 0.40-5.95 mg L^{-1}, which was below the level (35 mg L^{-1}) that inhibits the microalgae performance (Yan and Zheng, 2013). During cultivation in 20% AD effluent concentration, the DO value increased continuously during cultivation because the immobilized microalgae could grow and produce oxygen through photosynthesis activity. As the maximum DO value (5.95 mg L^{-1}) was lower than the saturation level at 30 ºC (7.6 mg L^{-1}) (Huguenin and Colt, 1989). This indicated that the produced oxygen mainly dissolved in the culture medium and did not affect the quality of biogas.

At 20% AD effluent concentration the COD removal efficiency ranged from 22.24% to 48.42% during cultivation. This indicated that the microalgae could consume organic carbon through heterotrophic metabolism (Ding *et al*., 2016). However, with the AD effluent concentration >40%, the COD removal became lower. It was obvious that high effluent concentration inhibited microalgae growth. In POME treatment through anaerobic digestion process, acetate is one of the products converted by a biological reaction from volatile fatty acids (VFA) via the process of acetogenesis. Afterwards, acetate is converted into methane and CO_2 by another biological reaction in the process of methanogenesis. Thus, the residual acetate in the downstream of anaerobic POME was found due to incomplete reaction of methanogenesis or the inhibition of methanogenesis (Ding *et al*., 2016). Therefore, it was possible that acetate could be mainly carbon source for microalgae. The TN removal using 20% AD effluent concentration was highest at 82.69% whereas with higher effluent concentration the TN removal became lower. Ding *et al*. (2016) found that about 70% of the nitrogen in anaerobic effluent was NH_4^+–N. Yuan *et al*. (2011) reported that high ammonium content might be toxic to microalgae growth. At 20% AD effluent concentration, total phosphorus was completely removed, followed by 40% AD effluent concentration (38.28%). As the dark color of undiluted effluent negatively affected the light penetration and led to lower the photosynthesis by the

microalgae, it could be concluded that the high content of some inhibitory compounds as well as low light penetration did inhibit the performance of the microalgae.

To increase the light availability, the light intensity was increased from 5.5 to 11.5 klux. The AD effluent concentration at 20% was used as culture medium. The biomass of microalgae increased from 2.19 to 2.90 g L^{-1} when the light intensity was increased from 5.5 to 9.5 klux. A further increase in light intensity did not increase more microalgal biomass. It was possible that at low light intensities (<9.5 klux) the photolimitation occurred and resulted in low biomass production. On the contrast, the microalgal biomass decreased at light intensity of 11.5 klux possibly due to photoinhibition effect. Zhao *et al*. (2013) found that excessive light intensity damaged microalgae by overloading the photosystem and bleaching the pigments. Therefore, it could be concluded that 9.5 klux was the optimum light intensity for the microalgae growth in 20% effluent concentration.

The results indicated that a high lipid production correspond to high biomass production. The lipid production increased from 0.61 g L^{-1} up to 1.03 g L^{-1} and lipid content increased from 27.73% up to 35.52% with increasing light intensity from 5.5 klux to 9.5 klux. It is well known that light energy is absorbed by the pigments and then converted to ATP or NADPH, and this chemical energy is stored ultimately as starch and/or lipids. Similarly to the cell growth, the production of lipids decreased when the light intensity was increased up to 11.5 klux. When high light energy was supplied into the microalgal culture, the light-harvesting chlorophyll molecules changed to unstable forms, which in turn react with dissolved oxygen species. These reactive oxygen species then react with free fatty acids to make lipid peroxidase in inactive form and this reduces the fatty acid synthesis which is known as photo-oxidative inhibition (Gim *et al*., 2016). Thus, the optimum light intensity for maximum total lipid production was found to be 9.5 klux. Under these condition, the lipid production was increased from 0.20 g L^{-1} up to 1.03 g L^{-1} with considerably high lipid content of 35.52%.

The pigment content increased with increasing light intensity from 5.5 klux to 9.5 klux, but decreased when light intensity was increased up to 11.5 klux. It was possible that with attenuating light the cells respond by synthesizing more chlorophylls. The chlorophylls content was as high as 45.88 mg g^{-1} biomass at light

intensity of 9.5 klux which was 3.37 fold of cultivation at light intensity of 5.5 klux (chlorophyll content of 13.63 mg g^{-1} biomass). The decrease in the chlorophyll content at high light intensity would be because the photosystem and pigments was destroyed and this phenomenon is known as photoinhibition. According to Cheirsilp and Torpee (2012) who mentioned that the necessary amount of light energy is completely absorbed by a growing number of cells, thus the cells require a lower content of cellular chlorophylls. For the production of carotenoids, some literature (García *et al*., 2005; Gim *et al*., 2016) indicated that when exposed to high light intensity, the microalgae cells might use the carotenoid synthesis pathway as a protective mechanism against photodamage. In this study, at higher light intensity (>9.5 klux) the carotenoid content decreased possibly due to the large damage on the photosystem. This suggested that carotenoid synthesis may not be enough to protect cells from high light stress. The highest carotenoid content was 26.34 mg g^{-1} biomass at 9.5 klux of light intensity which were 1.93, 2.04 and 8.96 fold of those with light intensity of 5.5, 7.5 and 11.5 lux, respectively.

It is well known that light intensity is an important factor for the photosynthetic activity of microalgae, which directly affects CO_2 removal efficiency. The CO_2 removal efficiency increased when the light intensity was increased from 5.5 to 9.5 klux. The maximum percent CO_2 removal and CO_2 removal rate were obtained at 88.24% and 4.58 g-CO_2 day^{-1} per 1 L-microalgal culture, respectively at light intensity of 9.5 klux. Consequently, the CH_4 content in biogas was increased up to 95.28%, which meets the standard of fuel requirements (Zhao *et al*., 2013). At too high light intensity (>9.5 klux), photoinhibition occurred and lowered both the photosynthesis activity and CO_2 removal efficiency.

The removal efficiency of COD, TN and TP increased when the light intensity was increased from 5.5 klux to 9.5 klux. The highest removal of COD (68.68%), TN (98.93%) and TP (100%) was obtained from the cultivation with 9.5 klux of light intensity. However, at light intensity >9.5 klux the pollutants removal decreased likely due to the photoinhibition on the performance of the microalgae. Thus, the light intensity at 9.5 klux was the optimal light intensity for cell growth, lipid and pigments production, CO_2 removal and pollutants removal. The DO value at 9.5 klux ranged from 2.65 to 6.35 mg L^{-1}, which were below the saturation level.

The 20% unsterile AD effluent concentration without pH-adjustment was used for microalgae cultivation. The results were compared with those using sterile and pH adjusted AD effluent. The biomass, lipids and pigments production using non-sterile AD effluent were close to those using sterile and pH adjusted AD effluent. Similarly, the CO_2 removal efficiency and CH_4 content after removing CO_2 in both sterile and non-sterile AD effluent were not significant different. Using non-sterile AD effluent, more than 88.24% of CO_2 was removed from biogas and the CH_4 content was upgraded up to 95.37%. The CO_2 removal rate was 4.6 g-CO_2 day^{-1} per 1 L-microalgal culture. Also, the COD removal of immobilized microalgae under using non-sterile AD effluent (71.25%) was slightly higher than using sterile AD effluent (68.68%). Interestingly, the total nitrogen and phosphorus were completely removed. The DO value of using non-sterile AD effluent ranged at 2.65-6.40 mg L^{-1}. Thus, this study indicated that using non-sterile and non-pH adjusted effluent can be used directly for cultivation of immobilized microalgae.

The lipids extracted from immobilized oleaginous microalga *Scenedesmus* sp. was converted to fatty acid methyl esters (FAMEs). The accumulated fatty acid chain lengths were in the range of C8 to C18 fatty acids. The main fatty acids were long-chain fatty acids with 16 and 18 carbon atoms including oleic acid (C18:1) as the predominant fatty acid (48.8%) followed by palmitic acid (C16:0; 17.94%) and linolenic acid (C18:2; 15.86%). The saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) of the microalgal lipids in this study were >75%. The SFA/UFA ratio was obtained at 0.25. The biodiesel fuel properties were calculated based on the fatty acid composition. The iodine value (IV), saponification value (SV), cetane number (CN), unsaturation degree (DU), LCSF (long chain saturated factor), cold filter plugging point (CFPP), high heating value (HHV) and oxidative stability (OS) were found to be 97.57 g I_2/100 g oil, 205.12 mg KOH/g oil, 50.95, 100.5, 3.13, -6.65 °C, 39.56 MJ/kg and 7.8 h, respectively. It could be concluded that the fuel properties of microalgal lipid are in accordance with the international standard ASTM D6751 (USA) and EN 14214 (European Organization). It then has high potential as biodiesel feedstocks.

Therefore, the use of immobilization technology in microalgae cultivation is the effective technique to reduce operational cost and energy input in

harvesting process due to the large sizes of the immobilized microalgae beads. The free cells of microalgae are smaller in range of 5-50 μm of diameter, which is required high energy input in harvesting process. Thus, the immobilized cells with larger size can be easily harvested by a simple sieving method without involving huge amounts of energy input. Based on this research, the use of immobilized microalgae are fairly cost-effective at estimated material around $0.07 per kg biomass. This cost was close to that the report of Cheirsilp *et al*. (2017), who found that material costs for immobilization at 25% beads volume to culture medium ratio was about $0.06 per kg microalgal biomass. Grima *et al*. (2003) have reported that the harvesting of free cells using centrifugation required nearly 0.3-8 kW h of electricity per m^3 culture volume and the harvesting costs would be $1.44-18 per kg microalgal biomass. While using electro-flocculation was estimated at $0.19 per kg microalgal biomass with 0.092 kW h of electricity per m^3 culture volume (Lee *et al*., 2013). Selesu *et al*. (2016) was also found that bio-flocculation with chitosan has still high cost up to $10 per kg microalgal biomass. Cheirsilp *et al*. (2017) reported that the immobilized microalgae beads could be reused for 2 cycles of cultivation. However, it is not possible in this research because immobilized beads had broken down when microalgal biomass increased higher than 3.0 g L^{-1}. Therefore, to enhance the economic potential of microalgal biomass production, using recycled Na-alginate after biomass harvesting for preparing new lot of immobilized microalgae beads is highly recommended. Based on these information, using the immobilized cells could then be economically attractive for microalgae cultivation than the free cells.

From this paper, it could be concluded that i) oleaginous microalga Scenedesmus sp. could be immobilized in alginate gel beads, ii) immobilized cells were effective in biogas upgrading and phytoremediation of effluent, iii) microalgae biomass with high lipids and pigments were obtained, and iv) The produced lipids have high potential as biodiesel feedstocks. The conclusion diagram for this paper is shown in Figure 4.

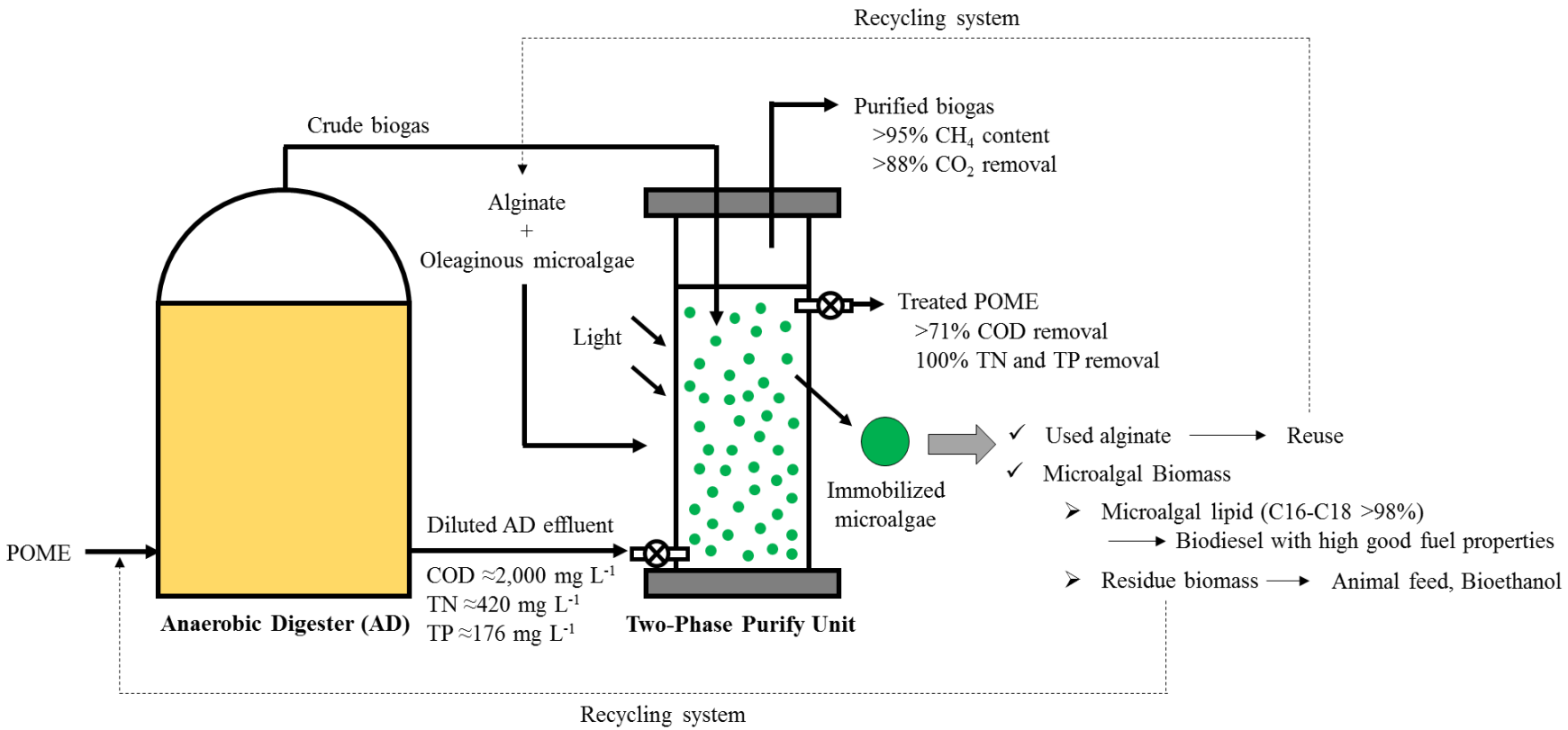


Figure 4 Conclusion diagram for Paper III.

4.4 Strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks: Nutrient starvations and cost-effective harvesting process (Paper IV)

In this study, several marine microalgae were locally isolated from Songkhla Lake, Thailand, based on their morphologies (i.e., cell shape and size) and quantitative analysis of lipid content. Among them, two isolates having lipid content >20%, SIT04 and SIT06, were selected. Isolate SIT04 and SIT06 were identified as clearly group with *Micractinium reisseri* and *Scenedesmus obliquus*. To determine the growth and biomass concentration of both microalgae strains, *M. reisseri* SIT04 and *S. obliquus* SIT06 were cultured in modified Chu13 medium at 28 °C and bubbled with air at a flow rate of 0.01 L min^{-1} under a 90 μmol m^{-2} s^{-1} light intensity with a 24 h full illumination for 10 days. The specific growth rates of *M. reisseri* SIT04 and *S. obliquus* SIT06 were comparable in the range of 0.8-0.9 day^{-1} while the final biomass concentration of *S. obliquus* SIT06 (1.83 g L^{-1}) was higher than that of *M. reisseri* SIT04 (1.29 g L^{-1}). The compositions of two microalgal biomass were also compared. *M. reisseri* SIT04 biomass contained slightly higher protein (39.53%) than did *S. obliquus* SIT06 (32.70%) whereas, their carbohydrate contents was similar in the range of 19%. The lipid content of *S. obliquus* SIT06 increased during cultivation and reached the maximum at 35.54% while that of *M. reisseri* SIT04 was stable at a level of 20–25%. It has been reported that the microalgal biomass collected during exponential growth phase contained high protein while those collected during stationary growth phase contained either high lipid or high carbohydrate. In addition to the strains used, the medium components and culture conditions are also crucial parameters affecting the biomass composition of the microalgae (Mata *et al*., 2010; Yeh and Chang, 2011). These results suggested that microalga *M. reisseri* SIT04 and microalga *S. obliquus* SIT06 could be promising sources for both lipids and proteins.

The relative percentage of fatty acid compositions in *M. reisseri* SIT04 and *S. obliquus* SIT06 lipids were determined through GC-MS analysis. Three main fatty acids found in *M. reisseri* SIT04 lipids were palmitic acid (C16:0, 39.87%), stearic acid (C18:0, 32.18%) and oleic acid (C18:1, 20.10%). Similarly, Karpagam *et al*. (2015) also reported that palmitic acid and oleic acid were the main fatty acids

found in *Micractinium* sp. M-13 lipids. However, in their study oleic acid content was higher than palmitic acid. The two main fatty acids found in *S. obliquus* SIT06 lipids were palmitic acid (62.94%) and steric acid (30.74%). While the two main fatty acids found in *S. obliquus* lipids in the study of Yang *et al*. (2016), were oleic acid (51.92%) and palmitic acid (20.42%). It should be noted that the higher content of saturated fatty acids in *M. reisseri* SIT04 and *S. obliquus* SIT06 lipids in this study indicated their potential use as biodiesel feedstocks because they could give higher oxidation stability and cetane number, decreased NO_x emissions and a shorter ignition delay time (Knothe, 2008; Toledo-Cervantes *et al*., 2013).

The effects of nutrient starvations on growth and lipid production of *M. reisseri* SIT04 and *S. obliquus* SIT06 were evaluated. Among three nutrient starvation tests, the P and Fe starvation slightly decreased the biomass while the N starvation drastically decreased the biomass of both strains. Among two strains, *S. obliquus* could survive under nutrient starvations better than *M. reisseri*. The severity of nutrient starvations on their growth are as follows: N starvation > P starvation > Fe starvation. These results confirmed the fact that nitrogen is the important nutrient for the cell proliferation and has significant impacts on cell growth of microalgae (Fan *et al*., 2014). The results in this study were consistent with those previously reported by Karpagam *et al*. (2014, 2015) who found that the nutrient starvations decreased cell growth and biomass productivity of *Meyerella* sp. N4 and *Chlamydomonas reinhardtii* CC1010. Yeesang and Cheirsilp (2011) and Fan *et al*. (2014) also reported that the nutrient starvations had negative effect on the cell growth of *Botryococcus* spp. and *C. pyrenoidosa*, respectively. However, the severity of each nutrient starvation on the cell growth was different depending on the species of the microalgae and also their origins.

In addition to the cell growth, the lipid content also contributes to the lipid productivity. During N starvation, the lipid contents of *S. obliquus* SIT06 and *M. reisseri* SIT04 obviously increased up to 56.87% and 36.72%, respectively. The lipid contents of both strains also increased during P and Fe starvations but with a lower level. The lipid contents of both strains increased up to 40.28% and 30.45% during P starvation and 39.75% and 29.60% during Fe starvation. The relative levels of these effects on lipid content are similar to those on the growth as follows: N

starvation > P starvation > Fe starvation. It should be noted that the N starvation was the most effective strategy to increase lipid content of the microalgae.

The two microalgae responded to each nutrient starvation differently. In case of *M. reisseri* SIT04, all types of nutrient starvation increased the content of palmitic acid (C16:0) and decreased the content of oleic acid (C18:1). Therefore, the total saturated fatty acids significantly increased. Usually high content of saturated fatty acids is preferential for increasing fuel quality of biodiesel and oxidative stability. While polyunsaturated fatty acids (PUFAs) showed great cold-flow properties but the biodiesel properties are interior oxidation stability (Karpagam et al., 2014; Talebi *et al*., 2013). In case of *S. obliquus* SIT06, Fe starvation had no significant effect on the fatty acid compositions while N and P starvations selectively increased the content of palmitic acid (C16:0). It has been reported that the changes in fatty acid composition relate to the activity of enzymes those involve in each fatty acid synthesis. It was therefore possible that the nutrient starvation might positively or negatively affect specific enzyme activities and increase or decrease the corresponding fatty acid content. Sharma *et al*. (2015) have reported that the palmitoyl thioesterase (PT) gene expression in *S. dimorphus* increased during N starvation and resulted in an increased content of palmitic acid. While the decrease of stearic acid could be affected by the decreased activity of stearoyl thioesterase (ST).These results indicate that the nutrient starvations not only increased lipid content but also increased the saturation level of the microalgal lipids.

To assess the suitability of microalgal lipids as biodiesel feedstocks, the fuel properties were calculated from their fatty acid profiles (Tongprawhan *et al*., 2014; Jawaharraj *et al*., 2016). Biodiesel fuel properties including iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), and cold filter plugging point (CFPP) were evaluated under different nutrient starvations. The IV of *M. reisseri* SIT04 and *S. obliquus* SIT06 lipids were found to be 0-26.3 and 0-10.3 g I_2/100 g oil, respectively, which meet the European standard (EN-14214) that defines a maximum IV value of 120 g I_2/100 g oil. It should be noted that the N starvation showed the lowest value of IV nearly 0 which indicates the most saturated level and have high stability against oxidation. The SV is the value of KOH (mg) required for the saponification of 1 g of oil. It is a measure of the average molecular

weight (namely chain length) of all the fatty acids present. A low SV indicates the presence of long chain fatty acids in the lipids. The SV of *M. reisseri* SIT04 and *S. obliquus* SIT06 were 208.02-214.51 and 210.95-216.73 mg KOH/g oil, respectively. The CN for *M. reisseri* SIT04 and *S. obliquus* SIT06 were 66.6-71.9 and 69.8-71.1, respectively. Cold filter plugging point (CFPP) of both microalgal lipids were found to be appropriate for use in hot regions (Karpagam *et al*., 2015a). The DU of *S. obliquus* SIT06 (0–7.9) was found to be lower than that of *M. reisseri* SIT04 (0-26.7) due to the lower unsaturation level in *S. obliquus* SIT06 lipids. It should be noted that the N starvation did decrease the value of DU to nearly 0%. In summary, *S. obliquus* SIT06 accumulated lipids higher than *M. reisseri* SIT04 and more positively responded to nutrient starvations. The biodiesel fuel qualities of *S. obliquus* SIT06 lipids are also superior to those of *M. reisseri* SIT04. Therefore, *S. obliquus* SIT06 was considered as potential biodiesel feedstocks and then chosen for further study.

The selected *S. obliquus* SIT06 was cultivated for 10 days and its culture broth added with bioflocculent. It should be noted that as the bioflocculent was added after the cultivation step and at a very small amount, it hardly influenced the fatty acid profiles of the microalgal lipids. Three biopolymers including chitosan, guar gum and starch was compared at the same concentration of 10 ppm with unadjusted final culture pH (around 10) and microalgal cell density of 0.65 g/L. After 60 min of flocculation time, the flocculation efficiency using chitosan (27.31%) was higher than those using other polymers (17-18%). The possible reason would be because chitosan possess the protonated amine groups (NH_3^+) which could effectively induce the flocculation of the microalgae biomass. While the ordered structures of guar gum and starch are destroyed during the solubilization process and resulted in the carboxyl groups (COO^-) (Liu *et al*., 2013).

The flocculation efficiency of the microalgal cells required the optimal levels of the factors including flocculent dosage, pH and cell density. The flocculation efficiency increased with increasing these factors up to the optimal levels and either reached a plateau or decreased when the factors were beyond their optimal levels. Based on the results of bioflocculent screening, chitosan was selected and used as bioflocculent in optimization study. Response surface methodology experiment

design was used to further determine the optimal combination of bioflocculent dosage, pH and microalgal cell density on flocculation efficiency of microalgae. The experimental results were concerned with flocculation efficiency of *S. obliquu*s SIT06 (%). The conditions at the center point were: chitosan dosage of 50 ppm, pH 7 and cell density of 0.35 g/L.

It was found that chitosan dosage of 100 ppm, pH of 4 and cell density of 0.35 g/L gave the maximum flocculation efficiency of 90.3%. The presence of chitosan and the acidic pH did increase the flocculation efficiency. This was because under acid condition chitosan having highly positive charge which could effectively bind to negative charge of microalgae cell surface and induced the large microalgae particles. Divakaran and Pillai (2002) have also reported that the presence of chitosan and the decreasing pH gave higher flocculation efficiency of *Synechocystis* sp. High chitosan dosage and high cell density did improve the flocculation of microalgae. However, increasing chitosan dosage is costly. Therefore, the strategies to use the interaction effect between factors to increase the flocculation efficiency should be established. When chitosan dosage was set at the center point of 50 ppm, the maximum flocculation efficiency was obtained when using the highest cell density tested and the lowest pH tested. Chen *et al*. (2013) reported that the optimal pH and chitosan dosage for flocculation of *Scenedesmus* sp. biomass were 5 and 80 ppm, respectively. However, the maximum flocculation efficiency was only 80%. Overall, the importance degree of three factors on flocculation efficiency of *S. obliquus* biomass in this study would be: chitosan dosage > cell density > pH and those of the interaction effects would be: chitosan dosage v.s. cell density > chitosan dosage v.s. pH > cell density v.s. pH.

The optimum conditions for maximizing flocculation efficiency of *S. obliquus* biomass were chitosan dosage of 64 ppm, pH of 4 and cell density of 0.65 g/L, which would give the flocculation efficiency as high as 99.50% and the harvesting cost would be 0.098 Bath per gram microalgae biomass. After optimization, the flocculation efficiency of *S. obliquus* biomass was increased 4.8-fold, comparing with that before optimization.

From this paper, it could be concluded that i) nutrient starvations positively affected lipid content of microalgae, ii) fatty acid compositions could be

manipulated by nutrient starvations, iii) nitrogen starvation most selectively increased saturated fatty acids, and iv) flocculation efficiency >99.5% could be achieved using pH-chitosan. The conclusion diagram for this paper is shown in Figure 5.

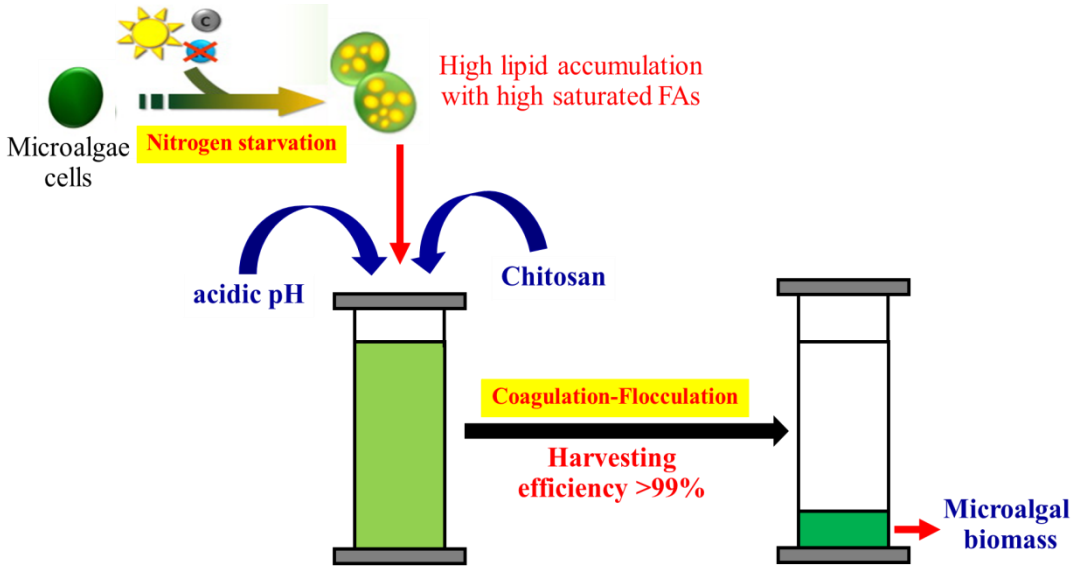


Figure 5 Conclusion diagram for Paper IV.

4.5 Photoautotrophic cultivation of oleaginous microalgae and co-pelletization with filamentous fungi for cost-effective harvesting process and improved lipid yield (Paper V)

In this study, two microalgae strains isolated from Songkhla Lake, Thailand, *M. reisseri* SIT04 and *S. obliquus* SIT06 were photoautotrophically cultivated in BG-11 medium and compared for their growth rate, lipid content, and fatty acid compositions. BG-11 medium was used for microalgae cultivation due to its balance of nutrients regarding both their presence and concentrations and the presence of citric acid and ferric ammonium citrate that could solubilize the salts, preventing precipitation and thus increasing their availability to cells (Sunda *et al.*, 2005; Harrison and Berges, 2005). The important factors for cultivating microalgae including light intensity, nitrogen source, and culture pH were simultaneously optimized through RSM. The biomass production in 10 days of *S. obliquus* SIT06 (2.01 g L^{-1}) was higher than that of *M. reisseri* SIT04 (1.86 g L^{-1}). The lipid contents in 10 days of *S. obliquus* SIT06 and *M. reisseri* SIT04 increased during cultivation

and reached the maximum at 34.4% and 27.7%, respectively. This was probably due to the decreasing of important nutrients for cell growth such as nitrogen source. As the cell growth is restricted the carbon flow is channeled into lipid accumulation and led to an increase in lipid content (Xin *et al*., 2010; Yeesang and Cheirsilp, 2011; Fan *et al*., 2014; Karpagam *et al*. 2015). Comparing among the two strains, the maximum lipid production by *S. obliquus* SIT06 (0.69 g L^{-1}) was higher than that of *M. reisseri* SIT04 (0.46 g L^{-1}) due to its higher biomass production and lipid content.

Based on the analysis of fatty acid compositions of two microalgae, it was found that palmitic acid was the main fatty acid found in *M. reisseri* SIT04 lipids (39.87%) followed by stearic acid (32.18%) and oleic acid (20.10%). While those found in *S. obliquus* SIT06 lipids were palmitic acid (62.94%) and steric acid (30.74%). The content of saturated fatty acids in *S. obliquus* SIT06 lipids (95.8%) was much higher than that in *M. reisseri* SIT04 (75.6%). *S. obliquus* SIT06 lipids tend to provide better fuel properties such as higher oxidation stability, higher CN, decreased NO_x emissions, and a shorter ignition delay time (Antolin *et al*., 2002). As *S. obliquus* SIT06 not only gives high biomass and lipid production but also has suitable fatty acid compositions for biodiesel production, it was then selected for further studies.

Three important factors for cultivating *S. obliquus* SIT06 including culture pH (5-9), light intensity (0-180 μmol proton m^{-2} s^{-1}) and nitrogen source ($NaNO_3$) concentration (0-3 g L^{-1}) were simultaneously optimized through RSM. Among the tested, under pH of 9, $NaNO_3$ concentration of 3 g L^{-1}, and light intensity of 90 μmol proton m^{-2} s^{-1} gave the highest biomass concentration of 2.28 g L^{-1} with a lipid content of 25.76%. The microalgae could not grow and accumulated low lipid content under the condition without light illumination (0 μmol proton m^{-2} s^{-1}) and the condition illuminated with too high light intensity (180 μmol proton m^{-2} s^{-1}). The maximum lipid content of 47.81% was obtained under the condition with high pH, low $NaNO_3$ concentration, and moderate light intensity.

The optimal level of $NaNO_3$ concentration for biomass production seems to be pH dependent. At a low pH, the saturation level of $NaNO_3$ was 1.5 g L^{-1}, and above this level, there was no further positive effect on biomass production. At a higher pH, the optimum level of $NaNO_3$ shifted to 2.7-3.0 g L^{-1}, which was close to those for other *Scenedesmus* spp. (Ren *et al*,. 2013; Arumugam *et al*., 2013). This

phenomenon was possibly because the culture pH is one of important factors affecting the solubility and the bioavailability of nutrients and also transport of substrates across the cytoplasmic membrane (Ren *et al*., 2013). In addition, a lower pH might also cause a loss of carbonate and result in a lower biomass. At a higher pH, the carbonate in the original medium is more stabilized and available for the microalgae. The optimal pH also slightly shifted from 7.0 to 8.0 when $NaNO_3$concentration was increased from 0 to 3.0 g L^{-1}. It has been reported that this pH range was also the suitable range for other microalgae, especially for *Scenedesmus* spp. (Hodaifa *et al*., 2010; Ren *et al*., 2013).

The biomass increased with increasing light intensity but sharply decreased when the light intensity was higher than 90 μmol proton m^{-2} s^{-1}. When the light intensity is insufficient, the microalgae growth would be limited but when the light intensity is above the saturation level, the photoinhibition would occur (Jeong *et al*., 2013). It seems that the optimal pH was not affected by the level of light intensity indicating that there was no interaction effect between these two factors. The effect of light intensity on biomass production was more obvious than that of $NaNO_3$ concentration. The optimal pH, $NaNO_3$ concentration and light intensity for biomass production were found to be 8.0, 2.7 g L^{-1} and 90 μmol proton m^{-2} s^{-1}, respectively.

The lipid content increased with decreasing $NaNO_3$ concentration. This was likely because the low nitrogen concentration could promote the lipid accumulation (Xin *et al*., 2010; Yeesang and Cheirsilp, 2011). However, the effect of pH on lipid content was insignificant. Similarly, Dayananda *et al*. (2006) also found that the lipid content in the microalgae was not much affected by the pH of the medium. With a fixed $NaNO_3$ concentration at 1.5 g L^{-1}, obviously the effect of pH on lipid content was less than that of light intensity. The optimal light intensity for lipid accumulation was close to that for biomass production at 88 μmol proton m^{-2} s^{-1}. However, in several studies, it was found that the optimal light intensities for cell growth and lipid accumulation were different. Cheirsilp and Torpee (2012) found that the cell growth of marine *Chlorella* sp. and *Nannochloropsis* sp. increased with increasing the light intensity up to 135 μmol proton m^{-2} s^{-1} but the lipid content of both strains drastically decreased. While

Thawechai *et al*. (2016) found that the optimal light intensity for the cell growth of *Nannochloropsis* sp. (70 μmol proton m^{-2} s^{-1}) was lower than that for lipid content (80 μmol proton m^{-2} s^{-1}). It could then be concluded that each microalga has a unique requirement for light intensity.

The moderate light intensity at 88 μmol m^{-2} s^{-1} was optimal for lipid accumulation, and there was no interaction effect between the light intensity and $NaNO_3$concentration. In many microalgae, a decrease in nitrogen concentration resulted in a significant favoring the accumulation of lipid. Although a deficiency of nitrogen favors lipid accumulation, nitrogen is required for microalgae growth. Yeesang and Cheirsilp (2011) compared the lipid content in *Botryococcus* sp. isolated from freshwater source in Thailand under nitrogen-rich and nitrogen-deficiency conditions. They found that the highest lipid content was obtained under nitrogen-deficiency conditions. Similarly, the microalgae *S. quadricauda* tended to accumulate high lipids under nitrogen-deficient conditions (Ahlgren and Hyenstrand, 2003). The optimal conditions for lipid content of *S. obliquus* SIT06 were found to be pH of 8.0 without the addition of $NaNO_3$ concentration and moderate light intensity of 88 μmol proton m^{-2} s^{-1}. The maximum lipid content obtained would be 45.7% based on its dried biomass.

From the previous section, the optimal pH for biomass and lipid content were the same at pH 8.0, and the optimal light intensity for both responses was 90 and 88 μmol proton m^{-2} s^{-1}, respectively. Although 2.7 g L^{-1} of $NaNO_3$ is needed for biomass production, there is no need to add $NaNO_3$ for lipid accumulation. Therefore, $NaNO_3$ should be added at initial and depleted during cultivation. The best conditions for compromising between biomass production and lipid accumulation were as follows: pH of 8.0, $NaNO_3$ concentration of 1.1 g L^{-1}, and light intensity of 87 μmol proton m^{-2} s^{-1}. The experimental biomass and lipid content were 1.99 g L^{-1} and 40.86%, respectively. With these conditions, the maximum lipid production of 0.81 g L^{-1} was obtained.

With harvesting process, the fungus was used as for harvesting microalgae biomass. Filamentous fungi have ability to form pellet and easily being harvested by sieving method. In this study, four filamentous fungi including *P. chrysogenum* TPU 4110, *F. saloni* TPU 4501, *C. echinulata* TPU 4652, and *M.*

alpine TPU 4880 were screened for their abilities to form pellets and accumulate lipids in the medium for microalgae added with 2% glucose. All fungi could form pellets within 24 h. The pellet sizes of *P. chrysogenum* TPU 4110 was smallest <1.0 mm, while those of *F. saloni* TPU 4501, *C. echinulata* TPU 4652, and *M. alpine* TPU 4880 were in the same range of 1.0-3.5 mm. At initial, the fungi biomass was approximately 0.1 g/L. *C. echinulata* TPU 4652 grew well and gave the highest biomass with the final pH increased higher than other fungi. The lipid yields of *F. saloni* TPU 4501 and *C. echinulata* TPU 4652 were obviously higher than those of other two fungi but less than those of the microalgae.

To harvest the microalgal cells by co-pelletization with filamentous fungi, the spores of fungi were added into the 10 days-cultured microalgae and the co-cultivation was conducted for 24 h. The microalgae-fungi pellets were rapidly formed within 24 h through co-pelletization process. The flocculation efficiency after 24 h was determined. The maximum flocculation efficiency of 92.65% was obtained when using *C. echinulata* TPU 4652 (the final pH was 7.96), followed by *F. saloni* TPU 4501 (45.53%, final pH of 7.15), *P. chrysogenum* TPU 4110 (41.25%, final pH of 6.8), and *M. alpine* TPU 4880 (40.90%, final pH of 6.82). There are several factors affecting the co-agglomeration between filamentous fungi and microalgae, such as the pH of the medium, pellet sizes, agitation speed, and carbon source addition (Zhou *et al*., 2012; Xie *et al*., 2013). It was possible that fungal hyphae and mycelia containing the positively charged polysaccharide and hydrophobic proteins can potentially neutralize the negative charges on the microalgae surface resulting attachment to the fungi cells (Prajapati *et al*., 2014; Gultom and Hu, 2013). Moreover, Muradov *et al*. (2015) have found that within fungi-pellets, microalgae cells did not just get entrapped within fungal filaments but also got attached to them. In this study, as *C. echinulata* TPU 4652 was the most suitable filamentous fungi for harvesting of microalgal cells, their microalgae-fungi pellets were analyzed for total biomass and lipid yield. It was found that the fungi not only co-agglomerated with the microalgal cells but also increased the overall biomass and lipid yield. The overall biomass and lipid yield were 4.45 and 1.21 g L^{-1}, which were 2.24 and 1.49 folds of the pure microalgae, respectively. This could be the effect of CO_2 addition by fungi to the microalgae. It is well known that some algae live in symbiotic association with fungi,

so called lichens. In the majority of lichens, the algae are entrapped in the fungal hyphae. It is possible that the microalgae could act as an oxygen generator for the fungi, while the fungi could provide CO_2 to the microalgae and together they would be able to carry out biomass and lipid production inside the pellets (Kitcha and Cheirsilp, 2014). As two metabolic reactions of CO_2 release and uptake were combined and complementary, this technique not only effectively harvested the microalgal cells but also contributed to the increase in lipid production. Since the production of lipid from microorganisms is subjected to the constraints of high operation costs, even a small improvement in the culture and harvesting techniques could result in substantial savings in the microbial lipid production.

The extracted lipids from the microalgae-fungi pellets were analyzed for their fatty acid compositions and fuel properties. The microalgae-fungi lipids were mainly composed of long-chain fatty acids of 16 and 18 carbon atoms, and the major fatty acids were palmitic acid (C16:0, 52%) and stearic acid (C18:0, 35%). It should be noted that these found in pure *S. obliquus* SIT06 lipids were also palmitic acid (62.94%) and steric acid (30.74%). The biodiesel fuel parameters including SV, IV, CN, DU, LCSF, and CFPP were calculated based on the fatty acid compositions. The SV of microalgae-fungi was found to be 209.5 mg KOH g^{-1} of oil. The CN was found to be 69.8 that indicating high combustion quality of the biodiesel. The IV was found to be 11.4-g iodine/100 g of oil. The DU was found to be 12.6%. Further, cold flow properties like LCSF and CFPP of fungi-algae were in accordance with the international standard revealing the good flow performance of biodiesel at low temperature. It should be noted that the fuel properties of biodiesel obtained from fungi *C. echinulata*-microalga *S. obliquus* were found to match with the biodiesel fuel specifications given by the regulatory international standard, ASTM D6751, and EN 14214. Thus, fungi *C. echinulata*-microalga *S. obliquus* pellets can be a promising alternative feedstock for biodiesel.

From this paper, it could be concluded that i) oleaginous microalga *S. obliquus* SIT06 was selected as potential biodiesel feedstocks, ii) the best culture conditions were as follows: pH of 8.0, $NaNO_3$ conc. of 1.1 g/L, and light intensity of 87 μmol proton/m^2/s, iii) *C. echinulata* TPU 4652 most effectively harvested the microalgal cells with the highest flocculation efficiency of 92.7%, and iv) lipids

extracted from microalgae-fungi pellets are suitable as biodiesel feedstocks. The conclusion diagram for this paper is shown in Figure 6.

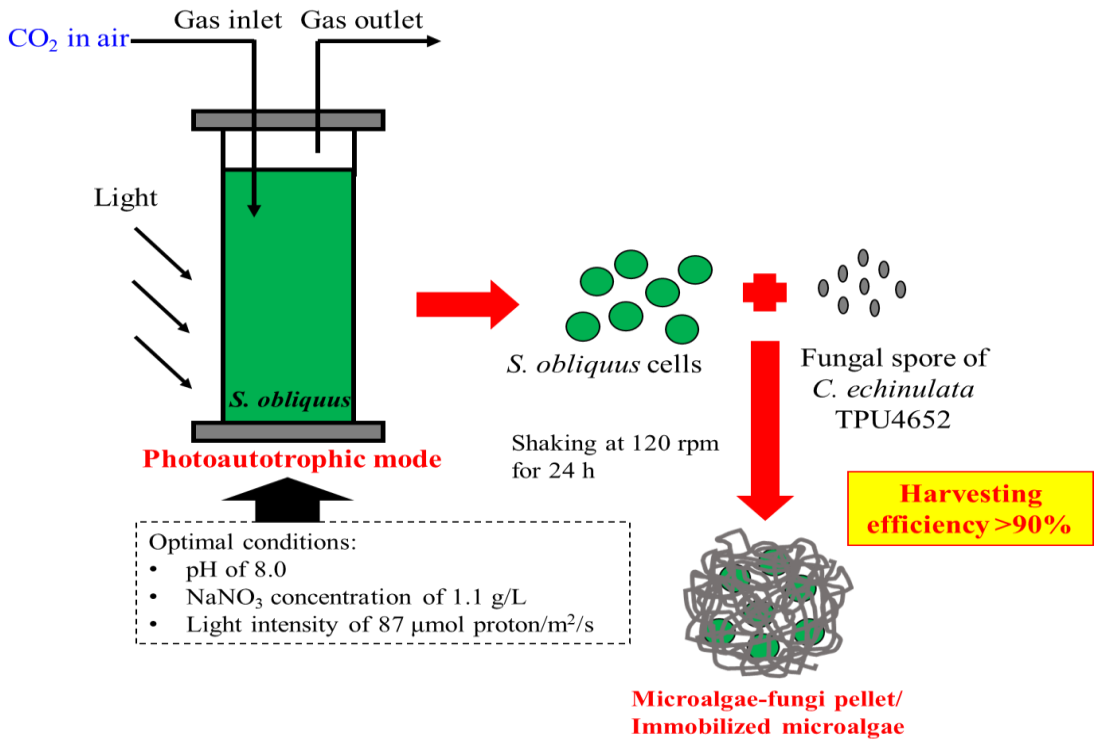


Figure 6 Conclusion diagram for Paper V.

4.6 A rapid method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi and the application in phytoremediation of secondary effluent (Paper VI)

This study aimed to use pellet-forming filamentous fungi for harvesting and immobilizing oleaginous microalgae for phytoremediation and lipid production. There are two strategies for filamentous fungi to entrap the microalgal cells: (1) the co-pelletization in which the microalgal cells are entrapped during the fungal pelletization and (2) the co-cultivation of microalgae with the fungal pellets, in which the microalgal cells are absorbed in the fungal pellets during co-cultivation. In this study, several filamentous fungi were tested for their ability to harvest oleaginous microalga *Scenedesmus* sp. through two techniques. The fungal strains including *A. niger* ATCC 6375, *T. reesei* QM 9414, and *A. oryzae* were investigated for pellets forming ability by inoculating their spores in potato dextrose broth and shaking. The effect of shaking speeds on the pellet-forming ability was evaluated from the

formation of firm pellets and the size of the pellets. *A. niger* ATCC 6375 formed loose and fluffy-like irregular aggregates rather than the pellets; nevertheless, the shaking speed was increased up to 150 rpm. While other two fungi formed the firm pellets at different sizes (0.25-8 mm in diameter) depending on the shaking speeds. A low shaking speed (80 rpm) was more suitable for the fungi to form large pellets. With increasing the shaking speed, the size of the pellets decreased. This was likely because high shaking speeds could cause high shear stress and the fungi could form large pellets (Purwanto *et al*., 2009). It should be noted that the sizes of the pellets formed and their distributions were mainly affected by both shaking speeds and types of the fungi. In addition to the pellet-forming ability, the cell growth and lipid production by the fungi were also compared. Among the strains tested, *A. niger* ATCC 6375 gave the highest biomass of 6.78 g L^{-1} and lipid production of 1.62 g L^{-1} after 24 h of cultivation followed by *T. reesei* QM 9414 and *A. oryzae*. However, as *A. niger* ATCC 6375 could not form firm pellets it was not selected. On the contrast, *T. reesei* QM 9414 which could form firm and adequate pellets and also had comparable high biomass and lipid yield was selected instead.

The selected filamentous fungus *T. reesei* QM 9414 was added directly into the microalgae culture to observe the possibility of co-pelletization with microalgal cells. Although the presence of organic carbon source and the pH of the medium have been reported as primary factors for pellet formation by the fungi (Zhou *et al*., 2012), in the medium for microalgae *T. reesei* QM 9414 could not form the pellets neither with nor without the addition of glucose as organic carbon source and at any pH tested (5-8). It was possible that the fungi needed other factors for their pellet formation. Therefore, the fungal pellets were firstly formed in PDA medium prior to their use for harvesting of microalgal cells. With this method, there was no need to adjust the pH of the microalgae culture which was at 7.8-8.0. It seems that the fungal spores germinated into hyphae and intertwined into pellets. After co-cultivation of fungal pellets with microalgae culture, it was obvious that the microalgal cells were entrapped in the hyphae of *T. reesei* QM 9414. Muradov *et al*. (2015) suggested that as the microalgae have a negative surface charge due to the presence of proton-active carboxylic, phosphoric, phosphodiester, hydroxyl, and amine functional groups and the fungal hyphae and mycelia contain polysaccharides

that have positive charge, the fungi may potentially neutralize the negative charges on the microalgal surface, enabling the attachment of microalgal cells to the fungal cell wall. As it has been reported that the fungal cells can secrete concentrated exopolysaccharides during interaction with other microorganisms (Selbmann *et al*., 2003; Miranda *et al*., 2015), it was also possible that the microalgal cells might not only get entrapped within fungal pellets but also get attached with the sticky exopolysaccharides secreted by the fungi.

In this study, three volume ratios of fungal pellets to microalgae culture (F/M ratio) at 1:10, 1:5, and 1:2 were tested to evaluate their effects on harvesting efficiency. The harvesting efficiency increased with increasing the harvesting time. When using 1:10 F/M ratio, the harvesting efficiency continuously increased up to 58.4% during first 2 h of harvesting time and slowly reached 69.3% at 4 h. When the F/M ratio was increased up to 1:5, the harvesting efficiency rapidly increased up to 83.6% within 1.5 h and saturated at this level. Interestingly, when using 1:2 F/M ratio the harvesting efficiency reached 94.5% within 10 min and almost all microalgal cells were entrapped in the fungal pellets within 30 min. The total biomass and lipid yield of the fungal pellets after harvesting of microalgal cells were 2.17 g L^{-1} and 0.60 g L^{-1}, respectively, when using 1:10 F/M ratio. With increasing the F/M ratio up to 1:5, the total biomass and lipid yield were increased up to 3.78 g L^{-1} and 0.75 g L^{-1}, respectively. At the highest F/M ratio of 1:2, the total biomass and lipid yield were 6.64 g L^{-1} and 1.70 g L^{-1}, respectively. It should also be noted that this study has shown the superior harvesting efficiency of the oleaginous microalgae than other studies. Zhang and Hu (2012) have evaluated the harvesting of *Chlorella vulgaris* using pellet forming fungi. However, in their study the flocculation efficiency was only about 60% and the total fatty acid was <0.3 g L^{-1}. Xie *et al*. (2013) also found that when using the F/M ratio of 1:2, nearly 99% of the *C. vulgaris* cells were removed from the liquid medium by co-cultivation with fungus *Cunninghamella echinulata* but the harvesting time required was as long as 2 days. The total lipid yields in their study were in the range of 0.75-1.0 g L^{-1}. Bhattacharya *et al*. (2017) found that when using 1:5 F/M ratio 99% of *Chlorella pyrenoidosa* could be harvested by the fungus *Aspergillus fumigatus* within 3 h but there is no available information regarding the lipid yield.

As this method requires low energy inputs and no addition of chemicals, it is therefore suitable as a new insight for immobilization of microalgal cells. According to the harvesting efficiency and initial microalgal cells in the culture (1 g L^{-1}), at 1:10 F/M ratio 0.693 g (693 mg) of microalgal cells would be entrapped in 100 mL of fungal pellet volume and this corresponded to 6.93 mg/mL microalgal cells in the pellets. This high concentration of microalgal cells could also be observed from the dark green color of the pellets. The microscopic photo also showed the high cell density of the microalgae inside the pellets. When using higher amount of fungal pellets at 1:5 F/M ratio, the color of pellets was lighter green and had less microalgal cells inside the pellets. At this F/M ratio, the approximate microalgal cells inside the pellets was (836 mg/200 mL =) 4.18 mg mL^{-1}. When using F/M ratio at 1:2, the green color of the pellets became much lighter and the approximate microalgal cells inside the pellets was (945 mg/500 mL =) 1.89 mg mL^{-1}. Therefore, the harvesting efficiency, harvesting time, and the density of microalgal cells inside the pellets could be manipulated by varying the volume ratio of fungal pellets to microalgae culture.

After harvesting of microalgal cells in the fungal pellets, the microalgae–fungal pellets were used as immobilized cells and applied for phytoremediation of effluent from seafood processing plants. The initial pH, COD, TN, and TP were 7.7, 1,239 mg L^{-1}, 144 mg L^{-1}, and 18.6 mg L^{-1}, respectively. The microalgae-fungal pellets were inoculated in sterilized and non-sterilized effluent. Although it was not possible to evaluate the quantitative change of the microalgal cells inside the pellets, the pollutant removal and pH change during the cultivation of microalgae-fungal pellets were observed. As the concentration of COD was much higher than those of total nitrogen and total phosphorus, the removal efficiency for COD was monitored in detail and the removal efficiency of TN and TP were determined after cultivation for 7 days. The pH increased from 7.4 to above 8.5 and there was no significant difference between those of sterilized and non-sterilized effluent. These results were consistent with those studied by Wrede *et al*. (2014) and Miranda *et al*. (2015) who reported that the industrial wastewaters are suitable as low-cost media for cultivation of the microalgae-fungal pellets. The biomass of the immobilized cells slightly increased during cultivation, indicating that the immobilized cells could grow and use the nutrients in the effluents. The immobilized

cells could reduce the COD better when using nonsterilized effluent (>74%) than sterilized effluent (>54%). The reduction of total nitrogen and total phosphorus were 63 mg L^{-1} and 18.6 mg L^{-1}, respectively. These corresponded to the removal efficiencies of 44% and 93%. The better removal efficiency of TP resulted from the much lower initial concentration of phosphorus (18.6 mg L^{-1}) compared to that of nitrogen (144 mg L^{-1}). It was possible that with non-sterilized effluent, other contaminated microorganisms might also use the nutrients in the effluent and contribute to a better pollutant removal. As the ratio of microalgae and fungi could not be quantitatively determined, the SEM analysis was performed to qualitatively determine the presence of microalgae and fungi in the pellets. The SEM images show that the microalgal cells not only get entrapped within the fungal pellets but also get attached to the fungal hyphae with sticky exopolysaccharides possibly secreted by the fungi. Interestingly, the indigenous bacteria from the effluent also got attached to the fungal hyphae. In this consortia system, a symbiosis between microalgae and aerobic microorganisms including fungi and bacteria would occur. As the microalgae could generate oxygen through their photosynthesis, this could satisfy the oxygen requirement by aerobic microorganisms. While the aerobic microorganisms could produce CO_2 and serve as carbon source for microalgae growth (Jia and Yuan, 2016). Similarly, Cao *et al*. (2017) and Wang *et al*. (2017) also found that the immobilized microalgal cells in fungal pellets could remove pollutants from the industrial effluents but there was no report on their lipid production. Wrede *et al*. (2014) and Miranda *et al*. (2015) studied the entrapment of microalgal cells by *Aspergillus fumigatus* and the further use of microalgae-fungi pellets for removal of nitrogen and phosphorus in swine wastewater. However, the lipid yield in their study was less than 0.3 g L^{-1}.

The lipids extracted from the microalgae-fungal lipids cultivated under nonsterilized effluent were converted to fatty acid methyl ester (FAME). The fatty acids profiles are composed mostly of palmitic acid (C16:0, 32.06%), oleic acid (C18:1, 24.51%), linolenic acid (C18:3, 14.16%), and arachidic acid (C20:0, 10.57%). The percentages of saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) observed in the microalgae-fungi lipids were 55.34 and 44.66, respectively. The important biodiesel properties such as IV, SV, CN, DU, LCSF, and CFPP that determine the quality of biodiesel are estimated based on the fatty acid compositions.

The SV of the microalgae-fungi pellets was found to be 204.36 mg KOH/g oil. The IV in this study was 71.64 g I_2/100 g oil which indicates the high stability against oxidation. The CN of this study was 56.89. The DU was found to be 64.84. The CFPP and LCSF were found to be 20.38 and 47.54 °C, respectively, which indicates poor performance in cold countries, but can be readily used in tropical regions.

From this paper, it could be concluded that i) *T. reesei* rapidly harvested *Scenedesmus* sp. cells within 10 min, ii) pollutants in effluent were effectively removed by the microalgae-fungi pellets, and iii) lipids extracted from microalgae-fungi pellets are suitable as biodiesel feedstocks. The conclusion diagram for this paper is shown in Figure 7.

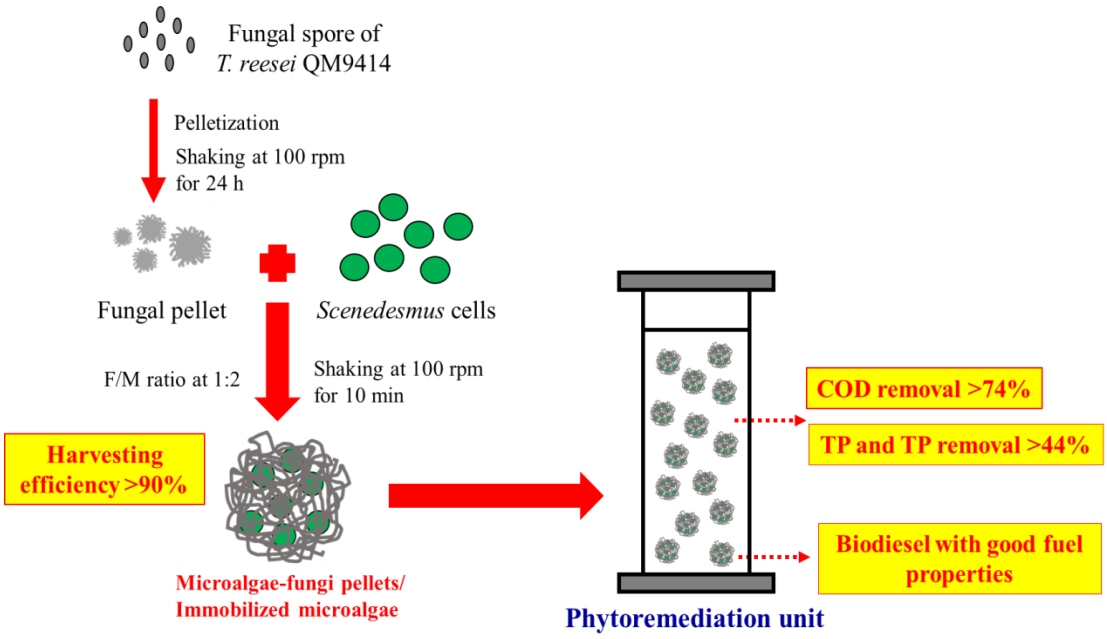


Figure 7 Conclusion diagram for Paper VI.

5. Concluding remarks

Among oleaginous microalgae tested, *Scenedesmus* sp. was found to be the most suitable strain for removing CO_2 from biogas and simultaneously producing lipids. The optimized conditions for maximizing the CO_2 removal efficiency and lipid productivity were: cultivation in modified Chu13 medium added with KNO_3 0.8 g L^{-1} as nitrogen source, inoculated with 10^7 microalgal cells mL^{-1}, illuminated at 5.5 klux light intensity and fed with biogas at a flow rate of 0.3 L h^{-1} per 1 L-microalgae culture. Under these conditions, >76% of CO_2 was effectively removed from biogas and the CH_4 content in biogas was increased from 60% up to >90% coupled with lipid productivity of 88.57 mg L^{-1} day^{-1}. The CO_2 removal rate was increased up to 4.00 g CO_2 day^{-1} per 1 L-microalgae culture which was 1.57 folds of the cultivation before optimization. The microalgal lipids contained mainly C16-C18 fatty acids which their prospect fuel properties meet the biodiesel specification. To increase the performance of *Scenedesmus* sp., the importance growth factors were stepwise increased. The most effective strategy was simultaneous stepwise-increasing of light intensity, nitrogen source addition and biogas flow rate. Through these strategy, >96% of CO_2 was continuously removed from biogas and the CH_4 content in the purified biogas was increased from 60% up to >98%. The lipid productivity was increased up to 139.38 mg L^{-1} day^{-1} which was 1.64 folds of the cultivation using constant growth factors. The CO_2 removal rate by this process was increased up to 6.50 g CO_2 day^{-1} per 1 L-microalgae culture which was 1.63 fold of the cultivation using constant growth factors. The microalgal biomass was also 1.42 folds increased from 3.12 g L^{-1} before optimization up to 4.40 g L^{-1} with relatively high lipid content of 34.10%.

Scenedesmus sp. was then immobilized in alginate gel beads in order to simultaneously purify biogas and treat effluent from anaerobic digester (AD). The immobilized microalgal cells can be harvested by a simple sieving method without involving huge amount of energy and cost. The optimal condition from immobilization of microalgal cells were: the use of initial cell concentration of 10^6 cells mL^{-1} and bead to medium volume ratio of 25% v/v. The optimal conditions for biogas purification and phytoremediation of AD effluent were: 20% AD effluent

concentration and light intensity at 9.5 klux, which could achieve 88.46% CO_2 removal from biogas and increase the CH_4 content in biogas from 60% up to 95.37%. The CO_2 removal rate was 4.63 g CO_2 day^{-1} per 1 L-microalgae culture. The immobilized microalgae also effectively removed 71.25% COD, 100% nitrogen and 100% phosphorus. The lipid productivity was 109.58 mg L^{-1} day^{-1}. The harvested microalgae biomass was 2.98 g L^{-1} with relatively high lipid content of 35.92%.

Apart from biogas purification, the cell growth and lipid production of the oleaginous microalgae were optimized. The microalgae were photoautotrophically cultivated in BG-11 medium and fed with CO_2 in air. The nutrient starvation was attempted to increase the lipid content. Starvation of either phosphorus (P starvation) or ferrous (Fe starvation) less affected cell growth but did stimulate lipid accumulation and increased the lipid content up to 39.75% and 40.28%, respectively, which was 1.2 folds of those with nutrient-rich condition. While nitrogen starvation severely limited cell growth but most effectively increased lipid content up to 56.80%, which was 1.6 folds of those with nutrient rich condition. Interestingly, the lipid accumulated during nitrogen starvation contained extremely high saturated fatty acids (almost 100%) which could make biodiesel with better fuel properties and higher oxidation stability. After microalgae cultivation, the pre-concentration by chitosan-induced flocculation was optimized. The maximum flocculation efficiency greater than 99% was achieved using minimal chitosan dosage of 98.5 g per kg microalgal biomass with adjusted pH to 4.0.

To develop a rapid method for harvesting and simultaneously immobilizing microalgae cells, the filamentous fungi was used as attractive bioflocculating agents for this purpose. There are two strategies for filamentous fungi to entrap the microalgae cells: i) the fungal spore-assisted flocculation and ii) the fungal pellets-assisted flocculation. The fungal *Cunninghamella echinulata* TPU4654 spore-assisted flocculation could effectively harvest the microalgae cells with the highest flocculation efficiency of 92.7% within 24 h. While the fungal *Trichoderma reesei* QM9414 pellets-assisted flocculation could rapidly harvest the microalgae cells up to 94% within 10 min. The obtained pellets after harvesting as immobilized cells were used for phytoremediation of secondary effluent from seafood processing plant. The immobilized microalgae-fungi could remove 74.99% COD, 44% nitrogen and

93% phosphorus from the unsterile secondary effluent. Moreover, the fungi not only effectively flocculated the microalgae cells but also increased the overall lipid yields which may offer a sustainable and efficient way to produce biofuels and simultaneously treat the industrial effluent. The extracted lipids from the immobilized microalgae-fungi were mainly composed of C16-C18 fatty acids (>83%) with their suitability as biodiesel feedstocks.

This study has shown that the oleaginous microalgae are the promising microorganisms that can be used not only for biogas purification but also production of lipids with high potential as biodiesel feedstocks. The effective strategies to increase microalgal growth, lipid content, CO_2 removal efficiency as well as the effective harvesting process and innovative immobilization of microalgae by filamentous fungi, have been proposed. Both oleaginous microalgae and microalgae-fungal pellets showed high potential for being used in lipid production and phytoremediation of industrial effluent. The extracted lipids have similar fatty acid compositions with those of plant oils indicating their suitable use as biodiesel feedstocks.

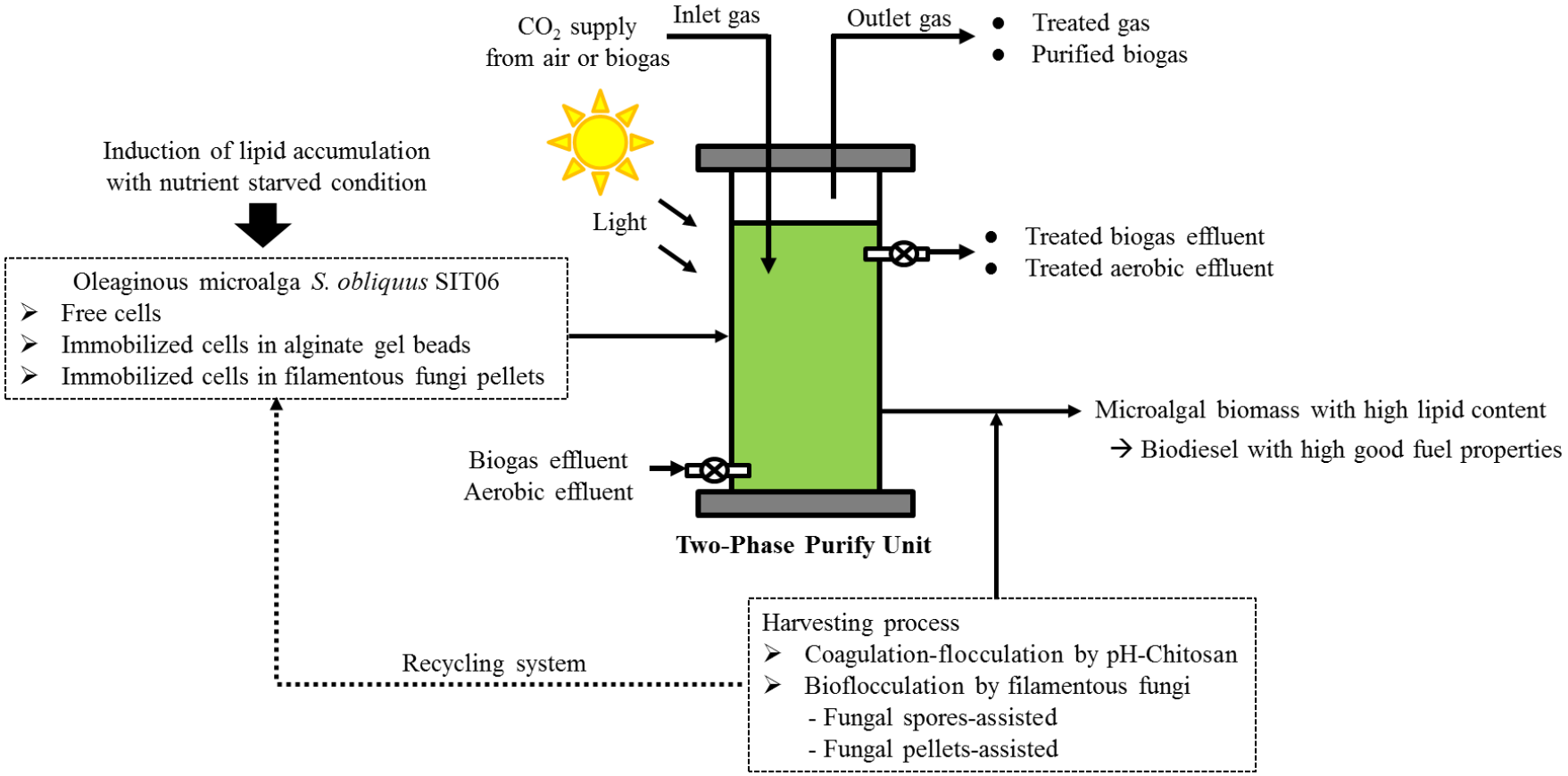


Figure 8 The proposed system scheme for simultaneous biogas upgrading, production of biodiesel feedstock and phytoremediation of wastewater using oleaginous microalgae.

6. Suggestions

1. The pilot scale of integrated outdoor microalgae cultivation with the anaerobic digester system should be investigated using biogas as CO_2 source and anaerobic digester effluent as nutrient source for further evaluation of process sustainability.

2. The process of using immobilized microalgae-fungi for combining biogas purification, phytoremediation of wastewater and production of biodiesel feedstocks should be studied in pilot scale and industrial scale.

3. The techno-economic analysis (TEA) and life cycle assessment (LCA) of microalgae cultivation for biogas upgrading, phytoremediation of effluent, harvesting process of microalgae cells and production of biodiesel feedstocks should be performed.

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Appendix

Paper I

Strategies to improve methane content in biogas by cultivation of oleaginous microalgae and the evaluation of fuel properties of the microalgal lipids

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Strategies to improve methane content in biogas by cultivation of oleaginous microalgae and the evaluation of fuel properties of the microalgal lipids

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ABSTRACT

This study aimed to improve methane content in biogas by feeding biogas through the culture of oleaginous microalgae. As oleaginous microalgae have ability to mitigate CO_2 into lipids and accumulate at the content >20%, this process not only contributes to CO_2 removal from biogas but also producing microalgal biofuel. Among the species tested, *Scenedesmus* sp. and marine *Chlorella* sp. are suitable for this purpose. However, *Scenedesmus* sp. was selected due to the higher CO_2 removal ability and the optimized conditions were as follows: gas flow rate of 0.3 L h^{-1} per 1 L- microalgal culture inoculated with 10^7 microalgal cells mL^{-1}, added with KNO_3 0.8 g L^{-1} as nitrogen source and illuminated at 5.5 klux light intensity. Under these conditions, methane content in biogas was increased from 60% up to >90% coupled with lipid productivity of 88.57 mg L^{-1} day^{-1}. With the strategy of stepwise-increasing gas flow rate to support the increasing biomass, the final biomass and lipid productivity were 1.25 and 1.79 folds increased. CO_2 removal rate was as high as 5.097 g-CO_2 day^{-1} per 1 L-microalgal culture. Fuel properties calculated based on fatty acid composition indicated high oxidation stability and high ignition quality of the microalgal lipids-derived biodiesel.

1. Introduction

In current time, biogas has been receiving increasing interest as a renewable and sustainable source of energy. Crude biogas produced by anaerobic digestion is mainly composed of 50–80% methane (CH_4) and 30–45% carbon dioxide (CO_2) and also has trace components such as water, oxygen, and hydrogen sulfide depending on the feedstock. For example, the CH_4 content in biogas from animal manure digester, industrial waste digester and household wastes have been reported to be in the range of 50–70%, 60–80% and 65%, respectively [1]. The CH_4 content in biogas is the most important criterion, especially for the combustion process in engines and electricity production. Only biogas with CH_4 content higher than 90% (v/v) meets the requirement to increase its calorific value [2], decrease the relative density of the gas and increase the Wobbe index [3]. There are several methods available for CO_2 removal and increasing CH_4 content in biogas. These include chemical absorption, water scrubbing, membrane separation and pressure-swing adsorption [4]. However, these methods require considerable large amount of energy, auxiliary materials, and chemicals. Nevertheless, these process also generate wastes and wastewater that would pollute the environment [5]. Recently, the biological method for CO_2 removal by microalgae cultivation was first developed by Mann et al. [6]. This method could improve the quality of biogas because as CO_2 is removed, the CH_4 content increases. The CO_2 removal by microalgae has received considerable attention because the CO_2 fixation capability of microalgae is high and it also gives high biomass productivity and high-value products. Meier et al. [2] found that 96% of CO_2 was removed from the biogas. However, due to the low solubility of O_2 in seawater medium most of the generated O_2 was desorbed to the gas phase. While Yan et al. [5] and Yan and Zheng [7] achieved good level of CO_2 removal >90% and only 0.2–1% O_2 was detected in the gas phase. This was because most of produced O_2 from the photosynthesis was dissolved in the medium and also used during photorespiration. Recent studies have demonstrated the use of specific microalgae strain, namely *Chlorella* sp., for CO_2 removal from biogas

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[5,7]. One of them has shown that it was also possible to use oleaginous microalgae for CO_2 removal from biogas coupling with lipid production [8]. However, only *Chlorella* spp. were tested in that research. In addition to *Chlorella*, *Nannochloropsis* and *Scenedesmus* are another well-known microalgal genera that are capable of mitigating CO_2 and accumulating lipids at a high level of 30–40% based on dry biomass [15e17]. Those should be investigated for this purpose as well.

For efficient CO_2 removal by the microalgae, the effects of light intensity, nitrogen concentration addition, initial cell concentration and gas flow rate should be intensively investigated for maximizing CO_2 removal by oleaginous microalgae. Statistical experimental design techniques, especially the Response Surface Methodology (RSM), are very useful tools for optimizing process parameters as they can provide statistical models which help us understand the interactions among parameters at varying levels and to calculate the optimal level of each parameter for a given target. Therefore, this study aimed to evaluate various species of oleaginous microalgae for CO_2 removal from biogas and simultaneously producing lipid. Four important operating parameters including initial cell concentration, gas flow rate, KNO_3 concentration as nitrogen source, and light intensity were simultaneously optimized and their combined effects were evaluated through response surface methodology (RSM). The stepwise-increasing of gas flow rate was studied to support the increased microalgal biomass and improve the CO_2 removal rate and lipid productivity. The fatty acid compositions and fuel properties of microalgal lipid were determined and their prospects for being used as biodiesel feedstocks were evaluated.

2. Materials and methods

2.1. Microalgae strains and media

Four oleaginous microalgae including marine *Chlorella* sp., freshwater *Chlorella* sp., *Nannochloropsis* sp. and *Scenedesmus* sp., were received from the National Institute of Coastal Aquaculture in Southern region of Thailand. One oleaginous *Botryococcus* sp. was obtained from a stock culture of Bioprocess Engineering Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Thailand. The modified Chu13 medium used as the basic medium in this study contained 0.2 g KNO_3 as a nitrogen source, 0.04 g K_2HPO_4 as a phosphorus source, 0.1 g citric acid, 0.01 g Fe citrate, 0.1 g $MgSO_4{\cdot}7H2O$, 0.036 g $NaHCO_3$, and 1 mL of trace metal solution per 1 L. The trace metal solution consisted of 2.85 g H_3BO_3, 1.8 g $MnCl_2{\cdot}4H_2O$, 0.02 g $ZnSO_4{\cdot}7H_2O$, 0.08 g $CuSO_4{\cdot}5H_2O$, 0.08 g $CoCl_2{\cdot}6H_2O$, and 0.05 g $Na_2MoO_4{\cdot}2H_2O$ per 1 L, pH was 6.8 [8].

2.2. Selection of oleaginous microalgae for CO_2 removal from biogas

Each microalga was precultured in 400 mL medium and aerated with air for 7 days. The microalgal cells were pelleted by centrifugation at 8500 rpm for 15 min and resuspended in 50 mL fresh medium and used as starter culture. The batch culture of the microalgae was performed by inoculating 10% of starter culture into 400 mL medium in a 500 mL Duran bottles. The initial cell concentration was fixed at 10^5 cell mL^{-1}. The cool-white fluorescent lamps were used for illumination at light intensity of 3.5 klux and photoperiod of 24 h. The agitation was performed by a magnetic stirrer at 150 rpm. The temperature was controlled at 30 ± 2 °C using waterbath temperature controller. The cultures were continuously aerated with 40% CO_2 in air or biogas with CO_2 content of 40% at a gas flow rate of 0.15 L h^{-1} per 1 L of culture medium for 7 day.

2.3. Optimization of the operating conditions for CO_2 removal from biogas and lipid production

The response surface methodology was employed to optimize the CO_2 removal from biogas and lipid production of the selected microalgae strain. The four operating parameters including light intensity, initial cell concentration, gas flow rate and KNO_3 concentration and were varied at three levels (−1, 0, +1) to determine their individual effects and combined effects on the four responses. These included microalgal biomass, lipid productivity, percent of CO_2 removal and CO_2 removal rate. The experiments were designed based on a Box–Benhken Design (BBD). The second order polynomial quadratic equation (Eq. (1)) was fitted to evaluate the main effect and interaction of each independent variable to the response.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y = predicted response, x_i and x_j = variables or parameters, β_0 = offset term, β_i = linear effect, β_{ij} = interaction effect and β_{ii} = squared effect.

2.4. Enhancing the CO_2 removal rate by stepwise-increasing of gas flow rate

To support the increased microalgal biomass during cultivation and enhance the CO_2 removal rate, the biogas was stepwise-increasingly fed. The optimal initial gas flow rate from optimization studies was used at initial and it was stepwise-increased by 0.03 L h^{-1} per 1 L-microalgal culture every 2 days and the performance was compared with the control using the constant gas flow rate.

2.5. Analytical methods

Cell concentrations was determined by using a hemocytometer. The microalgal biomass was harvested by centrifugation at 3500 × g for 15 min and the microalgal biomass were dried at 60 °C until constant weight. The specific growth rate (μ) was calculated using data in the exponential phase using the following equation:

$$\mu\ (day^{-1}) = (\ln X_2 - \ln X_1) / (t_2 - t_1) \quad (2)$$

where X_1 and X_2 are the concentrations of microalgal cells (g L^{-1}) at time t_1 and t_2, respectively.

Lipids of the dry microalgal biomass were extracted with a mixture of chloroform:methanol (2:1, v/v) and sonicated for 30 min. The suspension was centrifuged at 3500 × g for 15 min to obtain a clear supernatant. The solvent solution was evaporated overnight and the extracted lipid was determined gravimetrically. The lipid productivity (P_{lipid}) was calculated by the following equation:

$$P_{lipid}\ (mg\ L^{-1}\ day^{-1}) = (L_2 - L_1) / (t_2 - t_1) \quad (3)$$

where L_1 and L_2 were the lipid production (mg L^{-1}) at time t_1 (start point of cultivation) and t_2 (the point that the maximum lipid was obtained), respectively. The lipid content (*LC*,%w/w) was calculated with the following equation:

$$LC = [\text{ lipid production (g } L^{-1}) / \text{ dry microalgal biomass weight (g } L^{-1})] \times 100 \quad (4)$$

The compositions of biogas were determined using a Gas Chromatograph (GC 6890) with a cross-linked capillary HP-PLOT Q column (length 30 m, 0.54 mm I.D, 0.04 μm film thickness) and a

thermal conductivity detector. Helium was used as carrier gas. The percent of CO_2 removal from biogas and CO_2 fixation rate (g-CO_2 day^{-1} per 1 L-microalgal culture) were determined as follows:

$$\%CO_2 \text{ removal} = [\text{initial } CO_2\ (\%) - \text{final } CO_2\ (\%)) \,/\, \text{initial } CO_2\ (\%)] \times 100 \quad (5)$$

$$CO_2 \text{ fixation rate} = \text{gas flow rate (L day}^{-1}\text{ L}^{-1}) \times \text{initial } CO_2\,(\%) \times \%\ CO_2 \text{ removal} \times 1000 \text{ g-}CO_2/556.2 \text{ L} \quad (6)$$

The extracted lipids were converted to fatty acid methyl esters (FAMEs) by hydrolysis and esterification reactions [9]. The fatty acid compositions of the FAMEs were analyzed using a HP6850 Gas Chromatography equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm ID, 0.25 μm film thickness) and a flame ionization detector. Operating conditions were as follows: inlet temperature 290 °C; oven temperature initial 210 °C held for 12 min; then ramped to 250 °C at 20 °C/min; held for 8 min and the detector temperature was 300 °C. Fatty acids were identified by comparing their retention times with known pure standards [8]. The fuel properties were evaluated from their fatty acid composition using equations previously reported [10,11]. These properties include saponification value (SV), iodine value (IV), cetane number (CN), unsaturation degree (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP).

All experiments were performed at least in duplicates. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference ($p \leq 0.05$) was used to separate means, using the SPSS software.

3. Results and discussion

3.1. Selection of oleaginous microalgae under condition aerated with CO_2 in air

The growth and lipid production of five oleaginous microalgae including marine *Chlorella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., *Botryococcus* sp. and freshwater *Chlorella* sp. were studied in the modified Chu13 medium under condition aerated with 40% CO_2 in air (imitating CO_2 content in biogas). The results are shown in Fig. 1 and Table 1. Among the five strains tested, marine *Chlorella* sp. and *Scenedesmus* sp. grew fastest with the maximum specific growth rates of 0.34 ± 0.01 day^{-1} and 0.33 ± 0.01 day^{-1}, respectively, followed by freshwater *Chlorella* sp. and *Nannochloropsis* sp. The lipid content of these four strains were in the range of 17–28%. Although *Botryococcus* sp. accumulated lipid as high as 42.06 ± 2.91% of its dry biomass, it had a long lag time for adaptation with 40% CO_2, resulting in a low growth rate and reached a low lipid productivity. The increase in initial cell concentration may increase the tolerance against high levels of CO_2 and reduce the time for adaptation [12]. It has been reported that the lipid content of *Chlorella* sp. and *Scenedesmus* sp. could be increased up to >55% under optimized conditions [13,14].

When comparing the biomass obtained, marine *Chlorella* sp. gave the highest biomass of 0.87 g L^{-1} followed by *Scenedesmus* sp. (0.81 g L^{-1}) while those of other strains were in the range of 0.3–0.6 g L^{-1}. The maximum biomass concentrations of marine *Chlorella* sp. and *Scenedesmus* sp. were higher than those previously reported marine *Chlorella* sp. (0.6 g L^{-1}) and *Scenedesmus* sp. (0.4 g L^{-1}) [8] while were comparable to those of *Scenedesmus obliquus* (0.82 g L^{-1}) [15]. The lipid productivity of marine *Chlorella* sp. and *Scenedesmus* sp. (23–26 mg L^{-1} day^{-1}) were higher than those of other three strains. Yoo et al. [16] have compared the cultivation of three microalgae including *Botryococcus braunii*, *Scenedesmus* sp. and *Chlorella vulgaris* under condition aerated with 10% CO_2 at 0.3 air volume per medium volume per min (vvm). They found that *Scenedesmus* sp. gave the highest lipid productivity of 20.65 mg L^{-1} day^{-1} followed by *C. vulgaris* (6.91 mg L^{-1} day^{-1}). Several reports also confirmed that these two species were able to grow under high CO_2 content of 10–50% [15,18].

3.2. Selection of oleaginous microalgae using synthetic biogas

The tolerance to high CO_2 content and high CH_4 content in biogas are great important characteristics of oleaginous microalgae for being used in CO_2 removal from biogas. From the previous section, as marine *Chlorella* sp. and *Scenedesmus* sp. gave comparative high growth rate and high lipid productivity under condition aerated with 40% CO_2 in air, they were selected for the experiments using synthetic biogas. The synthetic biogas was prepared by mixing with CH_4 at a ratio of 40:60 v/v. The influence of using biogas on cell concentration, biomass, lipid production and lipid content of both strains are shown in Fig. 2. Although the biomass of marine *Chlorella* sp. was slightly higher than that of *Scenedesmus*

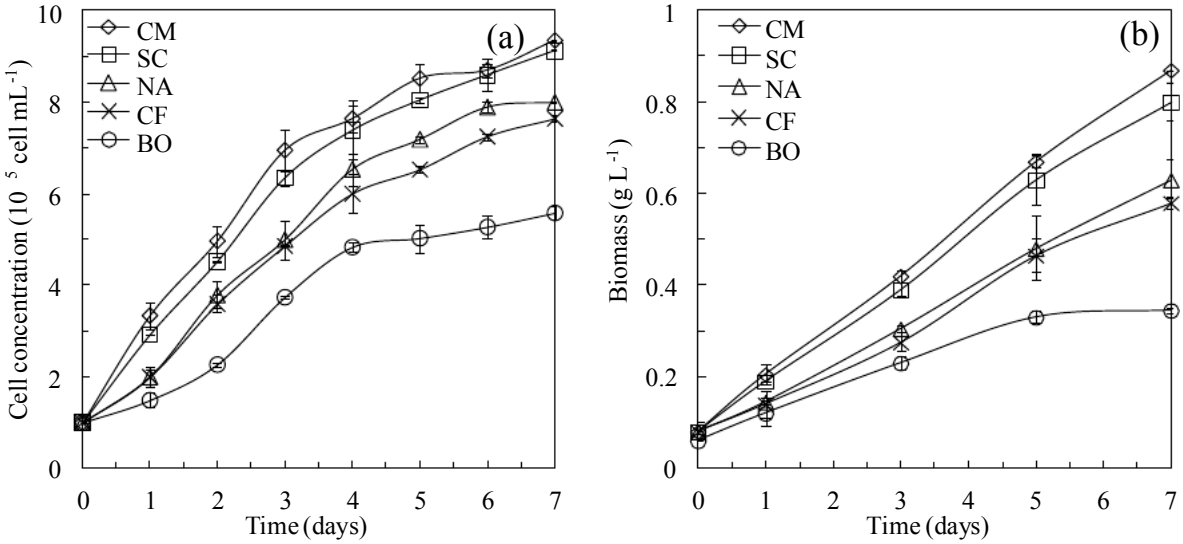

Fig. 1. Cell concentration (a) and Biomass (b) of five oleaginous microalgae strains cultivated in modified Chu13 medium and aerated with 40% CO_2 in air. CM: Marine *Chlorella* sp., SC: *Scenedesmus* sp., NA: *Nannochloropsis* sp., CF: Freshwater *Chlorella* sp. and BO: *Botryococcus* sp. Data are means of triplicates.

Table 1
Specific growth rate (μ), lipid content and lipid productivity of five oleaginous microalgae strains cultivated in modified Chu13 medium and aerated with 40%CO_2 in air.

Microalgae strain	μ (day^{-1})	Lipid content (%dry weight cell)	Lipid productivity ($mg\ L^{-1}\ day^{-1}$)
Marine *Chlorella* sp. (CM)	0.34 ± 0.01^{a}	21.84 ± 0.00^{c}	25.71 ± 0.00^{a}
Scenedesmus sp. (SC)	0.33 ± 0.01^{a}	27.60 ± 1.04^{b}	23.57 ± 1.01^{a}
Nannochloropsis sp. (NA)	0.29 ± 0.01^{b}	17.20 ± 1.18^{d}	12.86 ± 2.02^{c}
Botryococcus sp. (BO)	0.21 ± 0.02^{d}	42.06 ± 2.91^{a}	17.86 ± 1.01^{b}
Freshwater *Chlorella* sp. (CF)	0.25 ± 0.06^{c}	24.60 ± 0.57^{bc}	14.50 ± 0.71^{c}

Data in the same column with different superscript letters are significantly different ($P < 0.05$).

sp., its lipid content was much lower (Table 2). Their lipid productivity were then not significantly different. It should be noted that the performance of marine *Chlorella* sp. and *Scenedesmus* sp. using biogas were similar to those using 40% CO_2 in air. These results indicated that the CH_4 content in biogas at 60% had no negative effect on the performance of these two microalgae. Several researchers have also attempted to use microalgae for CO_2 removal from biogas [2,5]. Only Tongprawhan et al. [8] attempted to use oleaginous *Chlorella* spp. for this purpose. However, the lipid production in their studies (94.7 mg L^{-1}) [8] was much lower than the lipid productions by marine *Chlorella* sp. and *Scenedesmus* sp. in this study (300–320 mg L^{-1}). During 7 days of cultivation, CH_4 content in the outlet biogas increased higher than 98%. The percent CO_2 removal were 95% and 98% for marine *Chlorella* sp. and *Scenedesmus* sp., respectively. The percent CO_2 removal in this study are comparable to the results of Yan and Zheng [7] and Yan et al. [5] who used *Chlorella* sp. for biologically removing CO_2 from biogas. Comparing between two strains, the percent CO_2 removal and CO_2 fixation rate by *Scenedesmus* sp. were slightly higher than those by marine *Chlorella* sp. (Table 2). Thus, *Scenedesmus* sp. was selected as the most suitable strain for CO_2 removal from biogas coupled with lipid production.

3.3. Optimization of operating conditions through response surface methodology

3.3.1. Statistical analysis

Response surface methodology was used to optimize operating conditions for CO_2 removal from biogas and lipid production by the selected *Scenedesmus* sp. The independent variables for the process were gas flow rate ($L\ h^{-1}$ per 1 L-microalgal culture; A), initial cell concentration (log cells mL^{-1}; B), KNO_3 concentration ($g\ L^{-1}$; C) and light intensity (klux; D). Their values were varied in the ranges shown in Table 3 and coded at three levels of −1, 0 and + 1. The experimental results were concerned with biomass ($g\ L^{-1}$; Y_1), lipid productivity ($mg\ L^{-1}\ day^{-1}$; Y_2), percent CO_2 removal from biogas (%; Y_3) and CO_2 fixation rate ($g\text{-}CO_2\ day^{-1}\ L^{-1}$; Y_4) using the four-factor BBD experimental design (Table 3). The conditions at the center point were a gas flow rate of 0.3 $L\ h^{-1}\ L^{-1}$, an initial cell concentration of 10^6 cells mL^{-1}, KNO_3 concentration of 0.5 $g\ L^{-1}$ and a light intensity of 4.5 klux. The responses Y_1, Y_2, Y_3 and Y_4 were fitted using second order polynomial Eqs. (7)–(10), respectively.

$$Y_1 = -4.56 + 0.57A - 0.55B - 1.77C + 1.51D - 0.065A^2 + 0.092B^2 - 2.52C^2 - 0.25D^2 - 2.00 \times 10^{-3}AB + 3.33 \times 10^{-3}AC + 0.025AD + 0.47BC + 0.08BD + 0.45CD \quad (7)$$

$$Y_2 = 129.09 + 1.12A - 46.17B - 25.40C - 18.71D - 0.75A^2 + 8.00B^2 - 100.36C^2 + 1.35D^2 - 1.97AB + 0.093AC + 4.14AD + 14.23BC - 2.68BD + 7.98CD \quad (8)$$

$$Y_3 = 321.55 - 21.42A - 43.44B - 3.60C - 24.63D + 1.72A^2 + 2.63B^2 + 3.04C^2 - 0.24D^2 - 0.21AB - 1.23AC + 0.029AD - 4.92BC + 3.43BD + 11.08CD \quad (9)$$

$$Y_4 = 11.57 + 0.3625A - 2.4595B - 1.1392C - 1.1909D + 0.0285A^2 + 0.1605B^2 + 0.0699C^2 - 0.0259D^2 - 0.0337AB + 0.1372AC + 0.0037AD - 0.2589BC + 0.1812BD + 0.5955CD \quad (10)$$

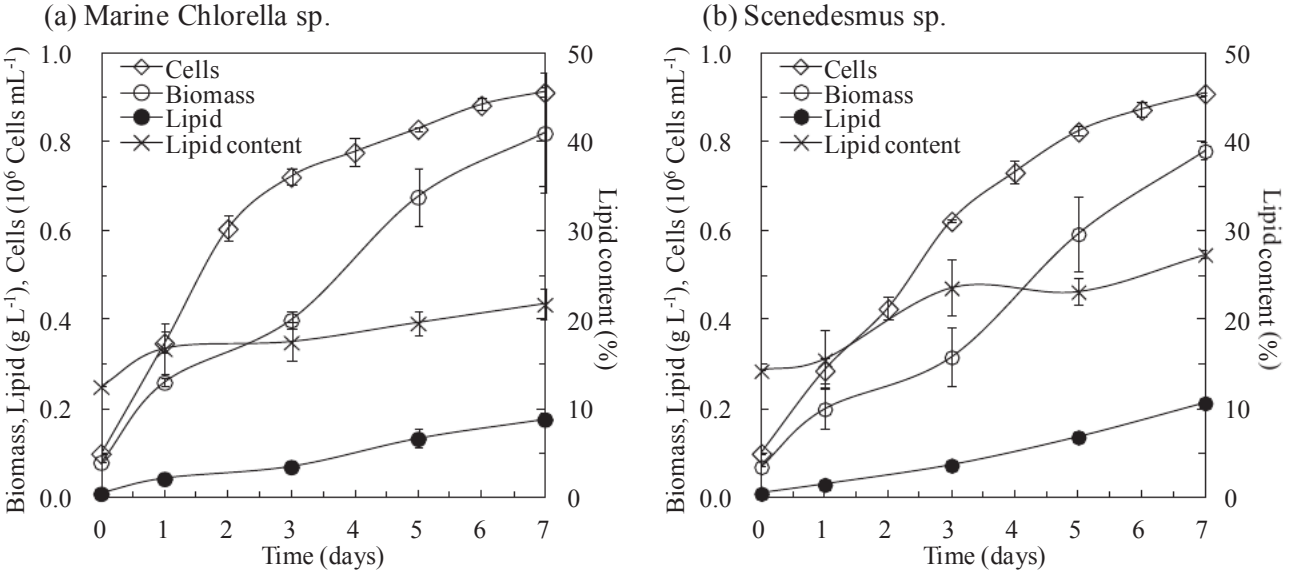

Fig. 2. Cultivation of Marine *Chlorella* sp. and *Scenedesmus* sp. in modified Chu13 medium and aerated with 40% CO_2 in biogas. Data are means of triplicates.

Table 2
Specific growth rate (μ), lipid content and lipid productivity of selected oleaginous microalgae strains cultivated in modified Chu13 medium and aerated with 40%CO_2 in biogas.

Microalgae strain	μ (day^{-1})	Lipid content (%dry weight cell)	Lipid productivity (mg L^{-1} day^{-1})	CH_4 content after CO_2 removal (%)	%CO_2 removal	CO_2 fixation rate (g-CO_2 $day^{-1}L^{-1}$)
Marine *Chlorella* sp. (CM)	0.33 ± 0.04[a]	21.74 ± 1.77[a]	25.24 ± 1.01[a]	98.21 ± 0.44[a]	95.55 ± 1.05[b]	2.47 ± 0.03[b]
Scenedesmus sp. (SC)	0.33 ± 0.01[a]	27.36 ± 1.95[b]	26.19 ± 0.82[a]	99.39 ± 0.22[a]	98.47 ± 0.53[a]	2.55 ± 0.01[a]

Data in the same column with different superscript letters are significantly different ($P < 0.05$).

The multiple correlation coefficients or R^2 of the regression equation from analysis of variance (ANOVA) were 0.96, 0.92, 0.90 and 0.95, respectively. This indicated that up to 96%, 92%, 90% and 95% of the variations in response can be explained by the model. These quadratic equations could appropriately describe the relationships between the factors and the responses. As shown in Table 4, the value of the adjusted determination coefficient (adjust R^2) is quite high, indicating a high significance of the model. In addition, the ANOVA quadratic regression model demonstrated that the model was highly significant, as evidenced from the Fisher's F-test with a very low probability ($P \leq 0.0001$). The *P-values* of lack of fit greater than 0.05, for the four responses indicated that the residual error to the pure error from the replicated experimental design points were insignificantly different (Table 4). The low coefficient value (C.V. <20) also indicated a high precision and reliability of the experiments.With the RSM, the effect of each variable on each response as well as the contribution of the joint effect of variables can be observed.

3.3.2. Effect on microalgal biomass

From experimental design in Table 3, the microalgal biomass ranged from 0.53 to 3.46 g L^{-1}. The optimum conditions for maximizing biomass, calculated by setting the partial derivatives of Eq. (7) to zero with respect to the corresponding variables, were gas flow rate of 0.317 L h^{-1} per 1 L-microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.75 g L^{-1}, and light intensity of 5.09 klux. The maximum calculated value for microalgal biomass was 3.37 g L^{-1}. Fig. 3 shows the three dimensional and contour plots based on Eq. (7) with two variables kept constant at their optimum levels and other two variables varied within the experimental range. The main objective of response surface analysis is to unravel the optimum combination of variables in order to maximize the response.

The significance of each coefficient was determined by probability values which are listed in Table 4. It was obvious that the significant variables for biomass production were initial cell concentration (B) and KNO_3 concentration (C). The initial cell concentration is a fundamental parameter that controlled the growth rate and fermentation time. The higher initial cell concentration would give a faster growth rate and hence higher biomass production [8]. Another major factor that affects the biomass is nitrogen source and its concentration. From Fig. 3, it could be observed that three important growth factors including light intensity, KNO_3 concentration and gas flow rate at high levels did improve the microalgal biomass and gave high biomass production. Arumugam et al. [19] reported that KNO_3 performed better than $NaNO_3$, Urea, $CaNO_3$, NH_4NO_3 and NH_4Cl for biomass production of *Scenedesmus*. This was because nitrogen and potassium in KNO_3 are

Table 3
Box–Benhken experimental design with experimental responses.

Run	Gas flow rate (L h^{-1} L^{-1})	Initial cell concentration (log cells mL^{-1})	KNO_3 concentration (g L^{-1})	Light intensity (klux)	Biomass (g L^{-1})		Lipid productivity (mg L^{-1} day^{-1})		%CO_2 removal		CO_2 fixation rate (g-CO_2 day^{-1} L^{-1})	
					Experiment value	Predicted value	Experiment value	Predicted value	Experiment value	Predicted value	Experiment value	Predicted value
1	0.30 (0)	5 (-1)	0.8 (1)	4.5 (0)	0.60	0.72	12.85	13.47	78.46	77.16	4.14	4.09
2	0.30 (0)	5 (-1)	0.5 (0)	3.5 (-1)	0.60	0.66	28.28	24.59	70.12	75.09	3.70	3.99
3	0.30 (0)	6 (0)	0.8 (1)	3.5 (-1)	1.47	1.42	37.14	41.67	70.86	70.11	3.73	3.68
4	0.45 (1)	5 (-1)	0.5 (0)	4.5 (0)	0.71	0.58	34.81	29.64	70.01	67.78	5.54	5.39
5	0.30 (0)	6 (0)	0.8 (1)	5.5 (1)	1.89	1.88	55.71	50.56	74.20	75.58	3.91	3.96
6	0.15 (-1)	6 (0)	0.8 (1)	4.5 (0)	1.47	1.43	38.57	38.64	99.02	99.59	2.61	2.59
7	0.15 (-1)	5 (-1)	0.5 (0)	4.5 (0)	0.58	0.45	17.14	17.00	95.38	96.38	2.51	2.54
8	0.30 (0)	6 (0)	0.5 (0)	4.5 (0)	2.25	1.96	55.00	69.80	62.36	68.72	4.01	3.62
9	0.30 (0)	6 (0)	0.2 (-1)	3.5 (-1)	1.33	1.36	50.00	48.86	67.03	68.56	3.55	3.60
10	0.45 (1)	7 (1)	0.5 (0)	4.5 (0)	2.69	2.84	96.00	89.85	64.89	66.80	5.13	5.23
11	0.15 (-1)	6 (0)	0.2 (-1)	4.5 (0)	1.24	1.11	42.00	41.18	85.69	89.55	2.25	2.36
12	0.30 (0)	6 (0)	0.5 (0)	4.5 (0)	2.25	1.96	55.00	55.00	69.80	68.72	4.01	3.62
13	0.30 (0)	7 (1)	0.5 (0)	5.5 (1)	3.12	3.11	90.00	98.74	73.38	73.96	3.88	3.91
14	0.45 (1)	6 (0)	0.5 (0)	3.5 (-1)	1.39	1.21	42.85	40.68	67.33	64.94	5.33	5.13
15	0.15 (-1)	7 (1)	0.5 (0)	4.5 (0)	2.58	2.73	98.00	96.88	92.34	97.48	2.43	2.69
16	0.45 (1)	6 (0)	0.5 (0)	5.5 (1)	1.62	1.52	62.86	65.50	67.86	63.90	5.39	5.10
17	0.30 (0)	7 (1)	0.8 (1)	4.5 (0)	3.46	3.26	98.00	92.06	75.87	74.27	4.01	3.91
18	0.30 (0)	7 (1)	0.2 (-1)	4.5 (0)	2.83	2.65	85.30	85.92	76.19	69.02	4.01	3.65
19	0.45 (1)	6 (0)	0.8 (1)	4.5 (0)	1.38	1.56	35.71	41.58	66.42	68.11	5.26	5.46
20	0.30 (0)	6 (0)	0.5 (0)	4.5 (0)	2.25	1.96	55.00	55.00	69.80	68.72	4.01	3.62
21	0.30 (0)	5 (-1)	0.2 (-1)	4.5 (0)	0.53	0.67	17.23	24.41	72.88	66.02	3.86	3.52
22	0.30 (0)	5 (-1)	0.5 (0)	5.5 (1)	0.74	0.68	32.86	34.05	62.64	67.06	3.31	3.55
23	0.30 (0)	6 (0)	0.5 (0)	4.5 (0)	2.25	1.96	55.00	55.00	69.80	68.72	4.01	3.62
24	0.45 (1)	6 (0)	0.2 (-1)	4.5 (0)	1.14	1.22	38.86	43.84	56.78	61.76	4.50	4.84
25	0.30 (0)	6 (0)	0.2 (-1)	5.5 (1)	1.21	1.27	59.00	48.17	57.07	60.73	3.00	3.18
26	0.30 (0)	7 (1)	0.5 (0)	3.5 (-1)	2.66	2.76	96.14	100.00	67.16	68.30	3.55	3.60
27	0.30 (0)	6 (0)	0.5 (0)	4.5 (0)	2.25	1.96	55.00	55.00	69.80	68.72	4.01	3.62
28	0.15 (-1)	6 (0)	0.5 (0)	5.5 (1)	1.16	1.28	38.57	41.98	99.46	93.39	2.61	2.41
29	0.15 (-1)	6 (0)	0.5 (0)	3.5 (-1)	1.18	1.22	60.00	58.60	99.22	94.72	2.61	2.49

Table 4
Model coefficient and analysis of response variance estimated by ANOVA.

Source	Microalgal biomass		Lipid productivity		$\%CO_2$ removal		CO_2 removal rate	
	Coefficient estimate	Probability	Coefficient estimate	Probability	Coefficient estimate	Probability	Coefficient estimate	Probability
Model intercept	−4.56	<0.0001[a]	129.09	<0.0001[a]	321.55	0.0001[a]	11.5728	<0.0001[a]
A-Gas flow rate	0.57	0.361	1.12	0.6476	−21.42	<0.0001[a]	0.3625	<0.0001[a]
B-Initial cell concentration	−0.55	<0.0001[a]	−46.17	<0.0001[a]	−43.44	0.9861	−2.4595	0.9648
C-KNO_3 concentration	−1.77	0.0206[a]	−25.40	0.6949	−3.6	0.0225[a]	−1.1392	0.0269[a]
D-Light intensity	1.51	0.1675	−18.71	0.5055	−24.63	0.7166	−1.1909	0.7203
A^2	−0.065	0.0004[a]	−0.75	0.2727	1.72	0.0002[a]	0.0285	0.1434
B^2	0.092	0.3068	8.00	0.0701	2.63	0.2467	0.1605	0.1828
C^2	−2.52	0.0199[a]	−100.36	0.0439 [a]	3.04	0.9018	0.0699	0.9570
D^2	−0.25	0.0119[a]	1.35	0.7459	−0.24	0.9122	−0.0259	0.8166
AB	−0.002	0.9644	−1.97	0.3598	−0.21	0.8538	−0.0337	0.5780
AC	0.0033	0.9822	0.093	0.9894	−1.23	0.7440	0.1372	0.4923
AD	0.025	0.5791	4.14	0.0659	0.029	0.9795	0.0037	0.9504
BC	0.47	0.2241	14.23	0.4248	−4.92	0.6027	−0.2589	0.6022
BD	0.08	0.4792	−2.68	0.6139	3.43	0.2366	0.1812	0.2359
CD	0.45	0.2402	7.98	0.6521	11.08	0.2499	0.5955	0.2485
R^2[b]	0.96	–	0.92	–	0.90	–	0.95	–
Adjusted R^2[b]	0.93	–	0.84	–	0.80	–	0.90	–
C.V.[c]	13.47	–	19.52	–	7.46	–	7.77	–
Lack of fit	–	0.9174	–	0.9810	–	0.4555	–	0.4571

[a] Significant level at 95%.
[b] R^2 = Regression coefficient.
[c] C.V. = coefficient value.

the two important nutrients for algal growth. In addition, they also suggested that the nitrogen concentration beyond 10 mM or 1.01 g L^{-1} exhibited poor microalgal biomass production, which may be due to the deleterious effect of nitrogen at high concentrations. The optimum nitrogen concentration for the growth of *Scenedesmus* in their study was 5–10 mM (0.5–1.0 g L^{-1}).

3.3.3. Effect on lipid productivity

From experimental design in Table 3, the lipid productivity ranged from 12.86 to 98.00 mg L^{-1} day^{-1} (Table 3). The lipid productivity significantly depended on the initial cell concentration. When using low initial cell concentration the lipid productivity was limited at 12.8–17.2 mg L^{-1} day^{-1} (Run 1, 7 and 21), while using high initial cell concentration the lipid productivity was as high as >85 mg L^{-1} day^{-1} (Run 10, 13, 15, 17, 18 and 26). In addition, Table 4 also shows that the linear term of initial cell concentration (B) significantly affected on lipid productivity. The three-dimensional and contour plots shown in Fig. 4 are based on Eq. (8) with two variables kept constant at theirs optimum level and the other two variables varied within the experimental range. The optimum conditions for maximizing lipid productivity were: slightly high gas flow rate of 0.413 L h^{-1} per 1 L microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.61 g L^{-1}, and light intensity of 5.43 klux. The maximum response value for lipid productivity was estimated to be 100.61 mg L^{-1} day^{-1}. Compared with the optimal conditions for biomass, higher gas flow rate with lower KNO_3, namely higher carbon to nitrogen ratio were more suitable for lipid production.

Nitrogen deficiency severely affects protein synthesis and reduces photosynthetic rates which result in metabolic flux towards lipid biosynthesis [20]. Yeh and Chang [21] reported that the nitrogen limitation is the key factor influencing the lipid accumulation of *Chlorella vulgaris*. Low nitrogen concentration could increase the lipid content of the microalgae. However, there is a tradeoff between biomass and lipid content. The microalgae could not grow well under nitrogen limitation and resulted in low biomass. Therefore to increase overall lipid productivity along with nitrogen stress, a two-step cultivation in which the condition for cell growth (low carbon to nitrogen ratio) is applied in the first step and the nitrogen stress (high carbon to nitrogen ratio) is applied in the second step, should be considered [20–22].

3.3.4. Effect on CO_2 removal efficiency

The percent CO_2 removal from biogas ranged from 56.78 to 99.46% (Table 3). The variables with a significant effect on CO_2 removal were the gas flow rate (A) and KNO_3 concentration (C) ($P < 0.05$) (Table 4). The quadratic term of gas flow rate (A^2) was also significant ($P < 0.05$), demonstrating that the percent CO_2 removal required a suitable gas flow rate. The optimum conditions for maximizing the percent CO_2 removal were gas flow rate of 0.15 L h^{-1} per 1 L microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.62 g L^{-1}, and light intensity of 5.28 klux. The maximum response value for percent CO_2 removal was estimated to be 100%. The response surface of percent CO_2 removal indicated that percent CO_2 removal increased with decreasing gas flow rate (Fig. 5a, b and c) and increasing KNO_3 concentration (Fig. 5b, d and f). This was consistent with other researchers who reported that the levels of gas flow rate and nitrogen concentration are important parameters for percent CO_2 removal [8]. As the gas flow rate increases, the size of gas bubble also increases and this reduces its retention time in the liquid and also its specific surface area per gas volume. These could then be the main reasons for the decrease in percent CO_2 removal at high gas flow rate [23]. Similar results were also reported by Chiu et al. [24]. Therefore, the maximum gas flow rate should be determined that could achieve 100% CO_2 removal.

It should be noted that the gas flow rate also affects the rate of CO_2 fixation. The CO_2 fixation rate is defined as the gram CO_2 removed per day per 1 L-microalgal culture, which is calculated based on Eq. (6). The CO_2 fixation rate was low (2.25–2.59 g-CO_2 day^{-1} L^{-1}) when using low gas flow rate (Run 7, 11 and 15), while the high CO_2 fixation rate (>5.18 g-CO_2 day^{-1} L^{-1}) were obtained when using high gas flow rate (Run 4, 14, 16 and 19). The three-dimensional and contour plots based on Eq. (10) with varing the two variables within the experimental rage was illustrated in Fig. 6. The CO_2 fixation rate increased with increasing gas flow rate. These results are in agreement with other researchers who reported that gas flow rate is an important parameter for the CO_2 fixation rate [23,25]. The optimum conditions for maximizing CO_2 fixation rate were: relatively high gas flow rate of 0.45 L h^{-1} per 1 L-microalgal

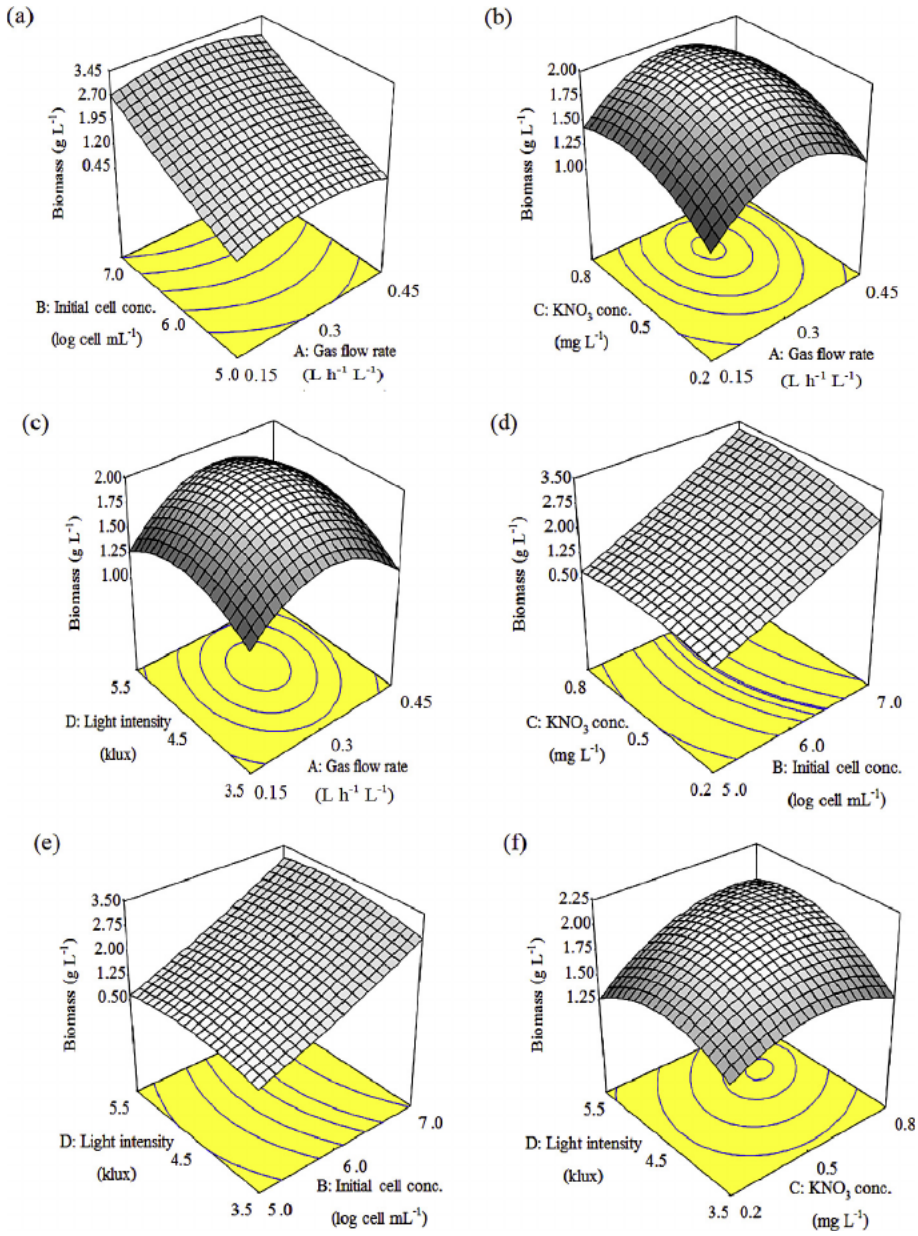

Fig. 3. 3D surface plots showing the effect of A: gas flow rate, B: initial cell concentration, C: KNO_3 concentration and D: light intensity on microalgae biomass production.

culture, initial cell concentration of $10^{6.07}$ cells mL^{-1}, KNO_3 concentration of 0.80 g L^{-1}, and high light intensity of 4.98 klux. The estimated maximum CO_2 fixation rate was as high as 5.54 g-CO_2 day^{-1} L^{-1}. However, with these conditions the biomass and lipid production were only 1.70 g/L and 69.39 mg/L/day, respectively.

3.3.5. Numerical optimization and verification of the model

The numerical optimization of culture conditions was carried out using Design-Expert statistical software based on the initial experimental results. When the target was set to maximize both percent CO_2 removal and lipid productivity. The compromised optimal conditions were: relatively low gas flow rate of 0.15 L h^{-1} per 1 L-microalgal culture, initial cell concentration of 10^7 cells mL^{-1}, KNO_3 concentration of 0.65 g L^{-1}, and light intensity of 5.28 klux. These conditions gave the maximum CO_2 removal of 99.33% and the maximum lipid productivity of 96.18 mg L^{-1} day^{-1}. The final microalgal biomass was 2.80 g L^{-1}. The methane content in the biogas under these conditions was increased from 60% up to 99.73%. However, these conditions gave relatively low CO_2 fixation rate of 2.59 g-CO_2 day^{-1} L^{-1}. The second target was then set to maximize CO_2 fixation rate and lipid productivity. The obtained optimal conditions were different. They were: higher gas flow rate of 0.3 L h^{-1} L^{-1}, higher KNO_3 concentration of 0.8 g L^{-1}, and slightly higher light intensity of 5.5 klux but with the same initial cell concentration of 10^7 cells mL^{-1}. With these conditions, the high CO_2 fixation rate (4.013 g-CO_2 day^{-1} L^{-1}) and acceptable methane content (90.73%) could be obtained. It should be noted that this methane content meets the standard (>90%) for efficient combustion with an increase in calorific value, decrease in relative density and increase in the Wobbe index. The adequacy of the model in predicting the response efficiently was verified by comparing the observed and predicted value of the responses (Table 5). The results of the measured responses were in close agreement with predicates value, and the deviation was found to be insignificant ($P >$

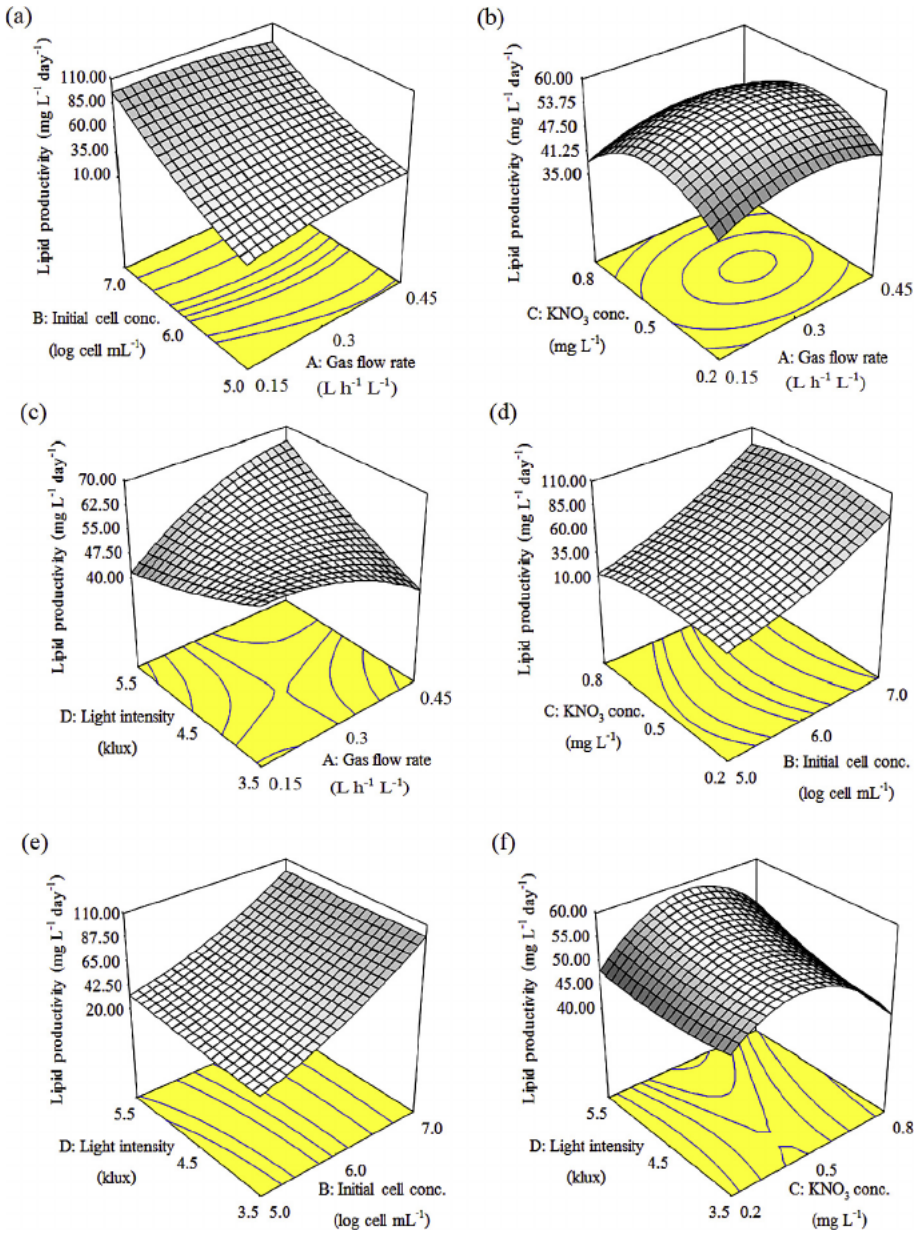

Fig. 4. 3D surface plots showing the effect of A: gas flow rate, B: initial cell concentration, C: KNO_3 concentration and D: light intensity on lipid productivity.

0.05). The final microalgal biomass and lipid productivity were 3.12 g L^{-1} and 88.57 mg L^{-1} day^{-1}, respectively.

3.4. Enhancing the process by stepwise-increasing of gas flow rate

Increasing gas flow rate can increase the amount of CO_2 which is the inorganic carbon source for the microalgae and this can lead to a higher biomass productivity. However, if the CO_2 supply is much higher than the CO_2 consumption by microalgae, the culture pH will drop rapidly. This acidic pH would then have an adverse effect upon the solubility and availability of nutrients, enzyme activity, and transport of substrate across plasma membrane and electron transport in respiration and photosynthesis [15]. Olaizola et al. [26] reported that the efficiency of CO_2 capture by microalgae is directly dependent on gas flow rate. Based on the RSM results from the previous section (Table 3, Run 11 and Run 24), the percent CO_2 removal increased when using low gas flow rate (0.15 L h^{-1} per 1 L-microalgal culture) but the CO_2 removal rate was so low at 2.25 g-CO_2 day^{-1} L^{-1}. When the gas flow rate was increased up to 0.45 L h^{-1} per microalgal culture, the CO_2 removal rate was as high as 4.50 g-CO_2 day^{-1} L^{-1}. As the percent of CO_2 removal is important for purifying and CO_2 removal rate is important for the productivity of the process, the strategy to improve both targets should be performed.

The stepwise-increasing of gas flow rate is one candidate for enhancing both percent CO_2 removal and CO_2 removal rate. At initial, the gas flow rate should be provided as a low rate which is suitable for low amount of microalgal cells. As the microalgae grew, the gas flow rate should be increased to supply more CO_2. Followed this strategy, the gas flow rate was stepwise-increased by 0.03 L h^{-1} per 1 L-microalgal culture every 2 days (Stepwise GFR) (Fig. 7b). The results were compared with the control that using a constant rate of 0.3 L h^{-1} per 1 L-microalgal culture (Fig. 7a). The optimum conditions from RSM was used in this experiment. The initial cell

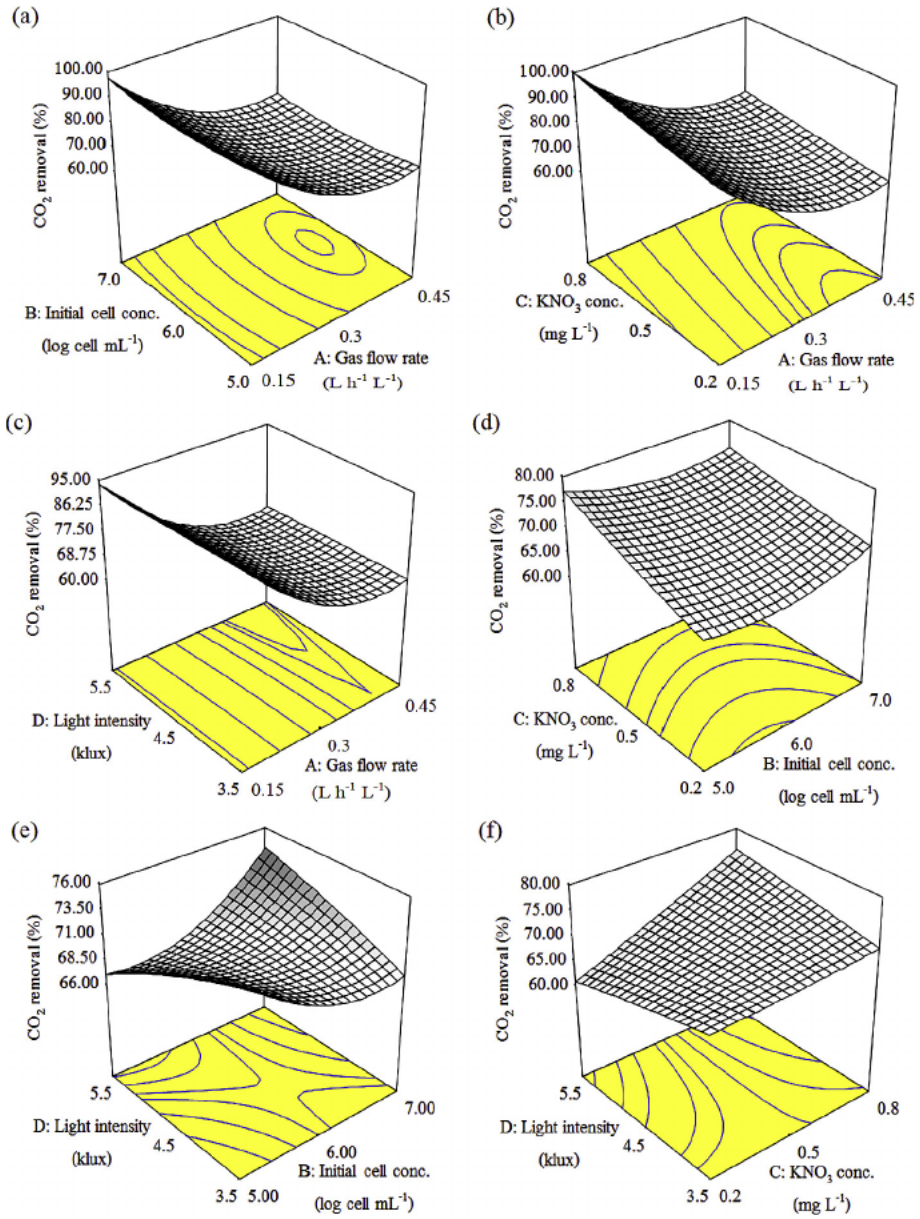

Fig. 5. 3D surface plots showing the effect of A: gas flow rate, B: initial cell concentration, C: KNO_3 concentration and D: light intensity on percent CO_2 removal.

concentration was 10^7 microalgal cells mL^{-1}. The culture was added with KNO_3 0.8 g L^{-1} as nitrogen source and illuminated at light intensity of 5.5 klux. With the Stepwise GFR, the final microalgal biomass and lipid productivity were increased by 1.25 and 1.79 folds, respectively. These results showed that increasing gas flow rate during cultivation is the effective strategy to increase the performance of the microalgae. The Stepwise GFR also showed higher lipid production (1.65 g L^{-1}) and higher lipid content (42.51%) than those of the control (0.98 g L^{-1} and 34.15%, respectively). These results were consistent with the results studies by Widjaja et al. [27] who reported the higher CO_2 flow rate used the higher lipid content obtained. The CO_2 removal rate by Stepwise GFR was increased up to 5.10 g-CO_2 day^{-1} L^{-1} with the acceptable percent CO_2 removal (Fig. 7c and d). After cultivation with the Stepwise GFR, the CH_4 content in the biogas increased from 60% up to 90.15%. Yan et al. [5] reported that the CH_4 content in biogas more than 90% (or CO_2 removal > 75%) could provide high-efficiency combustion. It should be also noted that the culture pH was in the range of 6.8–7.5 which had no significant effect on the CO_2 removal rate.

3.5. Fatty acid composition of microalgal lipid and its prospect as biodiesel feedstock

The lipids extracted from oleaginous *Scenedesmus* sp. cultivated under the optimal conditions for both CO_2 removal efficiency and lipid production, were converted to fatty acid methyl ester (FAME) and their compositions are shown in Table 6. Under both optimal conditions for percent CO_2 removal and CO_2 fixation rate, the major fatty acid of microalgal lipid were C16-C18 including palmitic acid, C16:0; heptadecanoic acid, C17:0; stearic acid, C18:0; oleaic acid, C18:1; linoleic acid, C18:2 and linolenic acid, C18:3. These fatty acids are favorable as biodiesel feedstocks [28]. The content of unsaturated fatty acids of the microalgal lipid in this study was >52%, which was similar to that of *Scenedesmus obliquus* SJTU-3 (>50%) [15]. The unsaturated, especially polyunsaturated, fatty

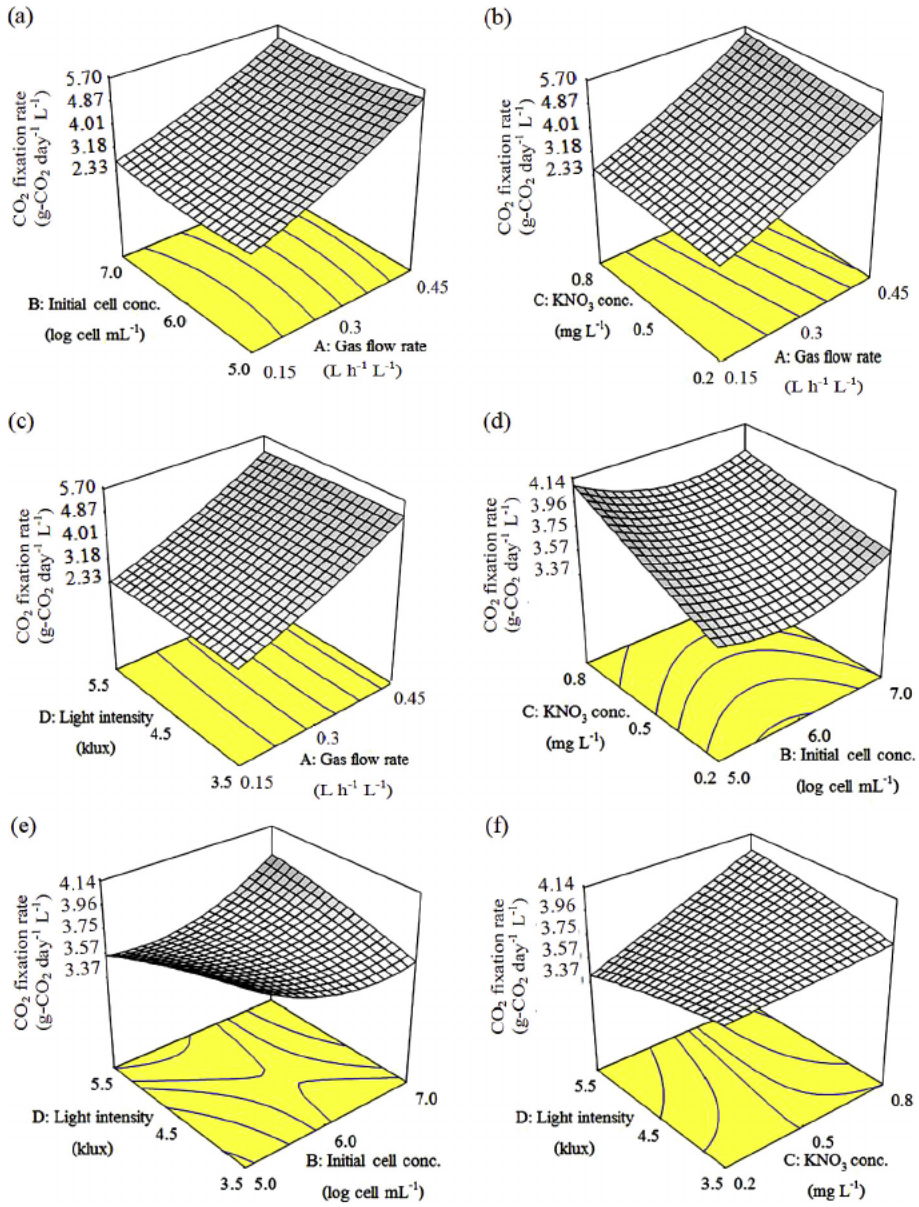

Fig. 6. 3D surface plots showing the effect of A: gas flow rate, B: initial cell concentration, C: KNO_3 concentration and D: light intensity on CO_2 fixation rate.

esters have lower melting points, which are desirable for the improvement of the low-temperature properties of biodiesel [29]. It seems that an increase in light intensity, KNO_3 concentration and gas flow rate did increase the amount of total unsaturated fatty acids, especially oleaic acid (C18:1) and linolenic acid (C18:3). While low gas flow rate was good for accumulation of saturated fatty acids including palmitic acid (C16:0) and short chain fatty acids (C14:0, C15:0). Similar trends were also found in the previous

Table 5
Response values under optimal conditions for CO_2 removal efficiency and lipid production.

Response	Optimal condition				Biomass (g L^{-1})		Lipid productivity (mg L^{-1} day^{-1})		%CO_2 removal		CO_2 fixation rate (g-CO_2 day^{-1} L^{-1})	
	Gas flow rate (L h^{-1} L^{-1})	Initial cell concentration (cells mL^{-1})	KNO_3 concentration (g L^{-1})	Light intensity (klux)	Predicted value	Experiment value[a]	Predicted value	Experiment value[a]	Predicted value	Experiment value[a]	Predicted value	Experiment value[a]
%CO_2 removal	0.15	10^7	0.65	5.28	2.83	2.80 ± 0.22	95.80	96.18 ± 0.01	99.46	99.33 ± 0.04	2.41	2.59 ± 0.03
CO_2 removal rate (mL L^{-1} min^{-1})	0.30	10^7	0.8	5.5	3.18	3.12 ± 0.22	93.70	88.57 ± 5.05	75.62	76.91 ± 0.90	3.94	4.01 ± 0.03

[a] Means of triplicate determination.

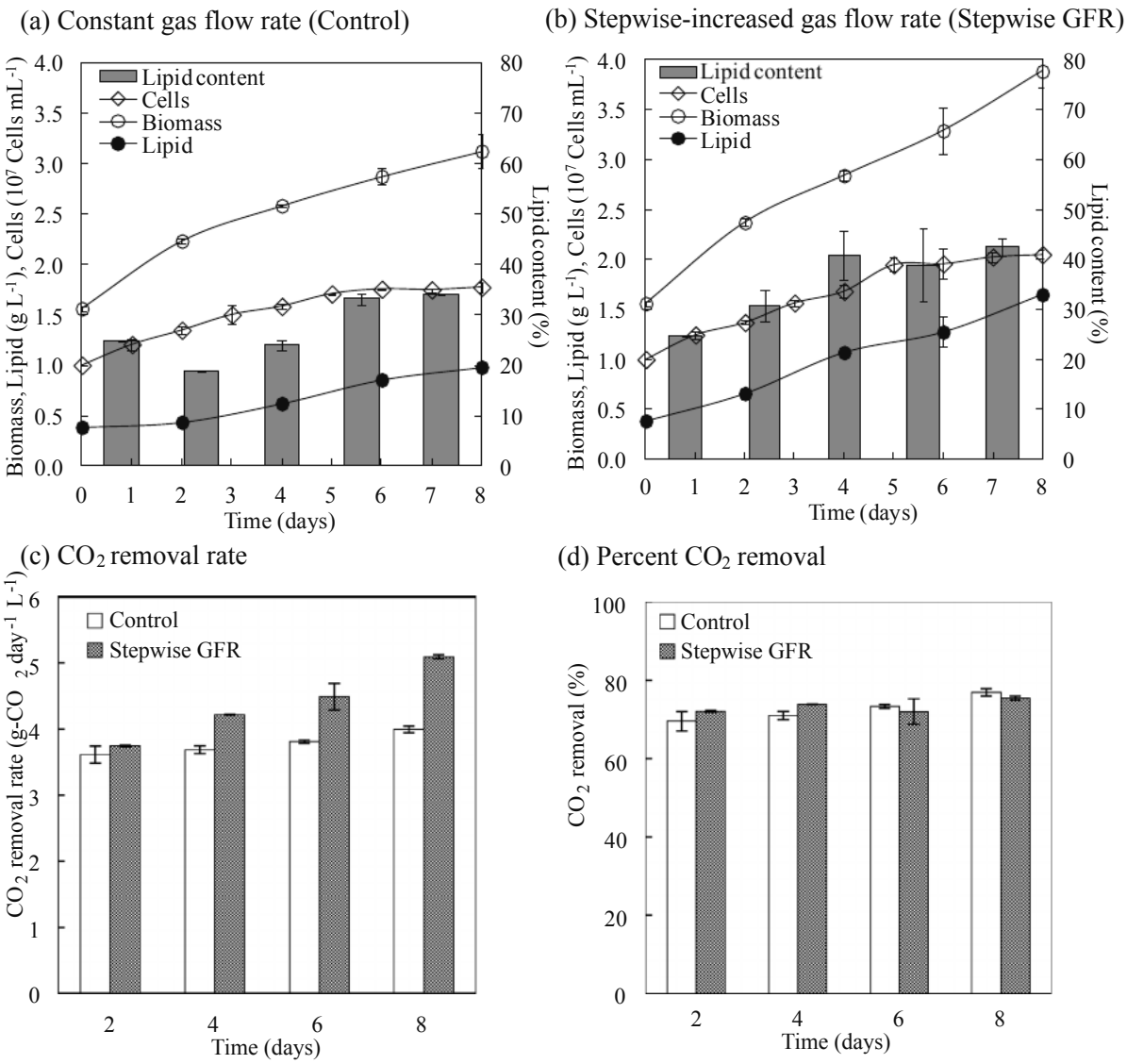

Fig. 7. Microalgae growth and lipid production under (a) constant gas flow rate (control) and (b) stepwise-increased gas flow rate (Stepwise GFR). The data of lipid content were the data at day 0, 2, 4, 6, 8. (c) and (d) are the results of CO_2 removal rate and percent CO_2 removal. Data are means of triplicates.

researches [30–33].

The biodiesel fuel properties namely, iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP) were calculated based on the fatty acid compositions (Table 7). Under optimum condition for percent CO_2 removal, the IV was found to be 111.18 g I_2/100 g oil which meets the European standard (EN-14214) (<120 g I_2/100 g oil). This indicates the microalgal biodiesel's stability against oxidation. The SV is the value of KOH (mg) required for the saponification of 1 g of oil. It is used to calculate the CN of the fuel [10,11]. The SV of biodiesel from both optimum condition were 207.24 and 205.95 mg KOH/g oil respectively, which are acceptable and close to that of *S. obliquus* biodiesel (217.5 mg KOH/g oil) [34]. Under optimum conditions for percent CO_2 removal, the CN was 47.62 which ensures the high ignition quality of the microalgae derived biodiesel. The minimum value of CN as per the international biofuel standards of EN-14214 and ASTM D675140 are 47. The DU of biodiesel from both optimum condition were in the range of 101–105 showing the oxidation stability of biodiesel [11]. Karpagam et al. [10] reported that when DU decreased, the CN and oxidation stability of biodiesel drastically increased. The LCSF and CFPP were in the range of 6–8 and 2–8 °C, respectively. Lower LCSF and CFPP attribute poor performance in tropical regions, but can be readily used in cold countries due to its optimum SV, CN, IV and DU [10]. These results suggested that microalgal lipid produced under optimal conditions for percent CO_2 removal showed better fuel properties than those produced under optimum conditions for CO_2 fixation rate.

The fatty acid composition of lipid obtained from Stepwise GFR were determined. As shown in Table 6, the fatty acids profiles are composed mostly of palmitic acid (C16:0), margaric acid (C17:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). More than 95% of fatty acids were covered with C16-C18 which is suitable for biodiesel production. Given the fatty acid profiles and its impact on the quality of produced biodiesel, it has been reported that palmitic acid and stearic acid are most favorable for biodiesel production [34]. The biodiesel fuel properties were calculated based on the fatty acid composition (Table 7). The IV, SV, CN, DU, LCSF and CFPP were found to be 115.81 g I_2/100 g oil, 206.51 mg KOH/g oil, 46.96, 96.43, 6.84 °C, and 5.03 °C, respectively. It could be

Table 6
Fatty acid composition of biodiesel derived from microalgal lipid.

Fatty acids	Relative amount of total fatty acids (%)		
	Under optimal conditions for %CO_2 removal from biogas	Under optimal conditions for CO_2 removal rate	Under stepwise-increasing of gas flow rate
Capric acid (C10:0)	–	0.13	–
Undecanoic acid (C11:0)	0.06	0.12	0.26
Lauric acid (C12:0)	0.22	0.30	0.51
Myristic acid (C14:0)	3.68	2.07	2.37
Pentadecanoic acid (C15:0)	0.31	0.14	0.17
Palmitic acid (C16:0)	32.58	25.57	26.09
Heptadecanoic acid (C17:0)	0.95	4.79	7.26
Stearic acid (C18:0)	4.36	3.12	3.77
Oleaic acid (C18:1)	11.26	19.67	19.96
Linoleic acid (C18:2)	23.96	9.82	7.33
Linolenic acid (C18:3)	20.98	33.09	30.90
Behenic acid (C22:0)	0.88	0.63	0.80
Erucic acid (C22:1)	0.32	–	–
Lignoceric acid (C24:0)	0.42	0.53	0.58
Saturated fatty acid	43.48	37.42	41.80
Unsaturated fatty acid	56.52	62.58	58.20
C_{16}-C_{18}	94.09	96.06	95.32
Lipid content (%w/w)	37.61	34.13	42.51

Table 7
Estimated properties of biodiesel fuel.

Conditions	IV	SV	CN	DU	LCSF	CEPP
Under optimal conditions for percent CO_2 removal from biogas	111.18	207.24	47.62	101.46	7.64	7.52
Under optimal conditions for CO_2 fixation rate	126.03	205.95	44.44	105.49	6.16	2.88
Under stepwise-increasing of gas flow rate	115.81	206.51	46.96	96.43	6.84	5.03

Biodiesel fuel parameters included iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP). The calculations for each property were: $IV = \sum[(254 \times F \times D)/MW]$; $SV = \sum[(560 \times F)/MW]$; $CN = [46.3+(5458/SV)]-(0.225 \times IV)$; DU = % MUFA+(2 × %PUFA); LCSF = (0.1 × C16)+(0.5 × C18)+(1 × C20)+(1.5 × C22)+ (2 × C24); CFPP = (3.1417 × LCSF) − 16.477, where D is the number of double bonds, F is the % of each type of fatty acid and MW is the molecular weight of corresponding fatty acid. MUFA is the weight percentage of the monounsaturated fatty acids (%wt) and PUFA is the weight percentage of the polyunsaturated fatty acids (%wt) [10].

concluded that the fuel properties of microalgal lipid are in accordance with the international standards (EN-14214 and ASTM D675140).

4. Conclusions

In present study, the oleaginous *Scenedesmus* sp. was found to be the most suitable strain for removing CO_2 from biogas and simultaneously producing lipid. A model predicted by RSM was successfully applied to determine the optimum conditions for effective CO_2 removal and lipid production. After optimization using multiple targets, the methane content in biogas could be upgraded up to the suitable level of >90% for being used as fuel. The final microalgal biomass and lipid productivity were 3.12 g L^{-1} and 88.57 mg L^{-1} day^{-1}, respectively. The stepwise-increasing of gas flow rate did enhance the CO_2 removal rate and the production of microalgal biomass. The main fatty acid compositions were C16-C18 (>95%) and their fuel properties were in accordance with the international standards. This integrated process represents as promising technology for economically viable production of microalgal biofuels.

Acknowledgements

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Paper II

Effective biogas upgrading and production of biodiesel feedstocks by strategic cultivation of oleaginous microalgae

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Effective biogas upgrading and production of biodiesel feedstocks by strategic cultivation of oleaginous microalgae

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ABSTRACT

This study has shown the strategies to upgrade biogas and produce lipids as biodiesel feedstocks by cultivation of oleaginous microalgae. Three important growth factors for microalgae including light intensity, nitrogen source and CO_2 supply, were strategically stepwise-increased during cultivation. The stepwise-increasing of CO_2 supply was suitable for cell growth and lipid production while the stepwise-increasing of light intensity was more suitable for CO_2 removal efficiency. Among the strategies attempted, the simultaneous stepwise-increasing of all three growth factors most effectively enhanced the performance of microalgae. Through this strategy, >96% of CO_2 was continuously removed from biogas and the CH_4 content in the purified biogas was >98%. This process also generated microalgal biomass at 4.40 ± 0.04 g L^{-1} with a lipid content of 34.10 ± 2.26%. The CO_2 removal rate by this process was as high as 6.50 ± 0.21 g-CO_2 day^{-1} per 1 L microalgal culture. The microalgal lipids contained long chain fatty acids (C16–C18) >94% and their prospect fuel properties indicated their suitable use as biodiesel feedstocks. The integrated processes and strategies in this study would contribute greatly to the production of biogas and biodiesel feedstocks.

1. Introduction

Biogas has been receiving increasingly interest as a renewable and sustainable source of energy. Biogas is produced from anaerobic digestion of organic wastes. Generally, crude biogas is composed of 40–75% CH_4, 25–60% CO_2 and trace amounts of other components [1]. But only biogas with CH_4 content >90% (v/v) meets requirement for being used as biofuel with high-efficiency combustion and high heating value [2,3]. The removal of CO_2 is an important key to increase CH_4 content in biogas. Despite physical/chemical absorption, water scrubbing, membrane separation, and pressure-swing adsorption supporting a removal of CO_2 from biogas, these technologies require considerable large amount of energy, auxiliary materials, and chemicals. Moreover, they also generate wastes and wastewater that can pollute the environment [4]. Recently, the biological removal of CO_2 using cultivation of microalgae has received considerable attention for improving CH_4 content in biogas. This is because the CO_2 fixation capability of the microalgae is high and this process also gives high productivity of microalgal biomass [5]. The CO_2 removal from biogas has been studied by using several species of microalgae i.e. *Scenedesmus obliquus*, *Nannochloropsis* sp., *Chlorella vulgaris* and *Neochloris oleoabundans*. The results showed that more than 90% of CO_2 could be removed from biogas while only 0.2–1% O_2 was detected in the gas phase. It should be noted that the use of specific microalgae that could produce high value products, would be more attractive in order to offset the costs of this process. However, a little report has evaluated the use of oleaginous microalgae for removing CO_2 from biogas [6,7]. Due to the high potential of this technique for environmental friendly and renewable energy production, the promising strategy to improve this technique should be developed to make it more viable at industrial scale.

The light intensity, nitrogen source and carbon dioxide concentration are main important growth factors that determine the CO_2 fixation capacity and lipid production of the microalgae. It has been reported that an insufficient light intensity could limit the microalgae growth and CO_2 fixation rate and the microalgae might even consume its storage lipid during photolimitation phase [8]. Nitrogen source is one of important growth factors for microalgae reproduction as it involves in the synthesis of nucleic acids, phospholipids and proteins. It has been reported that increasing

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nitrogen concentration could lead to the increase in biomass [10,11]. Although increasing the growth factors at initial could increase the ability of the microalgae, when their levels are above the optimal level the inhibition occurs. For example, an excessive light intensity could bleach the pigments and produce an overloaded photosystem which finally inhibited the microalgae growth and CO_2 fixation [7,9]. Similar trends have also been observed for the effects of nitrogen source and carbon dioxide concentration [10,11]. Although increasing of CO_2 supply could increase the microalgae growth, the CO_2 supply with high gas flow rate would rapidly lower the pH of the medium and decrease the performance of the microalgae. In addition, Kao et al. [12] also reported that the CO_2 utilization by microalgae decreased at high gas flow rate due to the decrease in retention time of large bubbles in the medium. Therefore, it is necessary to determine the optimal initial levels of light intensity, nitrogen source and CO_2 supply in order to avoid their inhibition effects. However, the more microalgae grow during cultivation the higher levels of these growth factors are needed. Hence, the strategies to efficiently provide these growth factors during cultivation should be determined.

In this study, oleaginous green microalga *Scenedesmus* sp. was used as potential microalgae to biologically remove CO_2 from biogas and simultaneously produce lipids for being used as biodiesel feedstocks. Firstly, the important growth factors including light intensity, nitrogen source (KNO_3) and CO_2 supply (biogas flow rate) were individually stepwise-increased to support the increased microalgae cells during cultivation. Secondly, the simultaneous stepwise-increasing of either two or three growth factors were attempted. Finally, the fatty acid compositions and prospect biodiesel properties of microalgal lipid were evaluated.

2. Materials and methods

2.1. Microalgae strain and culture medium

The microalgae strain used in this study was oleaginous *Scenedesmus* sp. from Bioprocess Engineering Laboratory at Prince of Songkla University, Thailand. The modified Chu13 medium used as basic medium consisted of 0.8 g KNO_3 as a nitrogen source, 0.04 g K_2HPO_4 as a phosphorus source, 0.1 g citric acid, 0.01 g Fe citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.036 g $NaHCO_3$, and 1 mL of trace metal solution per 1 L. The trace metal solution consisted of 2.85 g H_3BO_3, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.08 g $CoCl_2 \cdot 6H_2O$, and 0.05 g $Na_2MoO_4 \cdot 2H_2O$ per 1 L, pH was 6.8 [6].

2.2. Stepwise-increasing of growth factors for cultivation of microalgae

The oleaginous *Scenedesmus* sp. was cultured in 500 mL Duran bottles containing 400 mL of modified Chu13 and agitation was performed by a magnetic stirrer at 150 rpm. The culture was bubbled with simulated biogas (CO_2:CH_4 40:60) at a gas flow rate of 0.3 L h^{-1} per 1 L microalgal culture. The light illumination was provided by cool-white fluorescent lamps with photoperiod 24 h light: 0 h dark. The initial light intensity was set at 5.5 klux and the initial cell concentration was 10^7 cells mL^{-1}. The cultures were incubated at 30 °C for 8 days. Three growth factors including light intensity, nitrogen source and CO_2 supply each and their combination were stepwise-increased to support the increased biomass of the microalgae during cultivation. The stepwise-increasing of light intensity was performed by increasing the light intensity at a step of 0.5 klux every 2 days (Stepwise LI). The stepwise-addition of nitrogen source was performed by intermittently adding 0.2 g L^{-1} KNO_3 every 2 days (Stepwise N). The stepwise-increasing of CO_2 supply (Stepwise GFR) was performed by increasing biogas flow rate at a step of 0.03 L h^{-1} per 1 L microalgal culture every 2 days. The simultaneous stepwise-increasing of light intensity and nitrogen source (Simultaneous Stepwise LI + N) and those of three factors (light intensity, nitrogen source and CO_2 supply; Simultaneous Stepwise LI + N + GFR) were also performed.

2.3. Analytical methods

Cell concentration was determined by a direct microscopic count method using a hemocytometer and turbidimetrically at 660 nm using a spectrophotometer (Technical Cooperation, USA). The dry microalgal biomass was determined as follows: 10 mL of microalgal suspension was centrifuged at 3500 × g for 15 min and the supernatant was used to estimate the nitrate-nitrogen ($N\text{–}NO_3^-$) concentration by brucine method [13]. Then, the cell pellet was dried at 60 °C until constant weight and weighted. The specific growth rate (μ) was calculated using data in the exponential phase using the following equation:

$$\mu\ (day^{-1}) = (\ln X_2 - \ln X_1) / (t_2 - t_1) \quad (1)$$

where X_1 and X_2 are the concentrations of microalgal cells (g L^{-1}) at time t_1 and t_2, respectively.

The lipid content of the dried microalgal biomass was determined using liquid extraction by a mixed solvent solution of methanol and chloroform (2:1 v/v). Dry microalgal biomass was mashed and mixed with the solvent solution before sonication for 30 min. The suspension was centrifuged at 3500 × g for 15 min. The supernatant was collected and the cell pellets were extracted twice more. After extraction, the solvent solution was evaporated overnight and the extracted lipid was determined gravimetrically. The lipid productivity (P_{lipid}) was calculated by the following equation:

$$P_{lipid}\ (mg\ L^{-1}\ day^{-1}) = (L_2 - L_1) / (t_2 - t_1) \quad (2)$$

where L_1 and L_2 were the lipid production (mg L^{-1}) at time t_1 (start point of cultivation) and t_2 (the point that the maximum lipid was obtained), respectively. The lipid content (%w/w) was calculated with the following equation:

$$\text{Lipid content} = [\text{lipid production (g } L^{-1}) / \text{dried microalgal biomass weight (g } L^{-1})] \times 100 \quad (3)$$

The biogas compositions analysis, the compositions of biogas were determined using a Gas Chromatograph (GC 6890) with a cross-linked capillary HP-PLOT Q column (length 30 m, 0.54 mm I.D, 0.04 μm film thickness) and a thermal conductivity detector. Helium was used as carrier gas [6]. The percent of CO_2 removal from biogas and CO_2 removal rate (g-CO_2 day^{-1} per 1 L-microalgal culture) were determined as follows:

$$\%CO_2\ \text{removal} = [\text{initial } CO_2\ (\%) - \text{final } CO_2\ (\%) / \text{initial } CO_2\ (\%)] \times 100 \quad (4)$$

$$CO_2\ \text{removal rate} = \text{biogas flow rate (L day}^{-1}\ \text{L}^{-1}) \times \text{initial } CO_2\ (\%) \times \%CO_2\ \text{removal} \times 1000\ \text{g-}CO_2/556.2\ \text{L} \quad (5)$$

The extracted lipids were hydrolyzed and esterified to fatty acid methyl esters (FAME) [14]. The fatty acid profiles of the FAME were analyzed using a HP6850 Gas Chromatography equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm ID, 0.25 μm film thickness) and a flame ionization detector. Operating condition were as follows: inlet temperature 290 °C; oven temperature initial 210 °C held for 12 min, then ramped to 250 °C at 20 °C/min; held for 8 min and the detector temperature was 300 °C.

Fatty acids were identified by comparing their retention times with known pure standards. The biodiesel fuel properties of microalgae including saponification number (SN), iodine number (IN), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP) were obtained from its FAME profile using the following empirical Eqs. (6)–(11). Biodiesel fuel specifications are given by the regulatory international standards such as ASTM D6751 in the United States and EN 14214 in Europe [15].

$$SV = \sum [(560 \times F)/MW] \quad (6)$$

$$IV = \sum [(254 \times F \times D)/MW] \quad (7)$$

$$CN = [46.3 + (5458/SV)] - (0.225 \times IV) \quad (8)$$

$$DU = \%MUFA + (2 \times \%PUFA) \quad (9)$$

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24) \quad (10)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (11)$$

where D is the number of double bonds, F is the % of each type of fatty acid and MW is the molecular weight of corresponding fatty acid. MUFA is the weight percentage of the monounsaturated fatty acids (wt%) and PUFA is the weight percentage of the polyunsaturated fatty acids (wt%).

2.3. Statistical analysis

All experiments were performed at least in duplicates. The results are expressed as means plus standard deviations. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference was used to separate means, using SPSS software.

3. Results and discussions

3.1. Stepwise-increasing of growth factors for cultivation of *Scenedesmus sp.*

The optimum conditions for cultivation of oleaginous *Scenedesmus* sp. using simulated biogas (CO_2:CH_4 40:60) have been determined through response surface methodology [7]. They were: light intensity at 5.5 klux, initial KNO_3 concentration at 0.8 g L^{-1}, biogas flow rate at 0.3 L h^{-1} per 1 L microalgae culture and initial cell concentration of 10^7 cells mL^{-1} (Fig. 1a). Under these conditions, the microalgae biomass reached 3.12 ± 0.17 g L^{-1} and as more than 76% of CO_2 was removed. As a result, the methane content in biogas was increased up to 90.73%. The lipid productivity was 88.57 mg L^{-1} day^{-1} and the CO_2 removal rate was 4.0 g-CO_2 day^{-1} per 1 L microalgal culture. Several studies have reported that the supplementation of high levels of growth factors at initial negatively affected the performance of the microalgae [7–12]. Especially, light intensity is considered as the most important factor that affects both photosynthesis activity and the key enzymes involving in lipid biosynthesis process [16]. The use of high light intensity at initial may induce the photoinhibition and negatively affect the cell growth and lipid production [7,17,18]. However, the more microalgae grow the higher levels of growth factors are needed. Therefore, these growth factors should be efficiently provided during the growth of microalgae. In this study, three important growth factors including light intensity, nitrogen source and CO_2 supply were individually and simultaneously stepwise-increased in order to support the increased microalgae cells and their performance during cultivation.

3.1.1. Stepwise-increasing of light intensity

The stepwise-increasing of light intensity was performed by increasing the light intensity at a step of 0.5 klux every 2 days based on the growth curve of the microalgae (Stepwise LI, Fig. 1b). The microalgae grew better with the significant increase of cell concentration. The final biomass and lipid production were increased up to 3.80 ± 0.01 g L^{-1} and 1.48 ± 0.01 g L^{-1}, respectively which were 1.22 and 1.51 folds of the cultivation with constant light intensity at 5.5 klux (Table 1). The kinetic parameters for cultivation of oleaginous *Scenedesmus* sp. under stepwise-increasing of growth factors are shown in Table 2. The specific growth rate and lipid productivity were increased up to 0.28 ± 0.01 day^{-1} and 136.88 ± 0.88 mg L^{-1} day^{-1}, respectively. This was likely due to the increase of photoautotrophic growth with an increased light intensity during cultivation. These results were consistent with the results of Cheirsilp and Torpee [8], who found that the microalgae growth and lipid production of marine *Chlorella* sp. and *Nannochloropsis* sp. increased when applying stepwise-increasing of light intensity during cultivation. The lipid content of the microalgae in Stepwise LI was also increased from 34.15 ± 0.02% up to 39.0 ± 0.07%. As the microalgae growth was enhanced, the nitrogen concentration rapidly decreased. The low nitrogen concentration might lead to the higher ratio of carbon to nitrogen which could increase the lipid accumulation in the microalgae [19]. The CO_2 removal and CH_4 content also increased when applying Stepwise LI (Fig. 4b and c, Table 1). As 84.6% of CO_2 was removed from biogas, the CH_4 content in biogas was increased from 60% up to 93.6%. Wang et al. [20] reported that the CO_2 removal efficiency more than 75% (or CH_4 content in biogas >90%) is required for high-efficiency combustion and high heating value. The CO_2 removal rate by Stepwise LI was also increased up to 4.37 g-CO_2 day^{-1} per 1 L microalgal culture (Fig. 4a). These results indicate that the stepwise-increasing of light intensity is a promising strategy to improve the microalgae growth and lipid production.

3.1.2. Stepwise-addition of nitrogen source

To avoid the limitation by nitrogen depletion, the stepwise-addition of nitrogen source (Stepwise N) was attempted. The nitrogen source was added at 0.2 g L^{-1} every 2 days. Through this strategy, the nitrogen source could be efficiently supplied to support the cell growth. As shown in Fig. 2a, the Stepwise N showed higher cell concentration and microalgae biomass than the control (Fig. 1a). The microalgae biomass and specific growth rate reached 3.36 ± 0.11 g L^{-1} (Table 1) and 0.23 ± 0.01 day^{-1} (Table 2), respectively. Due to the improved cell growth, the CO_2 removal by Stepwise N was increased up to 79.24%. Consequently, the CH_4 content in biogas was increased up to 91.67%. The CO_2 removal rate was 4.12 ± 0.01 g-CO_2 day^{-1} per 1 L microalgal culture. From these results, it could be concluded that the stepwise-addition of nitrogen source positively affected microalgae growth and CO_2 removal. However, the lipid production was slightly lower than that of the control. It has been reported that the excessive nitrogen source could stimulate cell division but decrease acetyl-CoA carboxylase (ACCase) activity in lipid biosynthesis pathway [16]. Therefore, the strategy to balance between microalgae growth and lipid production should be performed.

3.1.3. Stepwise-increasing of biogas flow rate

In addition to light intensity and nitrogen concentration, CO_2 supply is also important factor for the microalgae growth and efficiency of CO_2 removal from biogas. It has been reported that the efficiency of CO_2 capture by microalgae is directly dependent on

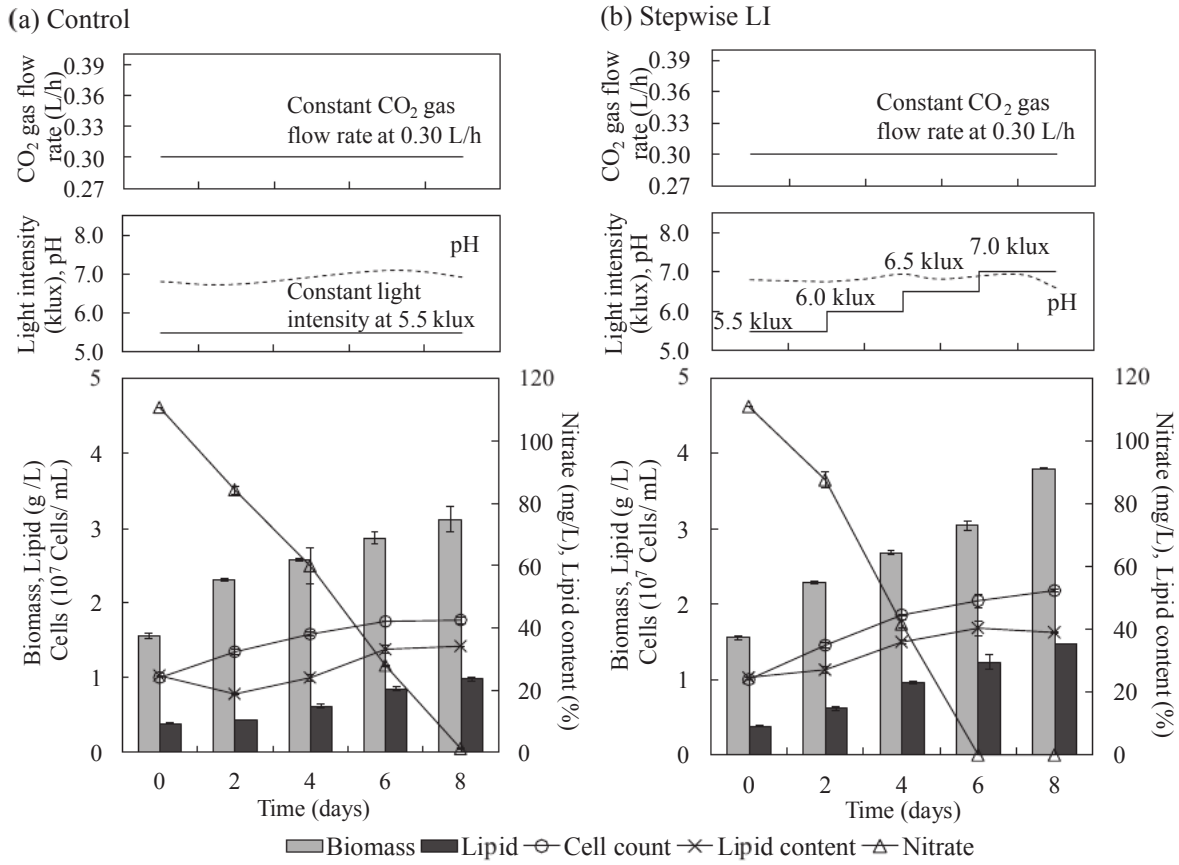

Fig. 1. Microalgae growth and lipid accumulation of oleaginous microalga *Scenedesmus* sp. cultivated under constant light intensity at 5.5 klux, initial KNO_3 concentration at 0.8 g L^{-1} and gas flow rate at 0.3 L/h per 1 L-microalgal culture (a, Control) and stepwise-increasing of light intensity from 5.5 to 7.0 klux (b, Stepwise LI).

Table 1
Microalgal biomass, lipid production, lipid content, CO_2 removal and CH_4 content after removing CO_2 of oleaginous *Scenedesmus* sp. cultivated under stepwise-increasing of growth factors.

Conditions	Biomass(g L^{-1}, fold)	Lipid production (g L^{-1}, fold)	Lipid content (%w/w, fold)	CO_2 removal (%, fold)	CH_4 content (%, fold)
Control	3.12 ± 0.17 (1.00)	0.98 ± 0.03 (1.00)	34.15 ± 0.02 (1.00)	76.91 ± 0.90 (1.00)	90.73 ± 0.36 (1.00)
Stepwise LI	3.80 ± 0.01 (1.22)	1.48 ± 0.01 (1.51)	39.00 ± 0.07 (1.14)	84.06 ± 0.19 (1.09)	93.60 ± 0.08 (1.03)
Stepwise N	3.36 ± 0.11 (1.08)	1.00 ± 0.07 (1.02)	29.81 ± 3.11 (0.87)	79.24 ± 0.19 (1.03)	91.61 ± 0.08 (1.01)
Stepwise GFR	3.77 ± 0.16 (1.21)	1.65 ± 0.00 (1.68)	43.77 ± 1.78 (1.28)	75.45 ± 0.92 (0.98)	90.15 ± 0.21 (0.99)
Stepwise LI + N	4.00 ± 0.16 (1.28)	1.29 ± 0.08 (1.32)	32.32 ± 3.38 (0.95)	88.47 ± 1.10 (1.15)	95.37 ± 0.44 (1.05)
Stepwise LI + N + GFR	4.40 ± 0.04 (1.41)	1.50 ± 0.08 (1.53)	34.10 ± 2.26 (0.99)	96.26 ± 3.16 (1.25)	98.50 ± 1.27 (1.09)

Table 2
Kinetic parameters for cultivation of oleaginous *Scenedesmus* sp. under stepwise-increasing of growth factors.

Conditions	Specific growth rate (1/day)	Lipid productivity (mg/L.day)	CO_2 removal rate (g-CO_2/day.L)
Control	0.20 ± 0.02	85.00 ± 0.05	4.00 ± 0.05
Stepwise LI	0.28 ± 0.01	136.88 ± 0.88	4.37 ± 0.01
Stepwise N	0.23 ± 0.01	76.88 ± 9.72	4.12 ± 0.01
Stepwise GFR	0.29 ± 0.02	158.13 ± 0.88	5.10 ± 0.03
Stepwise LI + N	0.31 ± 0.02	113.13 ± 9.72	4.60 ± 0.06
Stepwise LI + N + GFR	0.36 ± 0.01	139.38 ± 9.72	6.50 ± 0.21

Control: Constant light intensity at 5.5 klux, KNO_3 concentration at 0.8 g L^{-1} and gas flow rate at 0.3 L h^{-1} per 1 L-microalgal culture; Stepwise LI: Stepwise-increasing of light intensity; Stepwise N: Stepwise addition of nitrogen source; Stepwise GFR: Stepwise-increasing of gas flow rate; Stepwise LI + N: Simultaneous stepwise-increasing of light intensity and nitrogen source; Stepwise LI + N + GFR: Simultaneous stepwise-increasing of light intensity., nitrogen source and gas flow rate.

CO_2 gas flow rate and the culture pH [21]. In the previous study [7], it was found that when using low biogas flow rate the highly purified biogas could be obtained but the productivity of the process was low, namely the biogas was treated at a low rate. The CO_2 removal rate is an important parameter for biogas purification process. High CO_2 removal rate means that high amount of biogas could be treated at a high rate. Moreover, it was expected that the high gas flow rate would also provide the excessive carbon source which could enhance the lipid accumulation by the microalgae. The stepwise-increasing of biogas flow rate (Stepwise GFR) was then

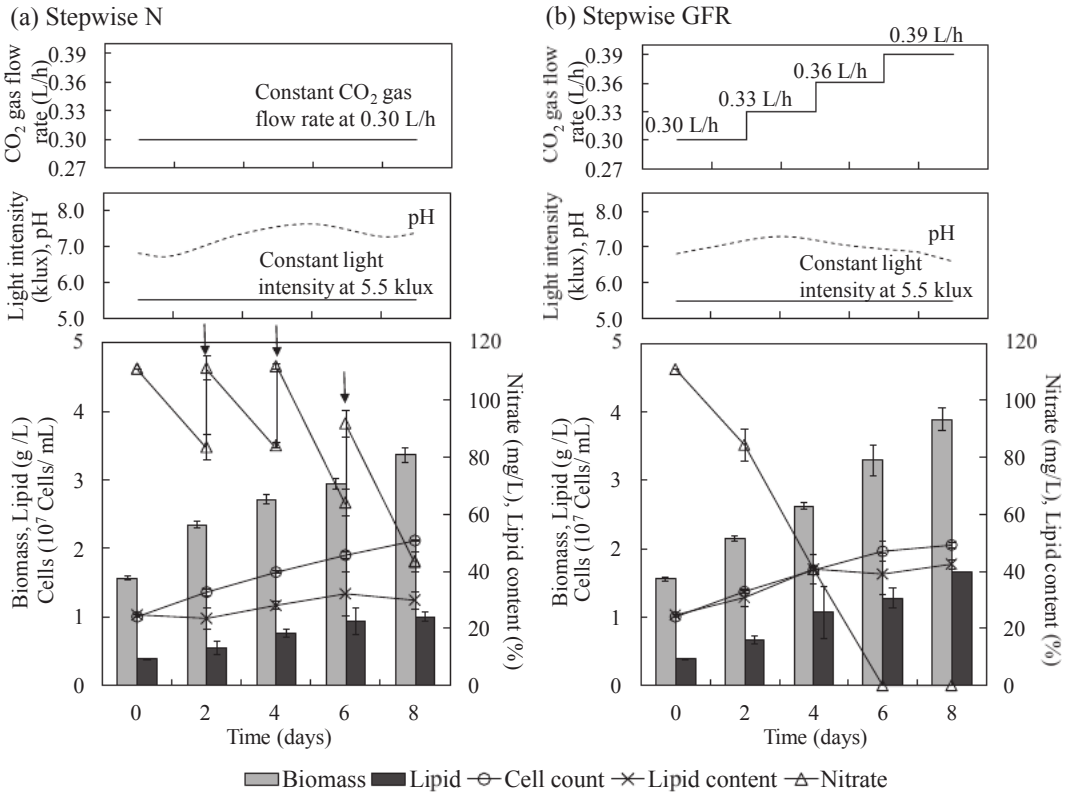

Fig. 2. Microalgae growth and lipid accumulation of oleaginous microalga *Scenedesmus* sp. cultivated under stepwise-addition of nitrogen source by adding 0.2 g/L KNO_3 every 2 days (a, Stepwise N) and stepwise-increasing of gas flow rate from 0.30 to 0.39 L/h per 1 L-microalgal culture (b, Stepwise GFR).

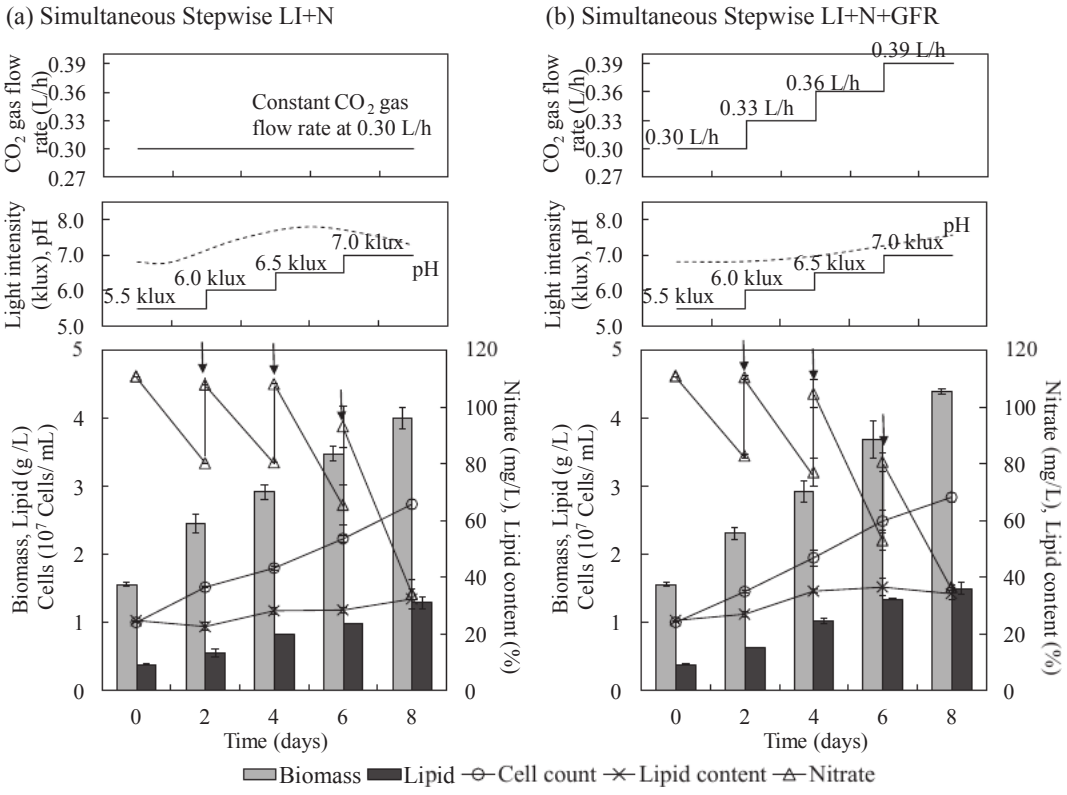

Fig. 3. Microalgae growth and lipid accumulation of oleaginous microalga *Scenedesmus* sp. cultivated under simultaneous stepwise-increasing of light intensity from 5.5 to 7.0 klux and nitrogen source concentration by adding 0.2 g/L KNO_3 every 2 days (a, Simultaneous Stepwise LI + N) and simultaneous stepwise-increasing of light intensity from 5.5 to 7.0 klux, nitrogen source concentration by adding 0.2 g/L KNO_3 every 2 days and gas flow rate from 0.30 to 0.39 L/h per 1 L-microalgal culture (b, Simultaneous Stepwise LI + N + GFR).

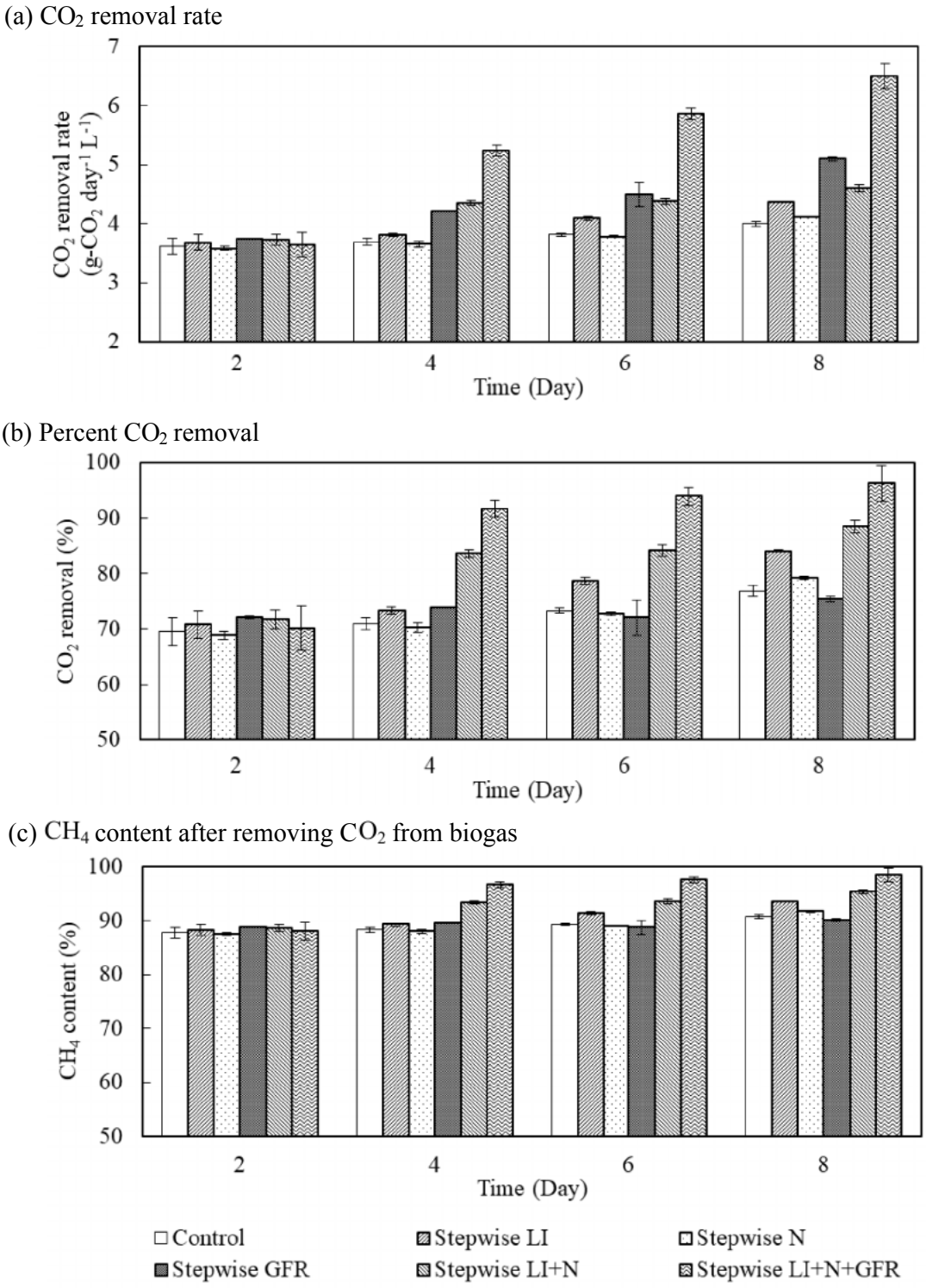

Fig. 4. CO_2 removal efficiency and CH_4 content after removing CO_2 from biogas by oleaginous microalga *Scenedesmus* sp. Control: Constant light intensity at 5.5 klux, KNO_3 concentration at 0.8 g L^{-1} and gas flow rate at 0.3 L h^{-1} per 1 L-microalgal culture; Stepwise LI: Stepwise-increasing of light intensity; Stepwise N: Stepwise addition of nitrogen source; Stepwise GFR: Stepwise-increasing of gas flow rate; Stepwise LI + N: Simultaneous stepwise-increasing of light intensity and nitrogen source; Stepwise LI + N + GFR: Simultaneous stepwise-increasing of light intensity., nitrogen source and gas flow rate.

attempted. The biogas flow rate was stepwise-increased by 0.03 L h^{-1} per 1 L microalgal culture every 2 days (Fig. 2b). The results showed that the Stepwise GFR was effective strategy to enhance not only cell growth but also lipid production (Table 1). Compared to the individual stepwise-increasing of growth factor, Stepwise GFR most enhanced the specific growth rate and lipid productivity of the microalgae (Table 2).

Among the three growth factors tested, it could be concluded that the stepwise-increasing of light intensity could effectively increase both cell growth and lipid production (Fig. 1b) while the

stepwise-addition of nitrogen source enhanced mainly cell division and cell growth but not lipid production (Fig. 2a). As the process productivity (CO_2 removal rate) depends mainly on gas flow rate, the most effective strategy to increase not only microalgae growth and lipid production but also the process productivity is the stepwise-increasing of gas flow rate.

3.2. Simultaneous stepwise-increasing of light intensity and nitrogen source

As the microalgae would require several growth factors to support their growth during cultivation, the simultaneous stepwise-increasing of light intensity together with nitrogen source (Stepwise LI + N) was then attempted. As shown in Fig. 3a, the cell concentration by this strategy was much higher than that of the control and that of the individual stepwise-increasing of growth factor. This indicated that the effective cell division required both increasing light intensity and nitrogen source. Stepwise LI + N also showed higher final biomass (4.0 ± 0.16 g L^{-1}) than that of the control and the individual stepwise-increasing of growth factor (Table 1). The specific growth rate was increased up to 0.31 day^{-1}. The CO_2 removal efficiency and CH_4 content were also higher than those of the control and the individual stepwise-increasing of growth factor (Fig. 4, Tables 1 and 2). However, the CO_2 removal rate by this strategy was lower than that by the Stepwise GFR. This was because the overall biogas flow rate by Stepwise LI + N was lower than that of Stepwise GFR.

3.3. Stepwise-increasing of three growth factors

To enhance the overall targets including microalgae growth, lipid production, CO_2 removal efficiency and CO_2 removal rate, all three growth factors including light intensity, nitrogen source and CO_2 supply were simultaneously stepwise-increased during microalgae cultivation (Simultaneous Stepwise LI + N + GFR). Through this strategy, the highest microalgae biomass of 4.4 ± 0.04 g L^{-1} and the lipid production of 1.5 ± 0.08 g L^{-1} were achieved. The specific growth rate and lipid productivity were 0.36 ± 0.01 day^{-1} and 139.38 ± 9.72 mg L^{-1} day^{-1}, respectively (Table 2). The CO_2 removal efficiency and CH_4 content were also highest at $96.26 \pm 3.16\%$ and $98.5 \pm 1.27\%$, respectively (Fig. 4). Interestingly, the CO_2 removal rate by this strategy was most increased up to 6.5 g-CO_2 day^{-1} per 1 L microalgal culture (Table 2). As the simultaneous stepwise-increasing of three growth factors showed the best performance, it could then be concluded that light intensity, nitrogen source and CO_2 supply all are importance growth factors that have to be increasingly supplied along with the cell growth of microalgae.

The performance of all stepwise-increasing of growth factors are compared with that of the control (Tables 1 and 2). Considering the increase in the microalgae biomass, the improvement levels of microalgae growth could be concluded as follows: Simultaneous Stepwise LI + N + GFR > Simultaneous Stepwise LI + N > Stepwise GFR > Stepwise LI > Stepwise N. While the improvement levels of lipid production were as follows: Stepwise GFR > Simultaneous Stepwise LI + N + GFR > Stepwise LI > Simultaneous Stepwise LI + N > Stepwise N. For CO_2 removal efficiency, the Simultaneous Stepwise LI + N + GFR gave the highest CO_2 removal efficiency (96.26%) followed by Simultaneous Stepwise LI + N (88.47%), Stepwise LI (84.06%), Stepwise N (79.24%) and Stepwise GFR (75.45%). The improvement levels of CH_4 content in biogas after removing CO_2 were in accordance with those of CO_2 removal efficiency. The Simultaneous Stepwise LI + N + GFR also gave the highest CO_2 removal rate of 6.5 g-CO_2 day^{-1} per 1 L microalgal culture which was 1.63 folds of the control. Finally, these results suggested that the simultaneous stepwise-increasing of three growth factors was the most suitable strategy to simultaneously upgrade biogas and produce lipid by oleaginous *Scenedesmus* sp.

3.4. FAME characterization and prospect fuel properties of microalgal lipids

The fatty acid profiling of microalgal lipids from each strategy were characterized and used to estimate their fuel properties. As shown in Table 3, the microalgal lipids were mostly composed of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). More than 94% of fatty acids were C16–C18, which are suitable for biodiesel production [22]. Among medium and long chain fatty acids, it has been reported that the saturated fatty acids, especially palmitic acid and stearic acid, are most favorable for biodiesel production with good fuel properties [23]. It should be noted that when the microalgae was cultivated through the stepwise-increasing of growth factors, the percentage of saturated fatty acids (SFA) increased while that of unsaturated fatty acids (USFA) decreased. The proportion of SFA and USFA affects key properties of biodiesel, especially cetane number, flash point, viscosity, oxidation stability, and fluidity at low temperature [24]. Higher ratio of SFA/USFA results in greater density and viscosity values and higher oxidative stability of the subsequent fuel [23]. Among individual stepwise-increasing of growth factor, the SFA/USFA ratio of lipids obtained from Stepwise GFR was higher than those of other strategies. Interestingly, the SFA/USFA ratio of lipids from Simultaneous Stepwise LI + N + GFR was highest at 0.77 (Table 3).

The fuel properties of microalgal lipids including saponification number (SN), iodine number (IN), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP) were calculated based on their FAME profiles. The content of saturated and monounsaturated fatty acids (SFA and MUFA) could give high value of cetane number (CN) which

Table 3
Fatty acid profiling of microalgae lipid under stepwise-increasing of growth factors.

Fatty acids	Control	Stepwise-increasing of growth factors				
		LI	N	GFR	LI + N	LI + N + GFR
C10:0	0.13	N.D.	0.13	N.D.	0.19	N.D.
C11:0	0.12	0.11	0.10	0.26	0.13	0.06
C12:0	0.30	0.22	0.26	0.51	0.32	0.22
C14:0	2.07	2.98	2.35	2.37	1.84	3.68
C15:0	0.14	0.17	0.19	0.17	0.15	0.31
C16:0	25.57	21.84	25.18	26.09	20.86	32.58
C17:0	4.79	8.59	5.22	7.26	11.24	0.95
C18:0	3.12	3.12	3.43	3.77	2.88	4.36
C18:1	19.67	14.03	17.76	19.96	10.35	11.26
C18:2	9.82	10.29	13.06	7.33	14.58	23.96
C18:3	33.09	37.25	30.58	30.90	36.14	20.98
C22:0	0.63	0.66	0.74	0.80	0.80	0.88
C22:1	N.D.	N.D.	0.47	N.D.	N.D.	0.32
C24:0	0.53	0.75	0.53	0.58	0.53	0.42
C16–C18	96.06	95.12	95.22	95.32	96.05	94.10
SFA	37.41	38.43	38.14	41.80	38.93	43.47
UFA	62.59	61.57	61.86	58.20	61.07	56.53
MUFA	19.67	14.03	18.23	19.96	10.35	11.58
PUFA	42.92	47.54	43.64	38.24	50.72	44.95
SFA/UFA	0.60	0.62	0.62	0.72	0.64	0.77

N.D.: Not detected; SFA: Saturated fatty acid; UFA: Unsaturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; Control: Constant light intensity at 5.5 klux, KNO_3 concentration at 0.8 g L^{-1} and gas flow rate at 0.3 L h^{-1} per 1 L-microalgal culture; Stepwise LI: Stepwise-increasing of light intensity; Stepwise N: Stepwise addition of nitrogen source; Stepwise GFR: Stepwise-increasing of gas flow rate; Stepwise LI + N: Simultaneous stepwise-increasing of light intensity and nitrogen source; Stepwise LI + N + GFR: Simultaneous stepwise-increasing of light intensity., nitrogen source and gas flow rate.

Table 4
Estimated properties of biodiesel fuel from microalgae lipid under stepwise-increasing of growth factors.

Conditions	IV	SV	CN	DU	LCSF	CEPP
Control	126.05	206.06	44.74	105.51	6.13	2.78
Stepwise LI	133.19	205.84	43.18	109.11	6.24	3.12
Stepwise N	123.66	205.90	45.29	105.50	6.41	3.66
Stepwise GFR	115.81	206.51	46.96	96.43	6.84	5.03
Stepwise LI + N	134.63	205.78	42.87	111.79	5.79	1.70
Stepwise LI + N + GFR	111.20	207.38	47.88	101.47	7.60	7.42

Biodiesel fuel parameters included iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), long chain saturation factor (LCSF) and cold filter plugging point (CFPP); Control: Constant light intensity at 5.5 klux, KNO_3 concentration at 0.8 g L^{-1} and gas flow rate at 0.3 L h^{-1} per 1 L-microalgal culture; Stepwise LI: Stepwise-increasing of light intensity; Stepwise N: Stepwise addition of nitrogen source; Stepwise GFR: Stepwise-increasing of gas flow rate; Stepwise LI + N: Simultaneous stepwise-increasing of light intensity and nitrogen source; Stepwise LI + N + GFR: Simultaneous stepwise-increasing of light intensity., nitrogen source and gas flow rate.

denotes better combustion quality and easy start of the engine. The unsaturation of the oil determines iodine number (IN) indicating the oxidative stability of the fuel. As shown in Table 4, the SV were found to be in the range of 205–208 mg KOH g^{-1} of oil. Consequently, the CN were found to be 42–48. It should be noted that the CN of microalgal lipids from Simultaneous Stepwise LI + N + GFR was ≥47, which meet the requirement for being used as biodiesel. The IV was found to be 111–135 g Iodine/100 g of oil. The microalgal lipids from Simultaneous Stepwise LI + N + GFR and Stepwise GFR meet with the standard value of IV (≤120 g Iodine/100 g of oil). The DU was found to be 96–112 which showed the high oxidative stability for longer storage in hot regions [23]. The cold flow properties like LCSF and CFPP of microalgae were in range of 5–8 and 1–8 °C, respectively which were in accordance with the international standard revealing the good flow performance of biodiesel at low temperature. In conclusion, the microalgal lipids from Simultaneous Stepwise LI + N + GFR showed the best fuel properties which meet the biodiesel fuel specifications given by the regulatory international standard of ASTM D6751 and EN-14214. It should be noted that after the extraction of microalgal lipid the residual biomass could be used in various applications such as animal feed, fertilizer and even as feedstocks for further production of bioethanol and biogas.

4. Conclusions

This study has shown that the oleaginous microalgae could purify biogas and simultaneously produce lipids. The stepwise-increasing of important growth factors including light intensity, nitrogen source and CO_2 supply successfully enhanced microalgae performance. The simultaneous stepwise-increasing of all three growth factors most enhanced not only biomass and lipid production but also CO_2 removal efficiency and CO_2 removal rate by the microalgae. The microalgal lipids contained mainly C16–C18 fatty acids which their prospect fuel properties meet the biodiesel specifications. The developed processes in this study could improve not only the quality of biogas but also the fixation of CO_2 into energy by the microalgae.

Control: Constant light intensity at 5.5 klux, KNO_3 concentration at 0.8 g L^{-1} and gas flow rate at 0.3 L h^{-1} per 1 L-microalgal culture; Stepwise LI: Stepwise-increasing of light intensity; Stepwise N: Stepwise addition of nitrogen source; Stepwise GFR: Stepwise-increasing of gas flow rate; Stepwise LI + N: Simultaneous stepwise-increasing of light intensity and nitrogen source; Stepwise LI + N + GFR: Simultaneous stepwise-increasing of light intensity., nitrogen source and gas flow rate.

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Paper III

Immobilized oleaginous microalgae as effective two-phase purify unit for biogas and palm oil mill effluent and production of biodiesel feedstock

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Manuscript

Immobilized Oleaginous Microalgae as Effective Two-Phase Purify Unit for Biogas and Palm Oil Mill Effluent and Production of Biodiesel Feedstock

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Abstract

The oleaginous microalga *Scenedesmus* sp. was immobilized in alginate gel beads and applied for phytoremediation of palm oil mill effluent (POME) and biogas purification. The optimal initial cell concentration and bead volume to medium volume ratio were 10^6 cells mL^{-1} and 25% v/v, respectively. The diluted effluent at 4:1 and light intensity at 9.5 klux were the optimal conditions to achieve 88.46% of CO_2 removal from biogas and increased the methane content up to 95.37%. The CO_2 removal rate was as high as 4.63 g-CO_2 day^{-1} per 1 L-microalgal culture. After process operation, the immobilized microalgae effectively removed COD (>71%), nitrogen (100%) and phosphorus (100%). The microalgal biomass of 2.98 g L^{-1} with high lipid content of 35.92% were harvested. The pigments including chlorophylls and carotenoids in biomass were 45.97 and 26.06 mg g^{-1} biomass, respectively. Fatty acid compositions of lipids were C16-C18 (>98%) and their fuel properties were in accordance with the international standards. These results indicate the potential use of immobilized microalgae for integrated systems for pollutants removal, biogas upgrading and production of microalgae based products.

Keywords: Biogas, CO_2 removal, Immobilized microalgae, Palm oil mill effluent, Lipid, Pollutants removal

1. Introduction

Currently, the environmental concerns and the increasing energy demand have stimulated scientists to find for environmentally friendly and renewable energy.

Biogas is seen as suitable and renewable source of energy because it is less harmful to the environments (Teo *et al*., 2014). Biogas produced from anaerobic digestion process is mainly composed of methane (CH_4, 40-75%), carbon dioxide (CO_2, 25-60%) with other minor components such as hydrogen sulfide (H_2S, 0.005-2%), nitrogen (N_2, 0-2%), oxygen (O_2, 0-1%) and ammonia (NH_3, <1%) (Posadas *et al*., 2015). The composition and concentration of biogas vary depending on its source. To be effective in energy conversion, the methane content in biogas should be upgraded to be higher than 90% before combustion (Zhao *et al*., 2015). Physical absorption, chemical conversion, membrane separation, pressure swing adsorption, and cryogenic separation have been applied for biogas upgrading process. However, these technologies are high cost and effective only removing CO_2 from biogas not converting (Meier *et al*., 2015). Photosynthetic CO_2 fixation by microalgae is an alternative way to remove CO_2 from biogas which is environmentally friendly and low-cost process (Posadas *et al*., 2015). Microalgae have attracted increasing interest in remove CO_2 in biogas because CO_2 in biogas is converted into microalgal biomass through photosynthesis with high-value products such as lipids, pigments and protein can be generated (Meier *et al*., 2015). Previous studies have demonstrated the feasibility of utilizing biogas as carbon source for cultivation of microalgae (Posadas *et al*., 2015; Zhao *et al*., 2015; Meier *et al*., 2015; Srinuanpan *et al*., 2017; Srinuanpan *et al*., 2018). These strategies have successfully attained the methane content more than 95% (v/v) with a low O_2 content of 0.1-2.0%. The upgraded methane content meets the standard (>90%) for efficient combustion with an increased calorific value (Zhao *et al*., 2013). Interestingly, our previous study (Srinuanpan *et al*., 2017; Srinuanpan *et al*., 2018) found that two oleaginous microalgae, marine *Chlorella* sp. and *Scenedesmus* sp. had high potential to upgrade biogas and simultaneously producing lipids (more than 27% of their biomass). The fatty acid profilings of extracted microalgal lipids were high long-chain fatty acids (C16-C18 >90%), indicating their potential use as biodiesel feedstocks. These results indicate that the oleaginous microalgae are not only able to remove CO_2 from biogas but also generate the 3rd generation renewable biofuels.

The palm oil industry is one of the vital agro-industry in Thailand, which is the third largest palm oil producer in the world. In the process of crude palm oil

(CPO) production from fresh fruit bunches, palm oil mill effluent (POME) was generated about 2.5 tons of effluent per ton of crude oil processed mainly from the process of sterilizing the fruits, extraction and clarification of oil (Pechsuth *et al*., 2001). POME is a thick brownish viscous liquid waste and characterized by high organic and inorganic contents that cause environmental concerns. POME normally is used as feedstock for biogas production through anaerobic digester. The effluent from anaerobic digester was then treated in a secondary treatment system as the effluent still contain high levels of carbon, nitrogen and phosphorus. On the one hand, these compounds need to be removed in the tertiary treatment, and on the other hand they are suitable and cost-effective for microalgae cultivation (An et al., 2003; Cheirsilp *et al*., 2017b; Xie *et al*., 2018). Several researches have indicated that the microalgae could be one of the candidates for treating secondary effluent due to their capability of consuming the organic and inorganic component in POME and generated biomass. Cheirsilp *et al*. (2017b) studies the use of secondary effluent from palm oil mill for semi-continuous cultivation of *Chlorella* sp. C-MR in photobioreactor with 1.5 L culture volume. They found that the microalgae could remove COD, nitrogen and phosphorus by 58-65%, 71-99% and 42-64%, respectively. The microalgal biomass of 0.45-0.73 g L^{-1} with lipid content of 20-43% were also obtained. Ding *et al*. (2016) compared the biomass production and nutrients removal by *Chlamydomonas* sp. cultured in different volume ratios of POME. POME ratio of 16.7% gave the highest biomass of 0.917 g L^{-1} after 9 days of cultivation. The nitrogen and phosphorus were removed more than 70%. They also found that higher volume ratio of POME decreased the light penetration and resulted in lower biomass production.Therefore, to achieve the maximum biomass and nutrient removal, the suitable combination of POME concentrations and light intensity should be investigated.

In recent years, with the aim of simplifying recovery of microalgal biomass for biofuel production, the use of immobilized microalgae has gained increasingly attentions. This technique could increase the retention time of microalgae cells within bioreactors and promoted the metabolism of the microalgae. Gel entrapment method is the most common way to immobilize microalgae cells, in which natural polysaccharides such as agars, carrageenans and alginates are preferably used due to their low toxicity and high transparency (Lam and Lee, 2012). Alginate is commonly

used for immobilization of microalgae and it also maintains the high viability of cells for extended periods of time (Ruiz-Marin *et al*., 2012). Lam and Lee (2012) have shown that a sodium rich alginate was crucial to ensure bead stability of *Chlorella vulgaris* in organic nutrients. They also found that the use of immobilized microalgae can enhanced the organic pollutants removal. The use of immobilized microalgae in the beads could also reduce the amount of energy input for biomass recovery because the beads can be recovered by a simple separation method (e.g. sieving). However, some components in the wastewater such as higher phosphate and citrate likely affected the strength of the gel matrix and ultimately dissolved it (Jimenez-Pere *et al*., 2004). Recently, it has been reported that the re-calcification of alginate beads can resolve this problem (Lam and Lee, 2012; Castro-Ceseña and Sánchez-Saavedra, 2015). Moreover, Covarrubias *et al*. (2012) reported that the immobilized *Chlorella sorokiniana* and *Azospirillum brasilense* in Ca-alginate beads significantly enhanced their populations in non-sterile secondary effluent. However, the possibility of using immobilized microalgae for removing CO_2 from industrial plants have been rarely reported. To develop the sustainable practices of immobilized microalgae, the immobilized microalgae beads should be attempted for simultaneous removing CO_2, treating effluent and contributing to microalgae based biofuel production.

This study aimed to evaluate the feasibility of using immobilized oleaginous microalgae for phytoremediation of palm oil mill effluent and purification of biogas in one step. The suitable bead volume and inoculum size were determined. The optimal effluent concentration and light intensity on pollutants removal, biogas upgrading, and lipid production, were investigated. Furthermore, the fuel properties of the microalgal lipids were also evaluated.

2. Materials and Methods

2.1 Microalgae strain and culture medium

The microalgae strain used in this study was oleaginous *Scenedesmus* sp. from Bioprocess Engineering Laboratory at Prince of Songkla University, Thailand. The modified Chu13 medium used as the basic medium for this study consisted of 0.8 g KNO_3 as a nitrogen source, 0.04 g K_2HPO_4 as a phosphorus source, 0.1 g citric acid,

0.01 g Fe citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.036 g $NaHCO_3$, and 1 mL of trace metal solution per 1 L. The trace metal solution consisted of 2.85 g H_3BO_3, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.08 g $CoCl_2 \cdot 6H_2O$, and 0.05 g $Na_2MoO_4 \cdot 2H_2O$ per 1 L, pH was 6.8 (Srinuanpan *et al*., 2017). The seed culture was bubbled with air containing 0.03% CO_2 in 400 mL Duran bottles under cool-white fluorescent lamps (3.5 klux) with full photoperiod 24 h. After 7 days of cultivation, the microalgal cells were recovered by centrifugation at 8,500 rpm for 15 min and resuspended in 50 mL fresh medium and used as seed culture.

2.2 Immobilization and cultivation of microalgae in alginate beads

3% w/v of sodium alginate and microalgae solution (initial cell concentration of 10^6 and 10^7 cells mL^{-1}) was mixed with 100, 200 and 300 mL sterilized medium to evaluate the effect of bead volume ratio. The mixture was dropped to a 500 mL calcium chloride solution at a concentration of 2% w/v and stirred with magnetic stirrer at 300 rpm. For every 10 mL of microalgae-alginate mixture, 344 of microalgae beads were produced with approximate diameter of 3 mm per bead. Consequently, the microalgae beads were stabilized in the calcium chloride solution for 1 h at 30±1 ºC before filtered with a sieve (mesh size 0.5 mm) and rinsed with distilled water (Lam and Lee, 2012).

The immobilized microalgae were inoculated into 500 mL Duran bottles containing 100, 200 and 300 mL modified Chu 13 medium. The cultures were continuously aerated with synthetic biogas (40% CO_2 was mixed with 60% CH_4) at a gas flow rate of 0.3 L h^{-1} per 1 L culture medium. The light intensity was 5.5 klux with a full photoperiod of 24 h.

2.3 Immobilized microalgae as two-phase purify unit for biogas and anaerobic digester effluent

The microalgae were mixed with sterilized distilled water and a 3%w/v of sodium alginate solution at optimized volumetric ratio from the above section. The immobilized microalgae beads were added into 500 mL bioreactor. The anaerobic digestor effluent was used directly and diluted with water. The effect of light intensity was varied in the range of 5.5-11.5 klux. The non-sterilized and non-pH adjust

effluent were also tested to evaluate the pratical use of this system. All the cultures were incubated at 30±1 °C and aerated with synthetic biogas (40% CO_2) at a gas flow rate of 0.3 L h^{-1} per 1 L culture medium and photoperiod 24 h for 8 days.

2.4 Analytical methods

To determine the biomass, approximately 10 mL of immobilized microalgae beads were solubilized with 1.5% w/v sodium carbonate anhydrous solution. The biomass were recovered by centrifugation and dried at 60 °C for 2 day before weighed.

The lipids of microalgae biomass was extracted using a mixture of chloroform/methanol (2:1, v/v) and sonication for 1 h. The extracted lipids were centrifuged to obtain a clear supernatant, and the solvent was removed by evaporation under vacuum and weighed. The lipid-free biomass was calculated by subtracting the lipid weight from the biomass weight (Cheirsilp *et al*., 2017b). The method for converting the extracted lipids to fatty acid methyl esters (FAMEs) involved hydrolysis of the lipids followed by esterification (Jham *et al*., 1982).

The fatty acid compositions of the FAME were analyzed using a HP6850 Gas Chromatograph equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm I.D, 0.25 µm film thickness) and a flame ionization detector. Operating condition were as follows: an inlet temperature was 290 °C; the initial oven temperature of 210 °C was held for 12 min, then ramped to 250 °C at 20 °C/min, held for 8 min, and the detector temperature was 300 °C. The fatty acids were identified by comparing their retention times with known pure standards. The fatty acid profiling was used to calculate the biodiesel properties of microalgae such as saponification value (SV), iodine value (IV), cetane number (CN), unsaturation degree (DU), long chain saturated factor (LCSF), cold filter plugging point (CFPP), high heating value (HHV) and oxidative stability (OS) as the following empirical Eqs. (1)-(8) (Katiyar *et al*., 2017).

$$SV = \sum[(560 \times F)/MW] \quad (1)$$

$$IV = \sum[(254 \times F \times D)/MW] \quad (2)$$

$$CN = [46.3 + (5458/SV)] - (0.225 \times IV) \quad (3)$$

$$DU = \%MUFA + (2 \times \%PUFA) \quad (4)$$

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24) \quad (5)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (6)$$

$$HHV = 49.43 - (0.041 \times SV) - (0.015 \times IV) \quad (7)$$

$$OS = [117.9295/(\%wt\ C18{:}2 + \%wt\ C18{:}3)] + 2.5905 \quad (8)$$

where D is the number of double bonds, F is the % of each type of fatty acid and MW is the molecular weight of corresponding fatty acid. MUFA is the weight percentage of the monounsaturated fatty acids (wt%) and PUFA is the weight percentage of the polyunsaturated fatty acids (wt%).

The compositions of synthetic biogas were determined using a Gas Chromatograph (GC6890) with a cross-linked capillary HP-PLOT Q column (length 30 m, 0.54 mm I.D, 0.04 µm film thickness) and a thermal conductivity detector. Helium was used as carrier gas. The percent of CO_2 removal from biogas and CO_2 fixation rate (g-CO_2 day^{-1} per 1 L-microalgal culture) were determined as follows:

$$\%CO_2 \text{ removal} = [\text{initial } CO_2\ (\%) - \text{final } CO_2\ (\%)) / \text{initial } CO_2\ (\%)] \times 100 \quad (9)$$

$$CO_2 \text{ fixation rate} = \text{gas flow rate } (L\ day^{-1}\ L^{-1}) \times \text{initial } CO_2\ (\%) \times \%CO_2 \text{ removal} \times 1000\ \text{g-}CO_2/556.2\ L \quad (10)$$

The wastewater used in this study was the effluent from anaerobic digester in palm oil mill (Surat Thani, Thailand). The effluent was filtered with a sieve through cheesecloth and was pretreated using sterilizer (SS-325; Tomy Seiko CO., Ltd, Japan). The COD, TN, and TP were analyzed using standard methods (APHA, 1995). Before sterilization, the pH, dissolved oxygen (DO), chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) of the effluent were 7.81, 0.4 mg L^{-1}, 10,000 mg L^{-1}, 2,100 mg L^{-1}, and 882 mg L^{-1}, respectively. After sterilization, the pH, DO, COD, TN, and TP slightly changed to 7.86, 0.4 mg L^{-1}, 9,500 mg L^{-1},

2,005 mg L^{-1}, and 800 mg L^{-1}, respectively. The nutrient removal efficiency i.e. COD, TN, TP was calculated as follow:

$$\%\text{Nutrient removal} = [1 - (C_i / C_0)] \times 100 \quad (11)$$

where C_0 is the nutrient concentrations in the cultures medium at time 0 and C_i is the nutrient concentrations in the cultures medium at time i.

All experiments were performed in triplicates. The results are expressed as mean plus standard deviations. Analysis of variance was performed to evaluate significant differences in treatment means, and the least significant difference was used to separate means, using SPSS software.

3. Results and discussion

3.1 biogas purification using free and immobilized oleaginous microalgae

3.1.1 Effect of initial cell concentration of microalgae

Among available, oleaginous microalgae *Scenedesmus* sp. has been selected as the most potential strain for removing CO_2 from biogas and simultaneously producing lipids (Srinuanpan *et al*., 2017). In this study, to increase the performance of the microalgae and simplify the harvesting process, the oleaginous microalga *Scenedesmus* sp. was immobilized in alginate gel beads and used for biogas purification. The results were compared between using free cells.

The immobilized microalgae cells at low initial cell concentration of 10^6 cells mL^{-1} gave the biomass of 1.78 g L^{-1} which was close to that of free cells (1.89 g L^{-1}). The results are shown in Fig. 1a and 1b. For the production of lipids, immobilized microalgae cells accumulated lipid content of 33.93% which was comparable to that of free cells. It was also found that the immobilized microalgae could grow without a long lag period after the beads were added in the medium when compared with the free cells, indicating no significant mass transfer limitation. Their results were consistent with those reported by Ruiz-Marin *et al*. (2012) and Cheirsilp *et al*. (2017a).

With the high initial cell concentration of 10^7 cells mL^{-1}, the free cells could grow and gave biomass increased up to 3.12 g L^{-1} with a lipid content of 34.15% (Fig.

1c). The immobilized cells grew similarly and gave the biomass of 2.99 g L^{-1} (Fig. 1a). However, at day 8 the beads spit and the cells leaded out from the beads with 0.2 g L^{-1} of biomass detected in the medium on day 8. It might be due to overloading of biomass occurred in the beads with high cell density. Lam and Lee (2012) reported that the mass transfer of gas and nutrients is the key factor for cultivating immobilized microalgae. In addition, the results of this study was controversial with those of Cheirsilp *et al*. (2017a), who found that a cell concentration of 10^7 cells mL^{-1} was suitable for the performance of oleaginous microalga *Nannochloropsis* sp. immobilized in alginate gel beads This might be due to the fact that the cells size of *Nannochloropsis* sp. (~3 µm) was much smaller than that of *Scenedesmus* sp. (>7.5 µm).

As shown in Fig. 2a, the immobilized microalgae with low initial cell concentration of 10^6 cells mL^{-1} contained chlorophylls and carotenoids at 16.54 and 9.67 mg g^{-1} biomass, respectively which were comparable to those of the free cells (17.98 and 10.91 mg g^{-1} biomass, respectively). While a high initial cell concentration (10^7 cells mL^{-1}), the production of pigments including chlorophylls and carotenoids were lower. These results are in consistent with those reported by Abinandan and Shanthakumar (2016) who found that the chlorophyll content decreased when the inoculum size increased. The amount of chlorophylls is an indicator of the rate of photosynthesis of microalgae and also the availability of the light (Cheirsilp *et al*., 2017a).

Fig. 2b and 2c show the performance of immobilized microalgae on CO_2 removal efficiency and CH_4 content after removing CO_2 from biogas compared with those of free cells. The percent CO_2 removal of free and immobilized microalgae at high initial cell concentration were higher than those using low initial cell concentration. The free and immobilized microalgae at high initial cell concentration (10^7 cells mL^{-1}) gave the maximum CO_2 removal of 75-77% and the CH_4 content was increased up to 90-91%. It should be noted that this methane content meets the standard (>90%) for efficient combustion with an increase in calorific value. CO_2 removal rate were as high as 3.9-4.0 g-CO_2 day^{-1} per 1 L-microalgal culture. Although the immobilized microalgae beads was broken, the CO_2 was also effectively removed due to both immobilized cells and laked cells from the beads could grow and

performed high CO_2 removal efficiency. At low cell concentration of 10^6 cell mL^{-1}, the percent CO_2 removal, CH_4 content and CO_2 removal rate by both free and immobilized microalgae were comparable to those at 10^7 cells mL^{-1}. More than 67-77% of CO_2 was removed from biogas and the CH_4 content was increased up to 86-91%. CO_2 removal rate were 3.5-4.0 g-CO_2 day^{-1} per 1 L-microalgal culture. It should be noted that the use of low initial cell concentration is more practical than the use of high initial cell concentration, initial cell concentration at10^6 cells mL^{-1} was then chosen for further experiments.

3.1.2 Effect of bead volume to medium volume ratio (B/M ratio)

The effect of bead volume on microalgae growth, lipid production and CO_2 removal efficiency were studied by varying the bead volume ratio of 25%, 50% and 75% medium volume. Among the three B/M ratio tested, the B/M ratio of 25% gave the maximum biomass of 2.00 g L^{-1} followed by the B/M ratio of 50% (1.78 g L^{-1}) and 75% (1.07 g L^{-1}), respectively (Fig. 3). It should be noted that a lower B/M ratio gave higher microalgae biomass. The lipid productions by immobilized microalgae at low B/M ratio were also higher than those using high B/M ratio. The lipid production and lipid content were found to be 0.12-0.67 g L^{-1} and 18.98-33.93%, respectively. The different performance depended on the amount of nutrients in liquid phase and solid phase and the rate of nutrient consumptions. The lower B/M ratio caused less mass transfer limitation of nutrients and gas.

The contents of pigments decreased with increasing the B/M ratio (Fig. 3d). This could be due to the lower light penetration through the beads. In addition, the mass transfer limitation between liquid and solid phase may also alter the photosynthetic metabolism and reduce the production of pigments in microalgal cells. Song *et al*. (2011) and Yeesang and Cheirsilp (2014) have also reported that the light penetration and nutrients availability in the culture medium did affect the photosynthetic efficiency of microalgae and then the production of pigments in the microalgal cells.

Fig. 3e and 3f shows the results of CO_2 removal efficiency and CH_4 content after biogas purification. These results were consistent with the microalgae growth. Fig. 3e and 3f demonstrate that lower B/M ratio achieved higher CH_4 content and

lower CO_2 content, indicating the better biogas upgrading efficiency. At B/M ratio of 25%, the CO_2 removal and CH_4 content were highest as well as the maximum CO_2 removal rate of 3.95 g-CO_2 day^{-1} per 1 L-microalgal culture. Possibly, the decreased CO_2 removal efficiency might also be due to the coalescence of gas bubbles that the decrease in bubble retention time in the medium. The decrease in surface area per unit gas volume of the bubbles also reduced the CO_2 removal efficiency (Kao *et al*., 2012). The optimal B/M ratio of 25% was in consistent with the results reported by Cheirsilp *et al*. (2017a). Therefore, the B/M ratio 25% was chosen for further experiment.

3.2 Two-phase purify unit for biogas and anaerobic digester effluent

3.2.1 Dilution of effluent

The immobilized microalgae were used in phytoremediation of anaerobic digester effluent. However, the effluent was in dark brown colour with high turbidity. The effluent concentration was then diluted. Park *et al*. (2009) and Khalid *et al*. (2016) also suggested that POME should be diluted before used as nutrient medium. Dilution of the effluent could improve light penetration and increase the photosynthesis activity and biomass production. Fig. 4 shows the cultivation of immobilized microalgae using biogas effluent and undiluted and diluted POME. The effluent concentration at 20% gave the highest biomass production (2.19 g L^{-1}), followed by 40% effluent concentration (1.28 g L^{-1}). The effluent concentration >40% gave poor microalgae biomass. There are two possible reasons. One might be due to inhibition from high concentration of some compounds and another one might be the low light penetration. Both high concentration of inhibitory compounds and low light penetration resulted in low microalgae growth and long lag time. Generally, POME contains phenolic compounds such as caffeic acid, tannic acid, ferulic acid, 4-hydroxybenzoic acid, catechol, and 3-methylcatechol (Khongkhaem *et al*., 2016) which could be inhibitory to microalgae growth and photosynthesis reaction in microalgae cells (Hadiyanto and Nur, 2012). Ding *et al*. (2016) also reported that the excessive nutrient content in undiluted wastewater could be toxic to microalgae and negatively affect their growth.

The lipid production by immobilized microalgae using different effluent concentration are shown in Fig. 4. With 20% effluent concentration, the lipid production was highest at 0.61 g L^{-1} corresponding to the lipid content of 27.73% while the effluent concentration >20% gave the lower lipid content than 22%. Liu *et al*. (2012) found that the lipid yield and lipid content of *Scenedesmus* sp. was low due to the low light penetration. Through photosynthesis, large amounts of ATP and NADPH are produced as well as CO_2 is converted to glyceraldehyde-3-phosphate (G3P). Then, G3P is converted to pyruvate and thereafter to acetyl-CoA, via a series of catalytic reaction, initiates the lipid biosynthetic pathway (He *et al*., 2015). Therefore, increasing light intensity could lead to significant increase in photosynthesis reaction and subsequent lipid synthesis. In addition, high carbon and nitrogen concentration may also inhibit the activity of key enzyme (ACCase: Acetyl CoA Carboxylase enzyme) in lipid synthesis pathway (Minhas *et al*., 2016).

Using 20% effluent concentration also gave higher chlorophyll and carotenoid contents than those using effluent concentration (Fig. 4f). It was possible that high effluent concentration had low light penetration and resulted in low photosynthesis activity. Kamyab *et al*. (2016) reported that the reduction of pigment content is directly related to the reduction of photosynthesis activity. Moreover, in the effluent the microalgae might also grow with heterotrophic regime using organic carbon without light together with photosynthetic regime which is so called mixotrophic regime. García *et al*. (2005) reported that the cellular photosynthetic components depended on the duration of time that cells remained in light and dark zones.

Fig. 5a and 5b show the effect of effluent concentration on CO_2 removal efficiency and CH_4 content in biogas after purification. Over time courses, the CO_2 content decreased, whereas the CH_4 content increased. Results indicated that lower effluent concentration achieved better biogas upgrading efficiency. The highest CH_4 content obtained was 90.75% when using 20% effluent concentration. The higher effluent used, the lower biogas upgrading efficiency obtained. Low light penetration and high concentration of inhibitory compounds may inhibit the carboxylating activity and restrain the oxygenating activity of Rubisco, resulted in decreased performance of microalgae cells and lower CO_2 removal process (Yan *et al*., 2016). Considering CO_2 removal rate, it was found that at 20% effluent concentration gave the highest values

at 4.0 g-CO_2 day^{-1} per 1 L-microalgal culture. Thus, it could be concluded that the use of effluent concentration at 20% was suitable for biogas upgrading by immobilized oleaginous microalgae.

The DO value in this research slightly increased during the experimental period, which was found to be in the range of 0.40-5.95 mg L^{-1} (Table S1), which was below the level (35 mg L^{-1}) that inhibits the microalgae performance (Yan and Zheng, 2013). During cultivation in 20% effluent concentration, the DO value increased continuously during cultivation because the immobilized microalgae could grow and produce oxygen through photosynthesis activity. As the maximum DO value (5.95 mg L^{-1}) was lower than the saturation level at 30 ºC (7.6 mg L^{-1}) (Huguenin and Colt, 1989). This indicated that the produced oxygen mainly dissolved in the culture medium and did not affect the quality of biogas.

As shown in Fig. 5c, at 20% effluent concentration the COD removal efficiency ranged from 22.24% to 48.42%. This indicated that the microalgae could consume organic carbon through heterotrophic metabolism (Ding *et al*., 2016). However, with the effluent concentration >40%, the COD removal became lower (Fig. 5c). It was obvious that high effluent concentration inhibited microalgae growth. In POME treatment through anaerobic digestion process, acetate is one of the products converted by a biological reaction from volatile fatty acids (VFA) via the process of acetogenesis. Afterwards, acetate is converted into methane and CO_2 by another biological reaction in the process of methanogenesis. Thus, the residual acetate in the downstream of anaerobic POME was found due to incomplete reaction of methanogenesis or the inhibition of methanogenesis (Ding *et al*., 2016). Therefore, it was possible that acetate could be mainly carbon source for microalgae.

Generally, pollutant removal by microalgae depends on the ability of microalgae to assimilate the pollutants. This study had shown that the diluted effluent exhibited as better cultivation medium for microalgae. Nitrogen is one of the most essential nutrients for microalgae assimilation and metabolism. Microalgae cells adsorbed the inorganic nitrogen in the form of nitrate after nitrification and also ammonia and organic nitrogen (Yan *et al*., 2016). Nitrogen source in anaerobic POME consisted of inorganic nitrogen such as NH_4^+–N, NO_3^-–N, and NO_2^-–N and organic nitrogen which have not been decomposed by the microorganisms. The total

nitrogen removal (TN) efficiency are shown in Fig. 5d. It was found that the TN removal using 20% effluent concentration was highest at 82.69% whereas with higher effluent concentration the TN removal became lower. Ding *et al*. (2016) found that about 70% of the nitrogen in anaerobic effluent was NH_4^+–N. Yuan *et al*. (2011) reported that high ammonium content might be toxic to microalgae growth. Phosphorus, especially ortherphosphate which is another crucial nutrient for microalgae growth and energy metabolism. Fig. 5d shows the total phosphorus removal efficiency in the different effluent concentrations. At 20% effluent concentration, total phosphorus was completely removed, followed by 40% effluent concentration (38.28%). As the dark color of undiluted effluent negatively affected the light penetration and led to lower the photosynthesis by the microalgae, it could be concluded that the high content of some inhibitory compounds as well as hard light penetration did inhibit the performance of the microalgae.

3.2.2 Effect of light intensity

To increase the light availability, the light intensity was increased from 5.5 to 11.5 klux. The effluent concentration at 20% was used as culture medium. The temperature was controlled at 30±1 °C. Fig. 6 shows the effect of light intensity on microalgal biomass when using 20% effluent concentration. The biomass of microalgae increased from 2.19 to 2.90 g L^{-1} when the light intensity was increased from 5.5 to 9.5 klux. A further increase in light intensity did not increase more microalgal biomass. It was possible that at low light intensities (<9.5 klux) the photolimitation occurred and resulted in low biomass production. On the contrast, the microalgal biomass decreased at light intensity of 11.5 klux possibly due to photoinhibition effect. Zhao *et al*. (2013) found that excessive light intensity damaged microalgae by overloading the photosystem and bleaching the pigments. Therefore, it could be concluded that 9.5 klux was the optimum light intensity for the microalgae growth in 20% effluent concentration.

Fig. 6 shows the lipid production of microalgae in 20% effluent concentration under different four light intensities. The results indicated that a high lipid production correspond to high biomass production. The lipid production and lipid content increased with increasing light intensity from 5.5 klux to 9.5 klux. It is well known

that light energy is absorbed by the pigments and then converted to ATP or NADPH, and this chemical energy is stored ultimately as starch and/or lipids. Similarly to the cell growth, the production of lipids decreased when the light intensity was increased up to 11.5 klux. When high light energy was supplied into the microalgal culture, the light-harvesting chlorophyll molecules changed to unstable forms, which in turn react with dissolved oxygen species. These reactive oxygen species then react with free fatty acids to make lipid peroxidase in inactive form and this reduces the fatty acid synthesis which is known as photo-oxidative inhibition (Gim *et al*., 2016). Thus, the optimum light intensity for maximum total lipid production was found to be 9.5 klux. Under these condition, the lipid production was increased up to 1.03 g L^{-1} with considerably high lipid content of 35.52%.

As shown in Fig. 6e, the pigment content increased with increasing light intensity from 5.5 klux to 9.5 klux, but decreased when light intensity was increased up to 11.5 klux. It was possible that with attenuating light the cells respond by synthesizing more chlorophylls. The chlorophylls content was as high as 45.88 mg g^{-1} biomass at light intensity of 9.5 klux. The decrease in the chlorophyll content at high light intensity would be because the photosystem and pigments was destroyed and this phenomenon is known as photoinhibition. According to Cheirsilp and Torpee (2012) who mentioned that the necessary amount of light energy is completely absorbed by a growing number of cells, thus the cells require a lower content of cellular chlorophylls. For the production of carotenoids, some literature (García *et al*., 2005; Gim et al., 2016) indicated that when exposed to high light intensity, the microalgae cells might use the carotenoid synthesis pathway as a protective mechanism against photodamage. In this study, at higher light intensity (>9.5 klux) the carotenoid content decreased possibly due to the large damage on the photosystem. This suggested that carotenoid synthesis may not be enough to protect cells from high light stress. The highest carotenoid content was 26.34 mg g^{-1} biomass at 9.5 klux of light intensity.

It is well known that light intensity is an important factor for the photosynthetic activity of microalgae, which directly affects CO_2 removal efficiency. The effect of light intensity on CO_2 removal efficiency and CH_4 content are shown in Fig. 7a and 7b. The CO_2 removal efficiency increased when the light intensity was increased from 5.5 to 9.5 klux. The maximum percent CO_2 removal and CO_2 removal

rate were obtained at 88.24% and 4.58 g-CO_2 day^{-1} per 1 L-microalgal culture, respectively at light intensity of 9.5 klux. Consequently, the CH_4 content in biogas was increased up to 95.28%, which meets the standard of fuel requirements (Zhao *et al*., 2013). At too high light intensity, photoinhibition occurred and lowered both the photosynthesis activity and CO_2 removal efficiency. The results are similar to those reported by Zhao *et al*. (2013) and Yan and Zheng (2013).

The removal efficiency of COD, TN and TP increased when the light intensity was increased from 5.5 klux to 9.5 klux (Fig. 7c and 7d). However, at light intensity >9.5 klux the pollutants removal decreased likely due to the photoinhibition on the performance of the microalgae. Similar results have been observed by Binnal and Babu (2017). Thus, the light intensity at 9.5 klux was the optimal light intensity for cell growth, lipid and pigments production, CO_2 removal and pollutants removal. The DO value at 9.5 klux ranged from 2.65 to 6.35 mg L^{-1} (Table S1), which were below the saturation level.

3.2.3 Performance under unsterile and non-pH adjustment

The unsterile effluent without pH-adjustment was used for microalgae cultivation. The results were compared with those using sterile and pH adjusted effluent (Table 1). The biomass, lipids and pigments production using non-sterile effluent were close to those using sterile and pH adjusted effluent. Similar results have been reported by Covarrubias *et al*. (2012). Similarly, the CO_2 removal efficiency and CH_4 content after removing CO_2 in both sterile and non-sterile effluent were not significant different. Using non-sterile effluent, more than 88.24% of CO_2 was removed from biogas and the CH_4 content was upgraded up to 95.37%. The CO_2 removal rate was 4.6 g-CO_2 day^{-1} per 1 L-microalgal culture. Also, the COD removal of immobilized microalgae under using non-sterile effluent (71.25%) was slightly higher than using sterile effluent (68.68%). Interestingly, the total nitrogen and phosphorus were completely removed. The DO value of using non-pretreated POME ranged at 2.65-6.40 mg L^{-1} (Table S1). Thus, this study indicated that using non-sterile and non-pH adjusted effluent can be used directly for cultivation of immobilized microalgae.

3.3 Estimation of fuel properties of microalgal lipids

The lipids extracted from immobilized oleaginous microalga *Scenedesmus* sp. was converted to fatty acid methyl esters (FAMEs) and their compositions are summarized in Table 2. The accumulated fatty acid chain lengths were in the range of C8 to C18 fatty acids. The main fatty acids were long-chain fatty acids with 16 and 18 carbon atoms including oleic acid (C18:1) as the predominant fatty acid (48.8%) followed by palmitic acid (C16:0; 17.94%) and linolenic acid (C18:2; 15.86%). These fatty acids are similar to those of plant oils which contain mainly oleic acid and palmitic acids (Gui *et al*., 2008), indicating that microalgal lipids has potential as biodiesel feedstocks. These results are consistent with those of Breuer *et al*. (2015) who reported that the oleic acid found in *Scenedesmus* sp. was as high as 36%. The saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) of the microalgal lipids in this study were >75%. This indicates that the biodiesel from microalgal lipids could provide higher cetane number (CN), lower NOx emissions, shorter ignition delay time, and higher oxidative stability. The SFA/UFA ratio was obtained at 0.25. It is well known that SFA/UFA ratio also determines the quality of the biodiesel. Higher ratio of SFA/UFA results in greater density and viscosity values and higher oxidative stability of the subsequent fuel (Talebi *et al*., 2013). The biodiesel fuel properties were calculated based on the fatty acid composition (Table 2). The iodine value (IV), saponification value (SV), cetane number (CN), unsaturation degree (DU), LCSF (long chain saturated factor), cold filter plugging point (CFPP), high heating value (HHV) and oxidative stability (OS) were found to be 97.57 g I_2/100 g oil, 205.12 mg KOH/g oil, 50.95, 100.5, 3.13, -6.65 °C, 39.56 MJ/kg and 7.8 h, respectively. It could be concluded that the fuel properties of microalgal lipid are in accordance with the international standard ASTM D6751 (USA) and EN 14214 (European Organization). It then has high potential as biodiesel feedstocks.

3.4 Significance and possible economic impact of the process

The primary requirements for the growth of microalgae are nutrients and CO_2. Cultivating microalgae with high nutrients from industrial effluent is interesting as it serves dual purpose of treatment of effluent as well as cost-effective production of

large quantity of microalgae biomass. Those strategy is effective not only in biomass production and removal of pollutants but also reduces the production cost. The use of commercial medium for microalgae cultivation would cost about $19 per m^3 culture volume from the cost of chemical substances. While using anaerobic digester effluent is more cost-effective. The dilution of effluent by using final effluent is one strategy to reduce the use of freshwater.

The use of immobilization technology in microalgae cultivation is the effective technique to reduce operational cost and energy input in harvesting process due to the large sizes of the immobilized microalgae beads. The free cells of microalgae are smaller in range of 5-50 μm of diameter, which is required high energy input in harvesting process. Thus, the immobilized cells with larger size can be easily harvested by a simple sieving method without involving huge amounts of energy input. Based on this research, the use of immobilized microalgae are fairly cost-effective at estimated material around $0.07 per kg biomass. This cost was close to that the report of Cheirsilp *et al*. (2017a), who found that material costs for immobilization at 25% beads volume to culture medium ratio was about $0.06 per kg microalgal biomass. Grima *et al*. (2003) have reported that the harvesting of free cells using centrifugation required nearly 0.3-8 kW h of electricity per m^3 culture volume and the harvesting costs would be $1.44-18 per kg microalgal biomass. While using electro-flocculation was estimated at $0.19 per kg microalgal biomass with 0.092 kW h of electricity per m^3 culture volume (Lee *et al*., 2013). Selesu *et al*. (2016) was also found that bio-flocculation with chitosan has still high cost up to $10 per kg microalgal biomass. Cheirsilp *et al*. (2017a) reported that the immobilized microalgae beads could be reused for 2 cycles of cultivation. However, it is not possible in this research because immobilized beads had broken down when microalgal biomass increased higher than 3.0 g L^{-1}. Therefore, to enhance the economic potential of microalgal biomass production, using recycled Na-alginate after biomass harvesting for preparing new lot of immobilized microalgae beads is highly recommended. The schematic diagram for reusing of Na-alginate is shown in Fig. S1. Based on these information, using the immobilized cells could then be economically attractive for microalgae cultivation than the free cells.

4. Conclusions

The immobilized oleaginous microalga *Scenedesmus* sp. in alginate beads has high potential for biogas upgrading, lipid production and phytoremediation of biogas effluent from palm oil mill. As the immobilized microalgae can be recovered by a simple sieving method, the harvesting cost could be reduced. Through this strategy, the methane content in biogas was upgraded up to the standard level for being used as fuel. The microalgal lipids has high potential as biodiesel feedstock due to the similar fatty acid compositions to those of plant oils and their prospect fuel properties were also in accordance with those of the international standards. The cultivation of immobilized microalgae for removing CO_2 from biogas and pollutants from industrial effluent would contribute greatly to the sustainability of the renewable bioenergy.

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Supplementary materials

Table S1 Variations of physical and chemical parameters in POME culture

Figure S1 Schematic diagram for reusing of Na-alginate in preparing immobilized oleaginous microalgae beads

Table 1 the performance of microalgae cultured in sterile and pH adjustment effluent (SPH) vs. unsterile and non-pH adjustment (USPH) on growth, lipid production, lipid content, pigments content, CO_2 removal efficiency, CH_4 content after purification and pollutants removal efficiency on days 8

Conditions	Biomass ($g\ L^{-1}$)	Lipid production ($g\ L^{-1}$)	Lipid content (%w/w)	Pigments production		CH_4 content in biogas after purification (%)	CO_2 removal efficiency (%v/v)	CO_2 removal rate ($g\text{-}CO_2\ day^{-1}$ per 1 L)	Pollutants removal efficiency		
				Chlorophylls	Carotenoids				COD removal (%)	TN removal (%)	TP removal (%)
SPH	2.90±0.01	1.03±0.01	35.52±0.01	45.88±1.94	26.34±1.26	95.28±0.83	88.24±2.08	4.58±0.11	68.68±0.37	98.93±0.14	100.0±0.0
USPH	2.98±0.01	1.07±0.02	35.92±0.88	45.97±4.45	26.06±0.33	95.37±0.50	88.46±1.26	4.60±0.07	71.25±1.06	100.0±0.0	100.0±0.0

Table 2 Fatty acid profiling and estimated fuel properties of microalgal lipids

Fatty acids	Relative content (%)		
Caprylic acid (C8:0)	0.28		
Capric acid (C10:0)	0.34		
Tridecanoic acid (C13:0)	0.36		
Myristic acid (C14:0)	0.56		
Palmitic acid (C16:0)	17.94		
Palmitoleic acid (C16:1)	6.41		
Stearic acid (C18:0)	2.67		
Oleic acid (C18:1)	48.80		
Linoleic acid (C18:2)	15.86		
Linolenic acid (C18:3)	6.78		
C16-C18	98.47		
Saturated fatty acid (SFA)	19.85		
Unsaturated fatty acid (UFA)	80.15		
Monounsaturated fatty acid (MUFA)	55.21		
Polyunsaturated fatty acid (PUFA)	22.64		
SFA/UFA	0.25		
Estimated fuel properties	Values	ASTM D6751	EN 14214
Saponification value (SV; mg KOH g^{-1})	205.12	NA	NA
Iodine value (IV; g $I_2 \cdot 100\ g^{-1}$)	97.57	NA	<120
Cetane number (CN)	50.95	>47	>51
Degree of unsaturation (DU; wt%)	100.50	NA	NA
Long chain saturation factor (LCSF; wt%)	3.13	NA	NA
Cold filter plugging point (CFPP; °C)	-6.65	NA	<5 to <−20
High heating value (HHV; $MJ \cdot kg^{-1}$)	39.56	NA	NA
Oxidative stability (OS; h)	7.80	NA	>6

NA is not available.

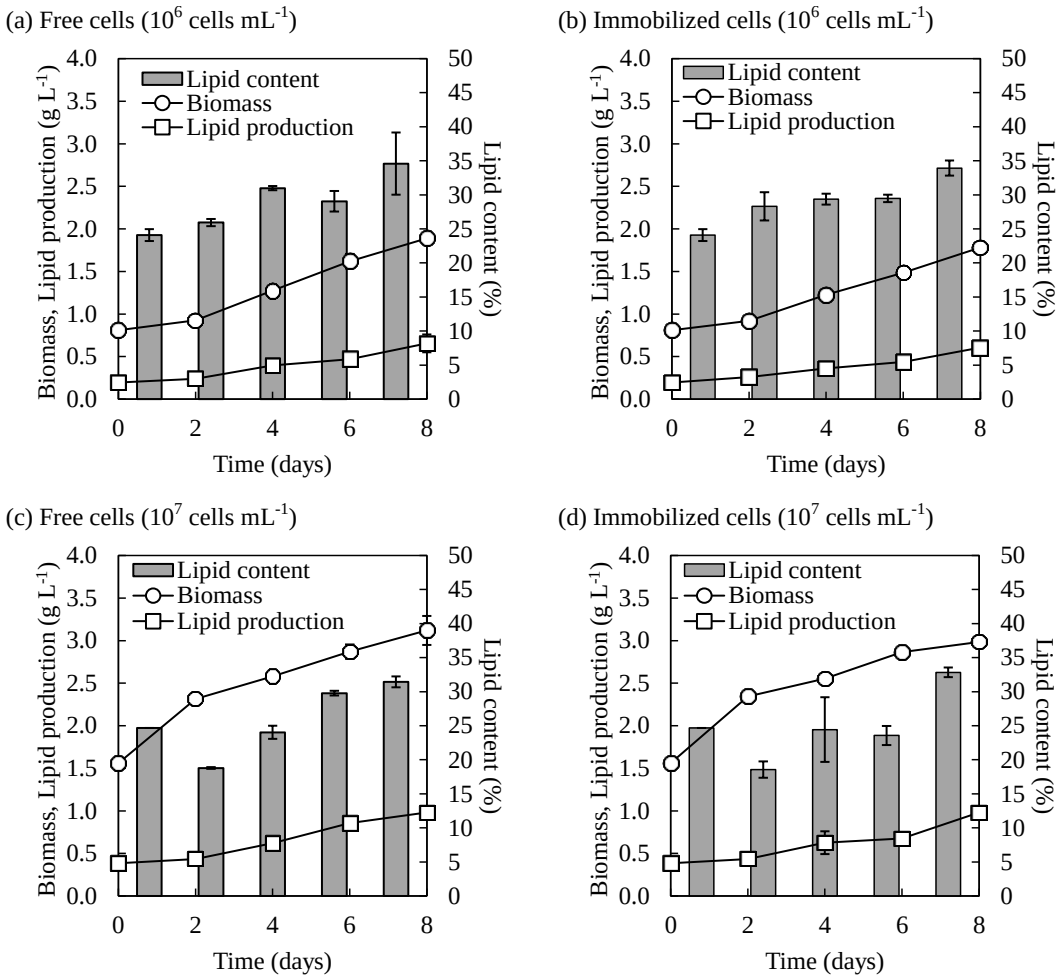

Figure 1 Effect of initial cell concentration on the microalgae growth, lipid production and lipid content of the immobilized microalgae (IC) compared with those of the free cells (FC). The data of lipid content were the data at day 0, 2, 4, 6, 8. Data are means of triplicates.

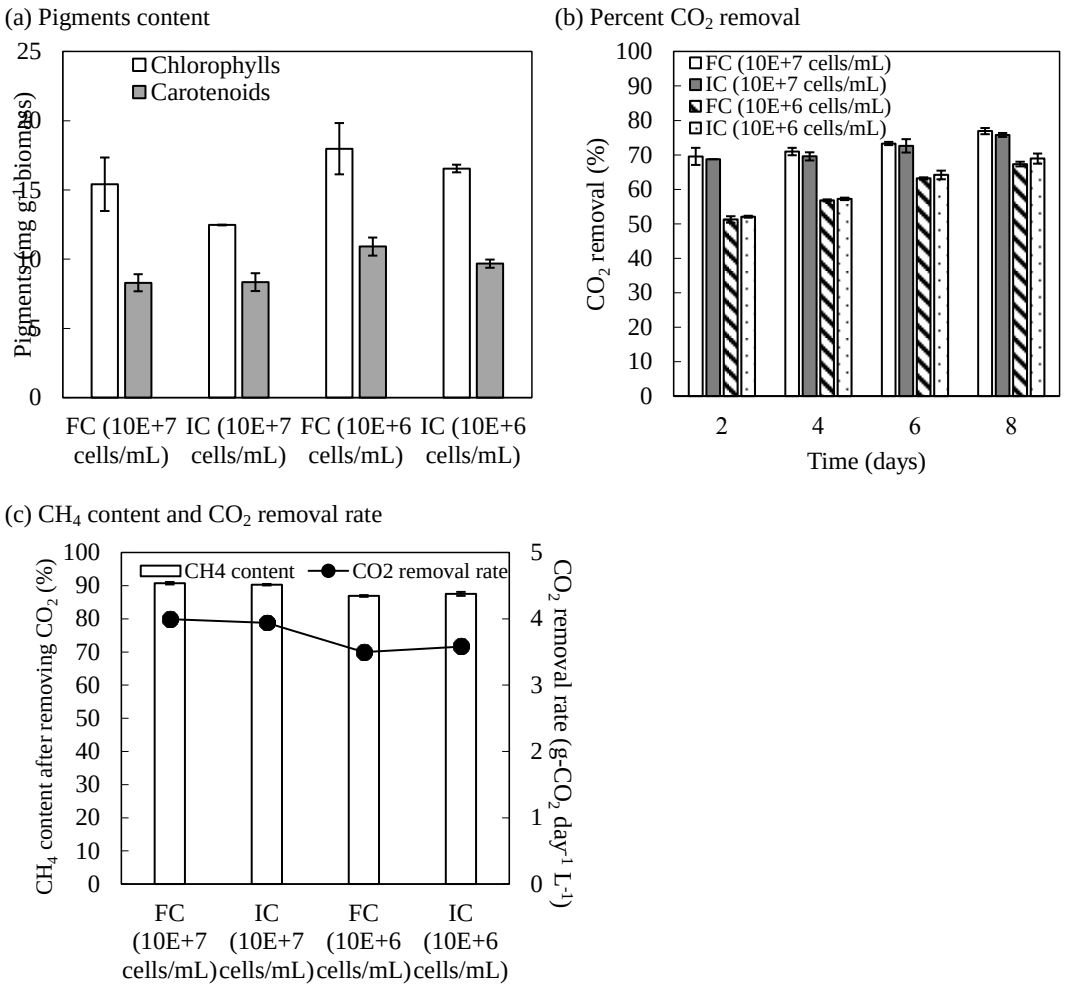

Figure 2 Effect of initial cell concentration on pigments content, CO_2 removal efficiency and CH_4 content after removing CO_2. FC and IC are free cell and immobilized cell, respectively. The data of pigments content, CH_4 content and CO_2 removal rate were the data at day 8. Data are means of triplicates.

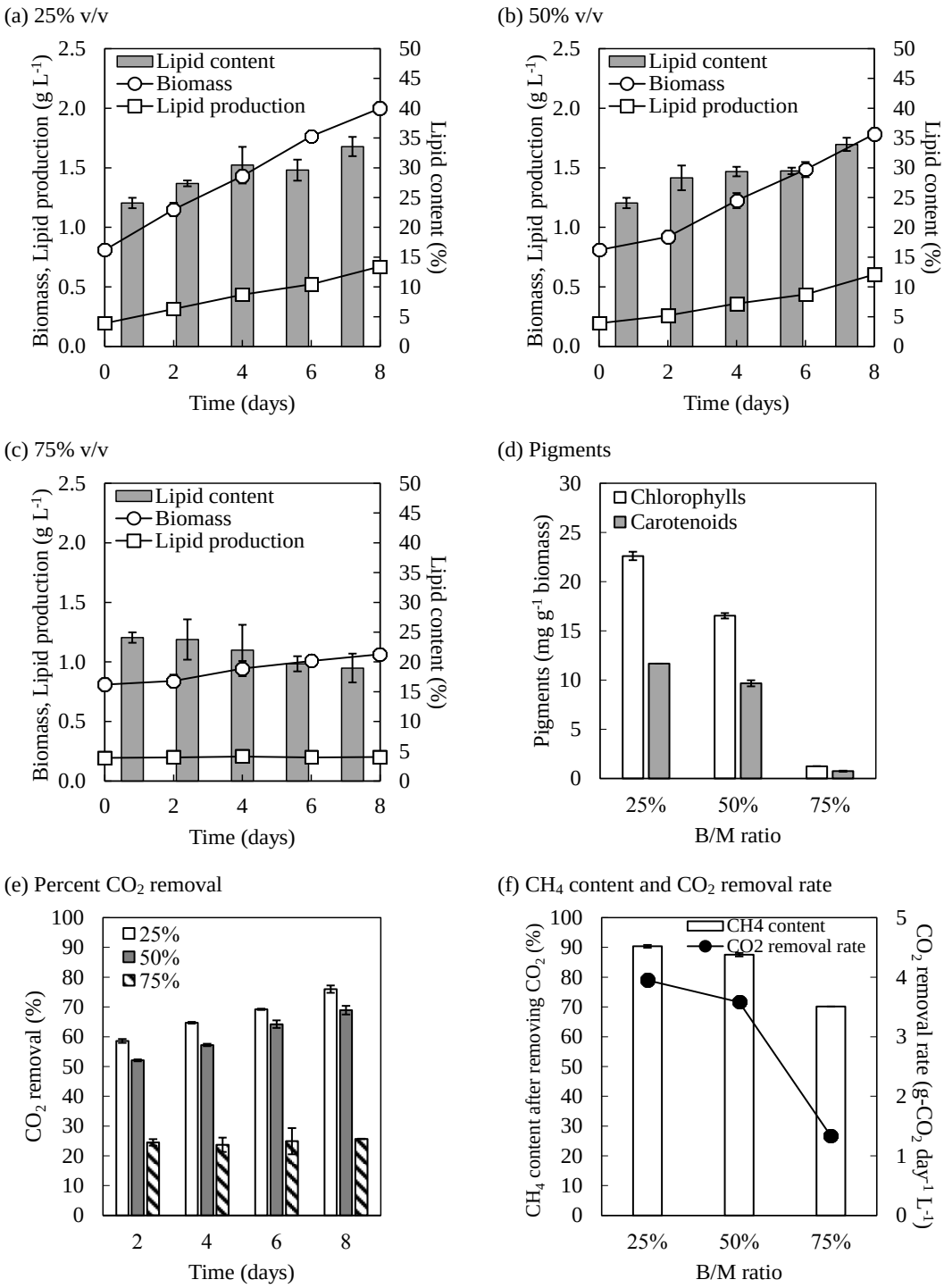

Figure 3 Effect of bead volume to medium volume ratio (B/M ratio) on the microalgae growth, lipid production, lipid content, pigments content, CO_2 removal efficiency and CH_4 content after removing CO_2 of the immobilized microalgae. The data of lipid content were the data at day 0, 2, 4, 6, 8. The data of pigments content, CH_4 content and CO_2 removal rate were the data at day 8. Data are means of triplicates.

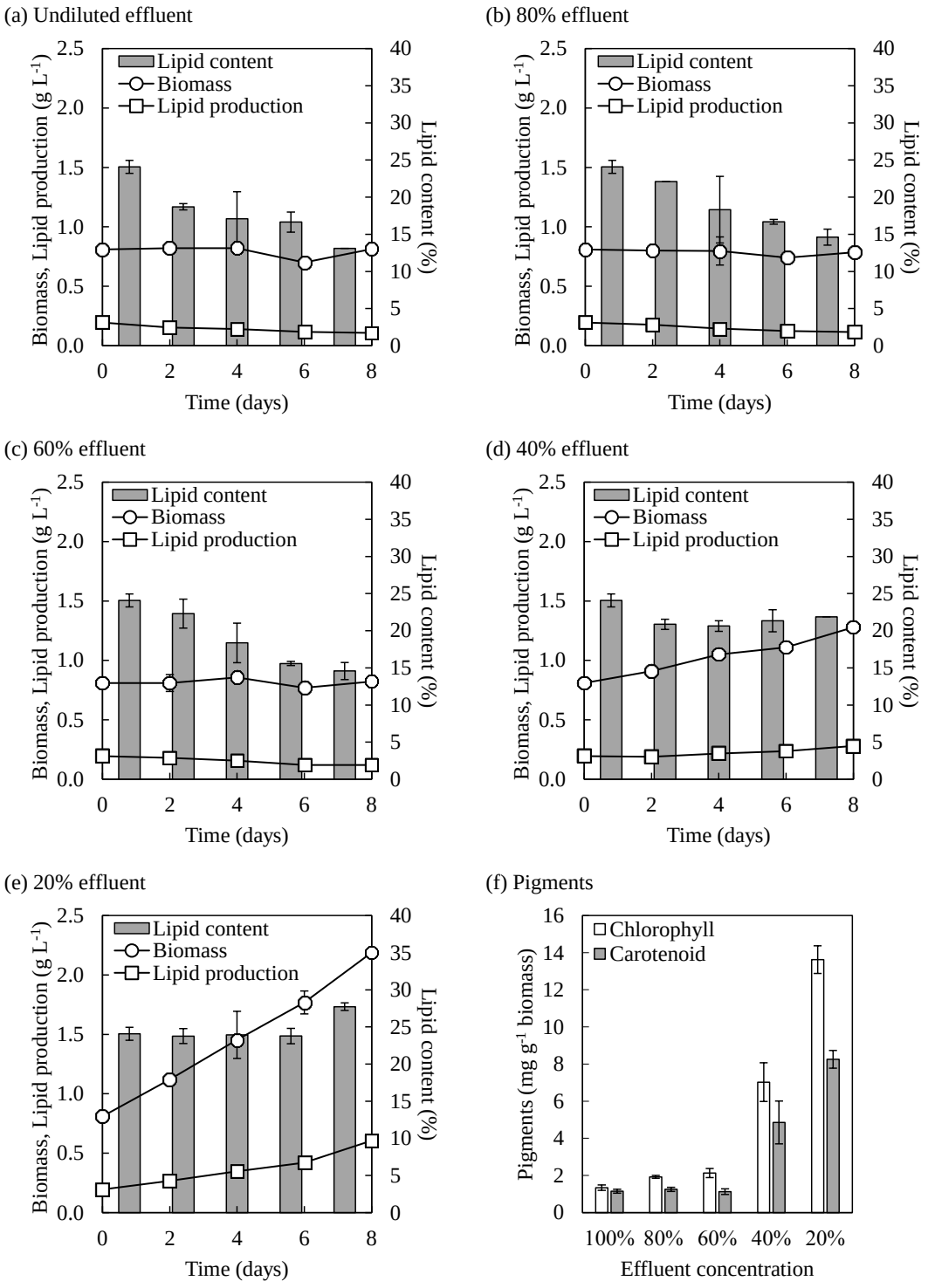

Figure 4 Effect of anaerobic digester effluent concentration on the microalgae growth, lipid production, lipid content and pigments production of the immobilized microalgae. The data of lipid content were the data at day 0, 2, 4, 6, 8. The data of pigments content were the data at day 8. Data are means of triplicates.

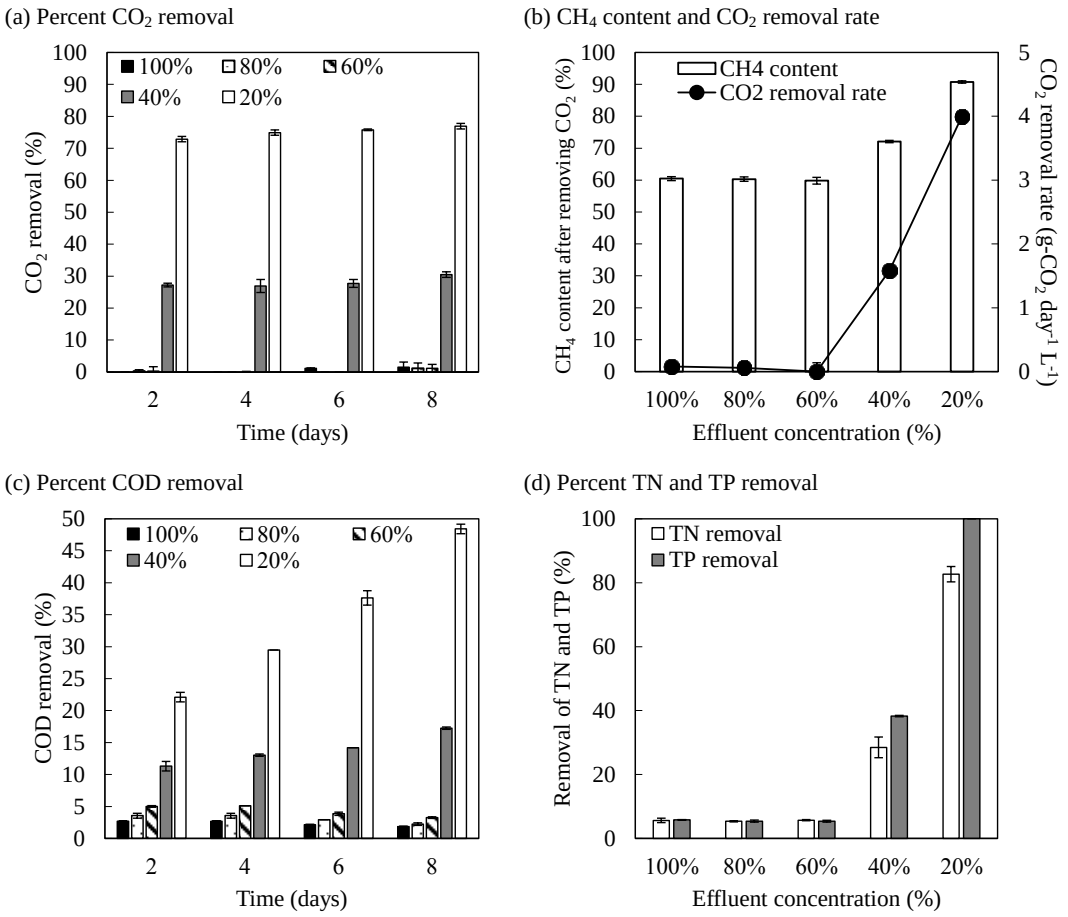

Figure 5 Effect of anaerobic digester effluent concentration on CO_2 removal efficiency, CH_4 content after removing CO_2, COD removal efficiency and the removal efficiency of TN and TP. The data of CH_4 content, CO_2 removal rate, TN removal and TP removal were the data at day 8. Data are means of triplicates.

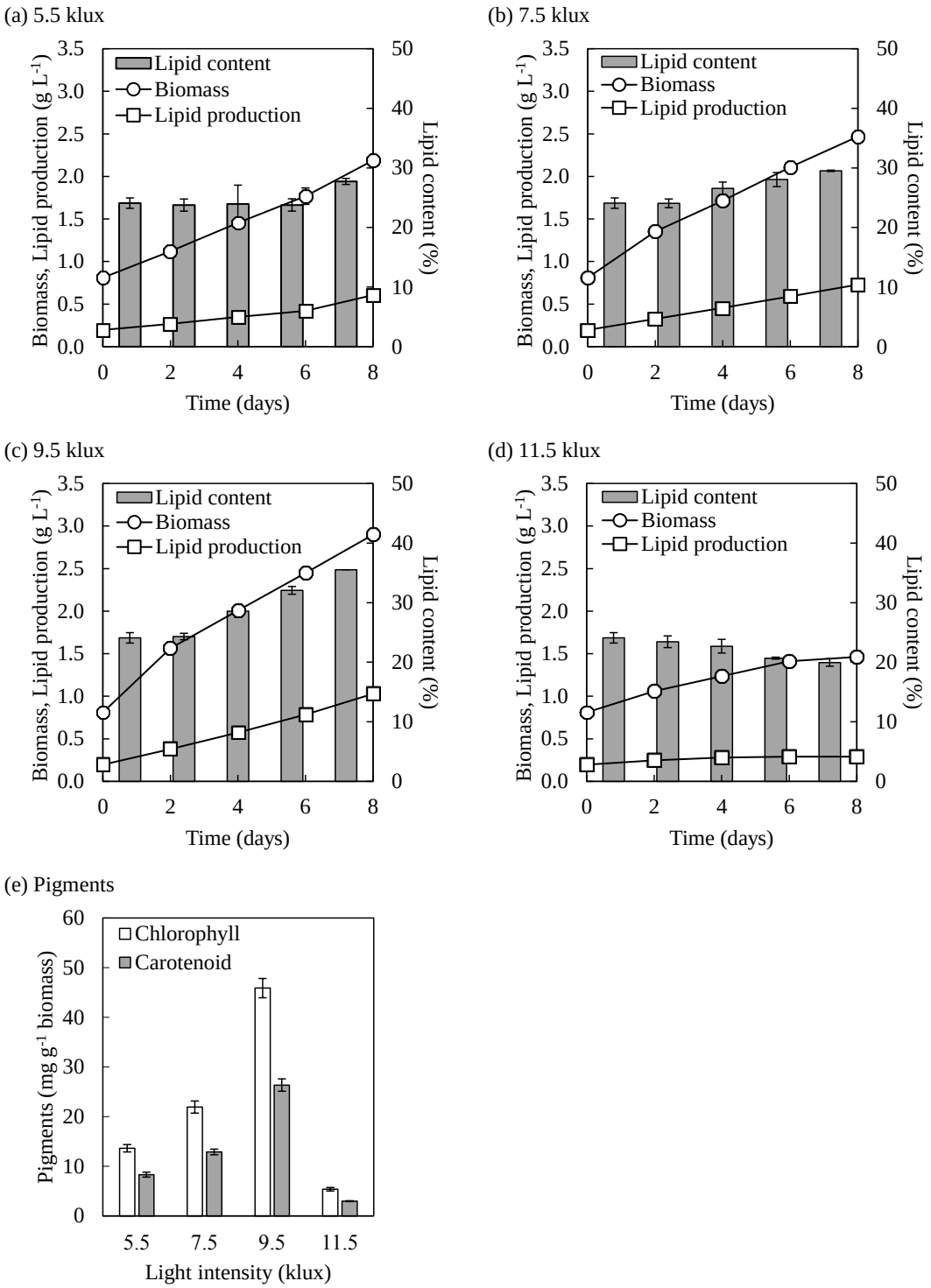

Figure 6 Effect of light intensity on the microalgae growth, lipid production, lipid content and pigments production of the immobilized microalgae. The data of lipid content were the data at day 0, 2, 4, 6, 8. The data of pigments content were the data at day 8. Data are means of triplicates.

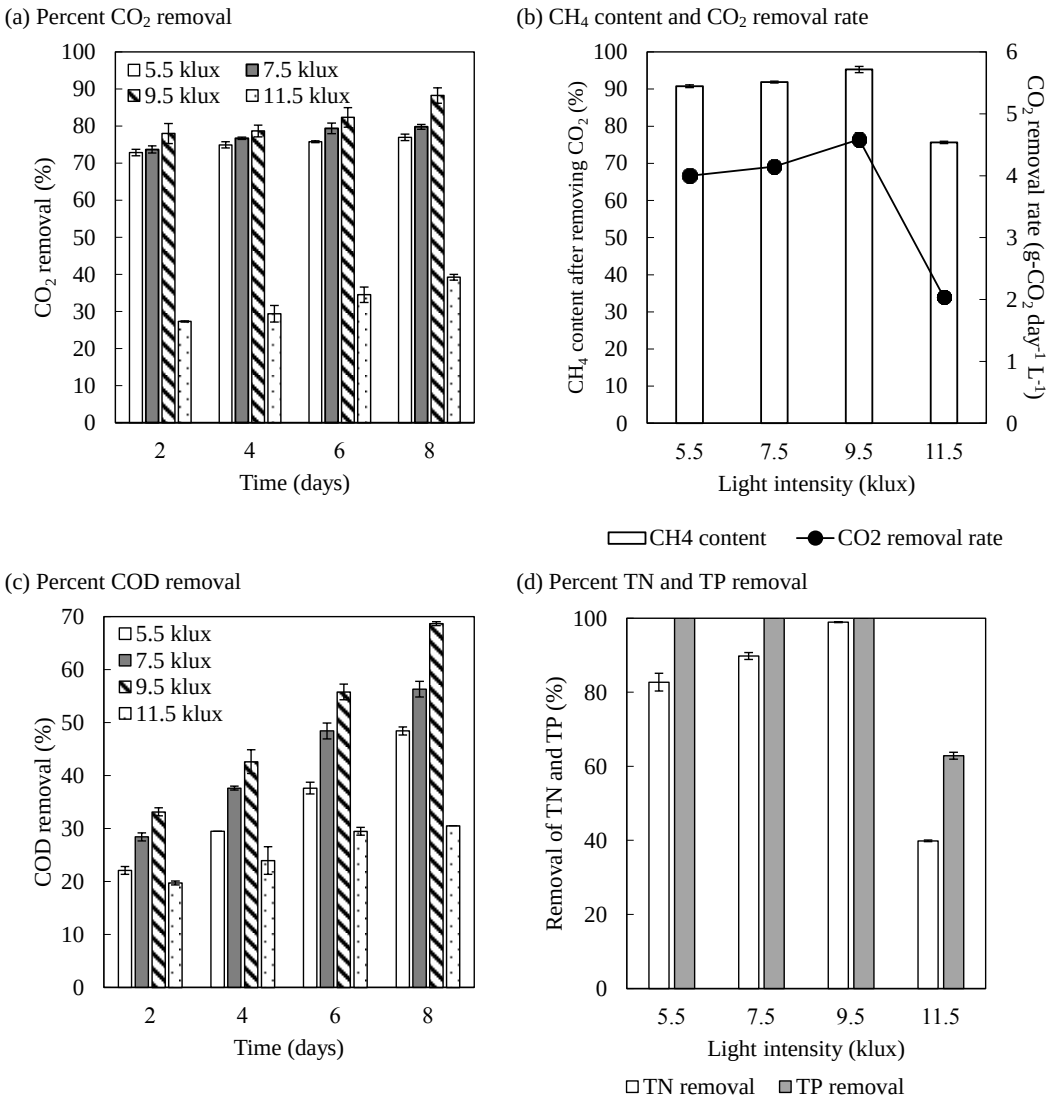

Figure 7 Effect of light intensity on CO_2 removal efficiency, CH_4 content after removing CO_2, COD removal efficiency and the removal efficiency of TN and TP. The data of CH_4 content, CO_2 removal rate, TN removal and TP removal were the data at day 8. Data are means of triplicates.

Table S1 Variations of physical and chemical parameters in POME culture.

Treatments	Physiochemical parameter	Experimental time (Days)				
		0	2	4	6	8
Effect of effluent concentration						
100%	pH	6.80±0.01	6.77±0.07	6.84±0.01	6.75±0.03	6.76±0.04
	DO (mg L^{-1})	0.40±0.01	0.50±0.01	0.50±0.01	0.50±0.01	0.50±0.01
80%	pH	6.80±0.01	6.82±0.01	6.73±0.04	6.72±0.04	6.74±0.04
	DO (mg L^{-1})	1.05±0.07	1.05±0.07	1.10±0.01	1.10±0.01	1.10±0.01
60%	pH	6.80±0.01	6.75±0.04	6.78±0.05	6.69±0.01	6.80±0.04
	DO (mg L^{-1})	1.55±0.07	1.60±0.01	1.65±0.07	1.60±0.01	1.60±0.01
40%	pH	6.80±0.01	6.91±0.56	6.90±0.52	7.39±0.05	6.88±0.01
	DO (mg L^{-1})	2.05±0.07	2.65±0.07	3.10±0.14	3.95±0.07	4.15±0.21
20%	pH	6.80±0.01	7.16±0.22	6.73±0.08	7.05±0.60	7.20±0.20
	DO (mg L^{-1})	2.65±0.07	4.25±0.07	5.25±0.21	5.90±0.14	5.95±0.07
Effect of light intensity						
5.5 klux	pH	6.80±0.01	7.16±0.22	6.73±0.08	7.05±0.60	7.20±0.20
	DO (mg L^{-1})	2.65±0.07	4.25±0.07	5.25±0.21	5.90±0.14	5.95±0.07
7.5 klux	pH	6.80±0.01	7.09±0.08	7.20±0.28	7.45±0.05	7.51±0.42
	DO (mg L^{-1})	2.65±0.07	4.85±0.07	5.00±0.01	6.20±0.14	6.35±0.21
9.5 klux	pH	6.80±0.01	7.75±0.08	7.79±0.03	7.72±0.02	7.23±0.01
	DO (mg L^{-1})	2.65±0.07	5.00±0.01	5.35±0.21	6.05±0.07	6.35±0.07
11.5 klux	pH	6.80±0.01	6.76±0.04	6.89±0.07	6.95±0.01	7.13±0.17
	DO (mg L^{-1})	2.65±0.07	4.50±0.01	4.80±0.01	4.95±0.07	5.05±0.07

Table S1 continued

Treatments	Physiochemical parameter	Experimental time (Days)				
		0	2	4	6	8
Effect of pretreated effluent						
Sterilized and pH adjusted effluent	pH	6.80±0.01	7.75±0.08	7.79±0.03	7.72±0.02	7.23±0.01
	DO (mg L^{-1})	2.65±0.07	5.00±0.01	5.35±0.21	6.05±0.07	6.35±0.07
non-sterilized and non-pH adjusted effluent	pH	7.91±0.02	7.69±0.05	7.60±0.03	7.56±0.05	7.55±0.01
	DO (mg L^{-1})	2.65±0.07	5.25±0.07	5.45±0.07	6.25±0.07	6.40±0.01

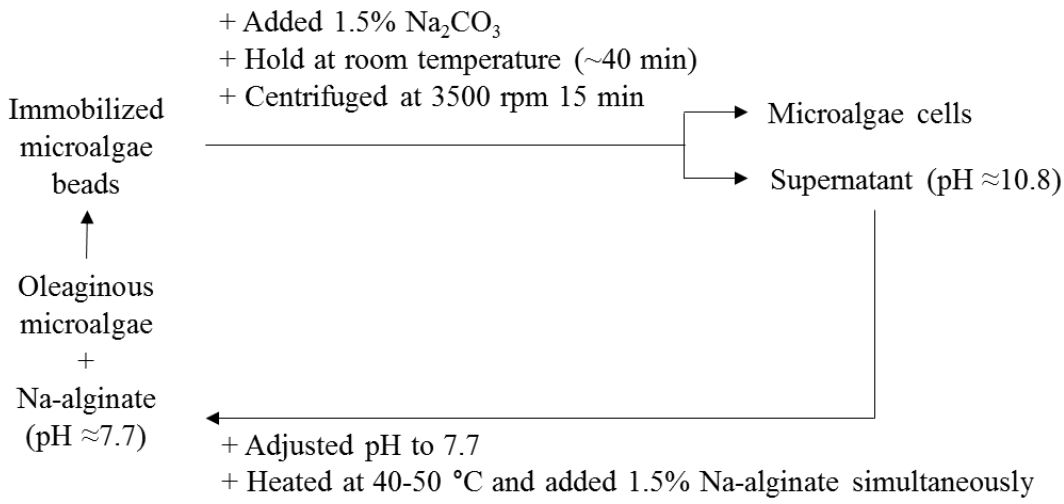

Figure S1 Schematic diagram for reusing of Na-alginate in preparing immobilized oleaginous microalgae beads

Paper IV

Strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks: nutrient starvations and cost-effective harvesting process

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Strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks: Nutrient starvations and cost-effective harvesting process

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ABSTRACT

Two locally isolated oleaginous microalgae from Songkhla Lake in Thailand were identified as *Micractinium reisseri* SIT04 and *Scenedesmus obliquus* SIT06. The effects of nutrient starvations on the responses of these two strains were intensively investigated in order to increase their lipid contents and manipulate their fatty acid compositions for suitable use as biodiesel feedstocks. Starvation of either phosphorus or ferrous less affected cell growth but did stimulate lipid accumulation of both strains by 1.2 folds. While nitrogen starvation severely limited cell growth but most effectively increased lipid content of both strains by 1.54 folds for *M. reisseri* SIT04 (up to 36.6%) and by 1.6 folds for *S. obliquus* SIT06 (up to 56.8%). The lipid accumulated during nitrogen starvation contained higher saturated fatty acids which could make biodiesel with better fuel properties and higher oxidative stability. The harvesting process through bioflocculation was optimized by Response Surface Methodology. The maximum flocculation efficiency greater than 99.5% was achieved using minimum dosage of chitosan as bioflocculant. This study has revealed the strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks and the cost-effective process for the harvesting of microalgal biomass.

1. Introduction

Microalgae are one of promising sources of fuels and foods, as they are renewable and can lead to the reduction of CO_2 emission [1]. Some microalgal biomass have been considered as sources for human food due to their high protein content [2] and some of them are oleaginous species which are suitable as biodiesel feedstocks due to their high lipid content and suitable fatty acid compositions [3]. To date, many studies have reported the crucial conditions for high lipid accumulation in microalgae such as nitrogen limitation [4,5], phosphorus and iron starvations [6,7]. However, their effects on only lipid accumulation are not enough to evaluate their potential use as biodiesel feedstocks. The effects on the fatty acid composition and fuel properties as biodiesel should also be investigated [8].

In addition to cultivation conditions, the effective harvesting process of microalgae biomass should also be evaluated to be more sustainable in developing microalgae biofuel industry [9]. The small size of microalgae cells and their low cell density make the harvesting process difficult and costly. Coagulation and flocculation are known as typical pre-concentration steps that can rapidly separate the microalgal biomass from the large amount of culture medium [10,11]. The coagulations can be induced by pH adjustment and/or coagulants. The coagulants destabilize the colloidal system by neutralizing the forces of different origin that keep it stable. While the flocculents increase the sizes of flocs and agglomerate the suspended particles [12]. Recently, flocculations by biopolymer have been increasingly used due to their effectiveness and environmental friendly. Divakaran and Pillai [13] successfully used chitosan for bioflocculation of *Spirulina*, *Oscillatoria*, *Chlorella*, and *Synechocystis* spp. They reported that the optimal chitosan dosage required to maximize flocculation depended on the type of microalgae and their cell density. Guar gum has also been used as bioflocculent in its both native and modified forms [14]. The

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combined uses of pH for coagulation and biopolymer for flocculation have been performed [11]. However, in those studies the optimizations have been done by one-factor-at-a-time method. Indeed, several factors could interactively involve in the process. Therefore, the important factors for bioflocculation should be simultaneously optimized in order to minimize the dosage use of bioflocculent and maximize the harvesting efficiency. These include the bioflocculent dosage, pH level and microalgal cell density. It was expected that with these simultaneous optimizations the coagulation-flocculation processes could be maximized and lead to technical- and cost-effective process for harvesting of microalgal cells.

In this study, two locally isolated oleaginous microalgae were characterized and their responses to nutrient starvations were intensively evaluated in terms of cell growth and lipid accumulation. The effects of nutrient starvations on fatty acid compositions and biodiesel fuel properties of microalgal lipid were also evaluated. After cultivation, the most suitable condition for bioflocculation of microalgae biomass was determined through Response Surface Methodology. The important factors to maximize the flocculation efficiency with minimum requirement of bioflocculant dosage were simultaneously optimized.

2. Materials and methods

2.1. Microalgae strains and medium

The microalgae strains were isolated from Songkhla lake in Thailand by morphologies isolation (i.e., cell shape and size) under a microscope [15]. The modified Chu13 medium was used in this study [16]. One liter of modified Chu13 medium contains 0.2 g KNO_3, 0.04 g K_2HPO_4, 0.1 g citric acid, 0.01 g Fe citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.036 g $NaHCO_3$ and 1 mL of trace metal solution. One liter of trace metal solution contains 2.85 g H_3BO_3, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.08 g $CoCl_2 \cdot 6H_2O$ and 0.05 g $Na_2MoO_4 \cdot 2H_2O$, pH was adjusted at 7.0.

2.2. Molecular identification of microalgae

The identity of the microalgal isolates was confirmed using molecular marker through 28S rRNA gene analysis. The genomic DNA of microalgae was extracted using DNeasy Plant Mini Kit (Quiagen). The genomic DNA within 28S rRNA region was amplified by PCR using two universal eukaryotic primers 5′-AGCGGAGGAAAAGAA.

ACTA-3′ as forward and 5′-TACTAGAAGGTTCGATTAGTC-3′ as reward, according to the PCR protocol described by Sonnenberg et al. [17]. The products were cloned into a T vector PMD20 (TAKARA, Japan) according to the manufacturer's protocol to create artificial standard clones. Overnight grown *Escherichia coli* JM109 cultures were used to extract plasmid DNA using the NucleoSpin® Plasmid QuickPure kit (Qiagen, USA) and sequenced with M13F and M13R primer. The DNA sequencing fragments were analyzed by ABI Prism 377 DNA sequencer. A search of GenBank with BLAST was used to identify the microalgae species by 28S rRNA gene sequence. The strain were registered and deposited in GeneBank with accession numbers. A phylogenetic tree was constructed using Kimura's two-parameter neighbor-joining algorithm, as implemented in the GENETYX Ver.12 program.

2.3. Culture condition and experimental design

Microalgae were pre-cultured in 400 mL of modified Chu13 medium in 500 mL glass bottle. The culture was incubated at 28 °C, bubbled with air at a flow rate of 0.01 L min^{-1} and illuminated under 90 μmol m^{-2} s^{-1} light intensity with 24:0 h light and dark cycles for 5–7 days. This pre-culture was used as seed culture. Experiments for characterization of microalgae were conducted in 500 mL glass bottle with 400 mL working volume (initial cell density at 660 nm of 0.2). The cultures were incubated under conditions mentioned above. For nutrient starvation studies, modified Chu13 medium without addition of KNO_3 (N starvation), K_2HPO_4 (P starvation) and Ferric citrate (Fe starvation) were used to cultivate the microalgae.

For bioflocculation of microalgal cells, natural polymeric substances i.e., chitosan, starch and guar gam were screened for their ability to flocculate microalgae biomass. The effects of three important factors including pH, flocculent dosage and microalgae cell density on flocculation efficiency were evaluated and optimized through Response Surface Methodology (RSM) (Design Expert version 6.0.2). Box-Benhken Design (BBD) was used to evaluate interactive effects of independent variables. Each independent variable was varied in the range as shown in Table 2 and coded at three levels (−1, 0, +1). The relationship of independent variables was determined by second order polynomial quadratic equation as follows:

$$Y = \beta 0 + \sum \beta ixi + \sum \beta ijxixj + \sum \beta jjx_j^2 \quad (1)$$

where *Y* is flocculation efficiency (%), *i* and *j* are linear and quadratic coefficients, respectively. β is regression coefficient, and *x* are independent variables including pH, flocculent dosage (ppm) and cell density.

2.4. Analytical method

Microalgal biomass was determined as follows: 10–20 mL of culture sample was centrifuged at 4000 × g for 15 min and the pellets was dried at 60 °C until constant weight. The lipid content of microalgal biomass was determined using solvent extraction method. Dried microalgal biomass was mashed and added with a mixed solution of methanol and chloroform (2:1 v/v), sonicated for 30 min and centrifuged at 4000 × g for 20 min. The supernatant was collected and the precipitate was extracted twice more. After extraction, the solvent solution was evaporated overnight and the extracted lipid was determined gravimetrically [16].

Extraction of protein was performed according to the modified procedure of Duong et al. [3]. Ten milligram of microalgae biomass was used to extract protein by incubation in 10 mL lysis buffer for 20 min. A 0.1 mL of this solution was transferred in a new Eppendorf tube and 0.1 mL Sodium Dodecyl Sulfate (SDS) solution was added. The mixture was then used for measurement of protein concentration following the protocol described in the Bio-Rad Protein Assay (Bio-Rad). The absorbance was measured and converted to protein concentrations using a standard curve of bovine serumal bumin (BSA). Carbohydrate content was determined by the phenol sulfuric acid method. Dried microalgae biomass (10 mg) were suspended in 0.5 mL deionized water and mixed with 0.25 mL of 5% phenol, followed by 1.25 mL of 98% sulfuric acid. The mixture was reacted at 30 °C for 5 min. After cooling, the absorbance of yellow-orange color was measured using spectrophotometer at 490 nm for hexose monosaccharide determination. The blank was prepared by replacing the sample with deionized water. The carbohydrate content was calculated using standard curve of glucose and expressed as amount of sugars [18].

Fatty acid methyl esters (FAME) were prepared by transesterification through acid catalysis [19]. The extracted lipid sample (10 mg) was dissolved in 0.5 mL toluene in a screw capped tube to

Table 1
The calculated properties of biodiesel based on microalgal fatty acid profiling.

Condition	Iodine value (mg of KOH g^{-1} of oil)		Saponification value (g of I_2 100 g^{-1} of oil)		Cetane number		Degree of unsaturation (%)		Cold filter plugging point (°C)	
	MI	SC	MI	SC	MI	SC	MI	SC	MI	SC
Control	26.30	10.30	208.50	211.10	66.60	69.80	26.70	7.90	46.60	54.40
N starvation	0	0	213.20	215.37	71.90	71.64	0	0	61.14	36.21
P starvation	1.06	0.06	214.51	216.73	71.47	71.47	1.18	0.07	47.67	31.30
Fe starvation	11.79	7.27	208.02	210.95	69.53	70.32	9.24	7.50	73.31	52.98

MI = *M. reisseri*, SC = *S. obliquus*. Control: Nutrient-rich condition.

Table 2
Experimental design and results of flocculation efficiency (%) of selected *S. obliquus* SIT06.

Run	Factor A: Chitosan concentration (ppm)	Factor B: pH	Factor C: Initial cell concentration (g/L)	Flocculation efficiency (%)	
				Predicted	Actual
1	100 (+1)	7 (0)	0.05 (−1)	17.09	13.3
2	100 (+1)	7 (0)	0.65 (+1)	79.04	85
3	50 (0)	4 (−1)	0.05 (−1)	57.34	59.6
4	50 (0)	7 (0)	0.35 (0)	69.8	62.4
5	100 (+1)	10 (+1)	0.35 (0)	33.4	29.7
6	50 (0)	7 (0)	0.35 (0)	69.8	70.3
7	50 (0)	4 (−1)	0.65 (+1)	96.79	89.3
8	50 (0)	7 (0)	0.35 (0)	69.8	75.3
9	0 (−1)	10 (+1)	0.35 (0)	7.52	6
10	50 (0)	10 (+1)	0.65 (+1)	49.16	46.9
11	50 (0)	7 (0)	0.35 (0)	69.8	71
12	0 (−1)	7 (0)	0.65 (+1)	11.81	15.6
13	0 (−1)	7 (0)	0.05 (−1)	5.96	0
14	50 (0)	10 (+1)	0.05 (−1)	20.81	28.3
15	100 (+1)	4 (−1)	0.35 (0)	88.77	90.3
16	50 (0)	7 (0)	0.35 (0)	69.8	70
17	0 (−1)	4 (−1)	0.35 (0)	36.3	40

which 1.5 mL of methanol and 50 μl of 35% conc. HCl (final concentration 0.39 M) were added and the contents were incubated at 100 °C for 1 h and 30 min. Transmethylated samples were cooled, to which 1 mL of hexane was added and vortexed. The hexane layer (FAME) was collected. FAME was analyzed by GC-MS (7890A GC System coupled with a 5975C inert XL EI/CI MSD with a Triple-Axis Detector operated at 70 eV; Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5 ms capillary column (0.25 mm i.d. × 30 m, 0.25 m film thickness; Agilent Technologies). As the fuel properties of biodiesel are mainly influenced by their fatty acid profiles, the empirical equations have been developed to calculate the biodiesel properties of the lipids based on their fatty acid compositions. These empirical equations are useful for primarily screening of the lipids [19]. The biodiesel properties of microalgal lipids such as saponification value (SV), iodine value (IV), cetane number (CN), unsaturation degree (DU), long chain saturated factor (LCSF) and cold filter plugging point (CFPP) were calculated based on the fatty acid profiles using the following empirical Eqs. (2)–(7).

$$SV = \sum[(560 \times F)/MW] \quad (2)$$

$$IV = \sum[(254 \times F \times D)/MW] \quad (3)$$

$$CN = [46.3 + (5458/SV)] - (0.225 \times IV) \quad (4)$$

$$DU = \%MUFA + (2 \times \%PUFA) \quad (5)$$

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24) \quad (6)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (7)$$

where D is the number of double bonds, F is the % of each type of fatty acid and MW is the molecular weight of corresponding fatty acid. MUFA is the weight percentage of the monounsaturated fatty acids (wt%) and PUFA is the weight percentage of the polyunsaturated fatty acids (wt%).

Microalgae biomass was harvested by flocculation method. The microalgal suspensions were left to settle for certain time without agitation. Subsequently, the optical density of the supernatant from half the height of the clarified layer and the sludge were measured at 660 nm (OD_{660}). Flocculation efficiency was calculated using Eq. (8) [11]:

$$\text{Flocculation efficiency}(\%) = (1 - A/B) \times 100 \quad (8)$$

where A is the OD_{660} of supernatant from half the height of the clarified layer after flocculation and B is the initial OD_{660} of the microalgal culture suspension.

All experiments were performed with three replicates. Analysis of variance was performed to calculate any significant differences in the treatment mean values, and the least significant difference, calculated using SPSS software, was used to separate means.

3. Results and discussion

3.1. Molecular identification and characterization of locally isolated microalgae

Among several isolated marine microalgae isolated from Songkhla Lake, Thailand, based on their morphologies (i.e., cell shape and size) and quantitative analysis of lipid content, two isolates having lipid content >20%, SIT04 and SIT06, were selected. Microscopic analysis allowed preliminary identification of isolates SIT04 and SIT06 as genus *Micractinium* and *Scenedesmus*, respectively. *Micractinium* specie was observed to be typical spherical cells with a thin cell wall, while *Scenedesmus* specie was small, non-motile, and colonial. Specie identification was further performed using molecular marker to confirm the morphology-based species identification. Microalgae isolates SIT04 and SIT06 were then identified as *Micractinium reisseri* SIT04 (base pair length 1030; maximum identity 95%; similarity to *Micractinium reisseri* JN169781) and *Scenedesmus obliquus* SIT06 (base pair length 867; maximum identity 98%; similarity to *Scenedesmus obliquus* FR7511678 as synonym of *Acutodesmus obliquus*), respectively. The sequenced 28S rRNA was deposited in the DDBJ database under GeneBank accession numbers LC153789 for *M. reisseri* SIT04 and LC153788 for *S. obliquus* SIT06 (Table 2). The species identification was also confirmed by the phylogenetic analysis of 28S rRNA sequence (Fig. 1). As shown in phylogenetic tree, isolate SIT04 and SIT06 were identified as clearly group with *Micractinium reisseri* and *Scenedesmus obliquus*.

3.2. Comparison of growth and lipid production under nutrient-rich condition

To determine the growth and biomass concentration of both microalgae strains, *M. reisseri* SIT04 and *S. obliquus* SIT06 were cultured in modified Chu13 medium at 28 °C and bubbled with air at a flow rate of 0.01 L min^{-1} under a 90 μmol m^{-2} s^{-1} light intensity with a 24 h full illumination for 10 days. The cell growth and lipid production of *M. reisseri* SIT04 and *S. obliquus* SIT06 under nutrient-rich condition are shown in Figs. 2a and 3a, respectively. The specific growth rates of *M. reisseri* SIT04 and *S. obliquus* SIT06 were comparable in the range of 0.8–0.9 day^{-1} while the final biomass concentration of *S. obliquus* SIT06 (1.83 g L^{-1}) was higher than that of *M. reisseri* SIT04 (1.29 g L^{-1}). The compositions of two microalgal biomass were also compared. *M. reisseri* SIT04 biomass contained slightly higher protein (39.53%) than did *S. obliquus* SIT06 (32.70%) whereas, their carbohydrate contents was similar in the range of 19%. The lipid content of *S. obliquus* SIT06 increased during cultivation and reached the maximum at 35.54% while that of *M. reisseri* SIT04 was stable at a level of 20–25%. It has been reported that the microalgal biomass collected during exponential growth phase contained high protein while those collected during stationary growth phase contained either high lipid or high carbohydrate. In addition to the strains used, the medium components and culture conditions are also crucial parameters affecting the biomass composition of the microalgae [20,21]. These results suggested that *M. reisseri* SIT04 and *S. obliquus* SIT06 could be promising sources for both lipids and proteins.

The relative percentage of fatty acid compositions in *M. reisseri* SIT04 and *S. obliquus* SIT06 lipids determined through GC-MS analysis are shown in Fig. 4. Three main fatty acids found in *M. reisseri* SIT04 lipids were palmitic acid (C16:0, 39.87%), stearic acid (C18:0, 32.18%) and oleic acid (C18:1, 20.10%). Similarly, Karpagam et al. [19] also reported that palmitic acid and oleic acid were the main fatty acids found in *Micractinium* sp. M-13 lipids. However, in their study oleic acid content was higher than palmitic acid. The two main fatty acids found in *S. obliquus* SIT06 lipids were palmitic acid (62.94%) and steric acid (30.74%). While the two main fatty acids found in *S. obliquus* lipids in the study of Yang et al. [1], were oleic acid (51.92%) and palmitic acid (20.42%). It should be noted that the higher content of saturated fatty acids in *M. reisseri* SIT04 and *S. obliquus* SIT06 lipids in this study indicated their potential use as biodiesel feedstocks because they could give higher oxidation stability and cetane number, decreased NO_x emissions and a shorter ignition delay time [22,23].

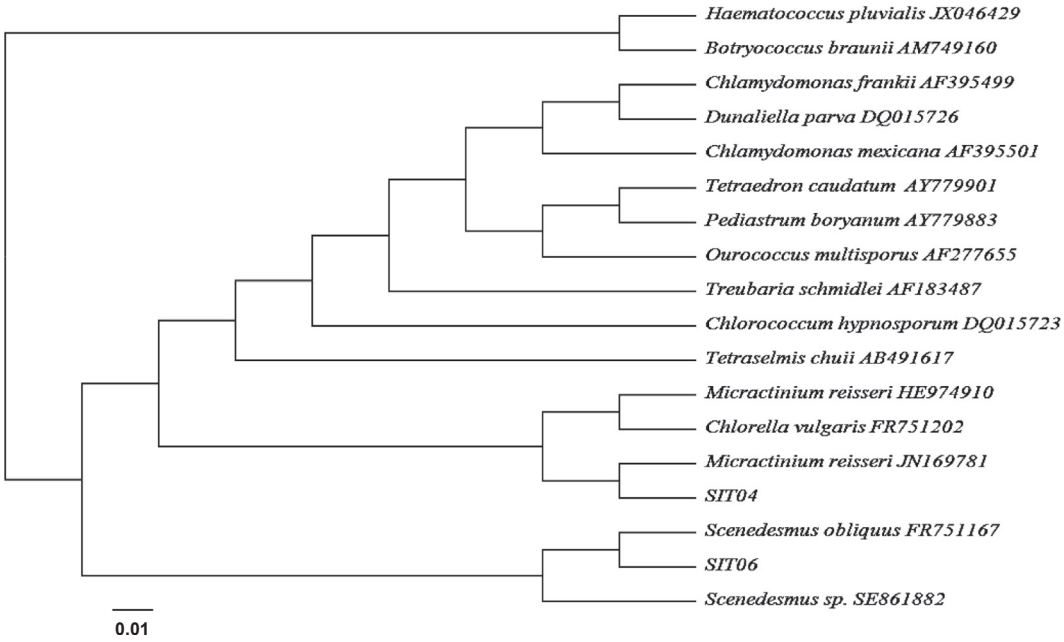

Fig. 1. Phylogenetic tree showing the relationships among 28S rRNA sequence of isolate SIT04 and SIT06 and the most similar sequences retrieves from NCBI nucleotide database.

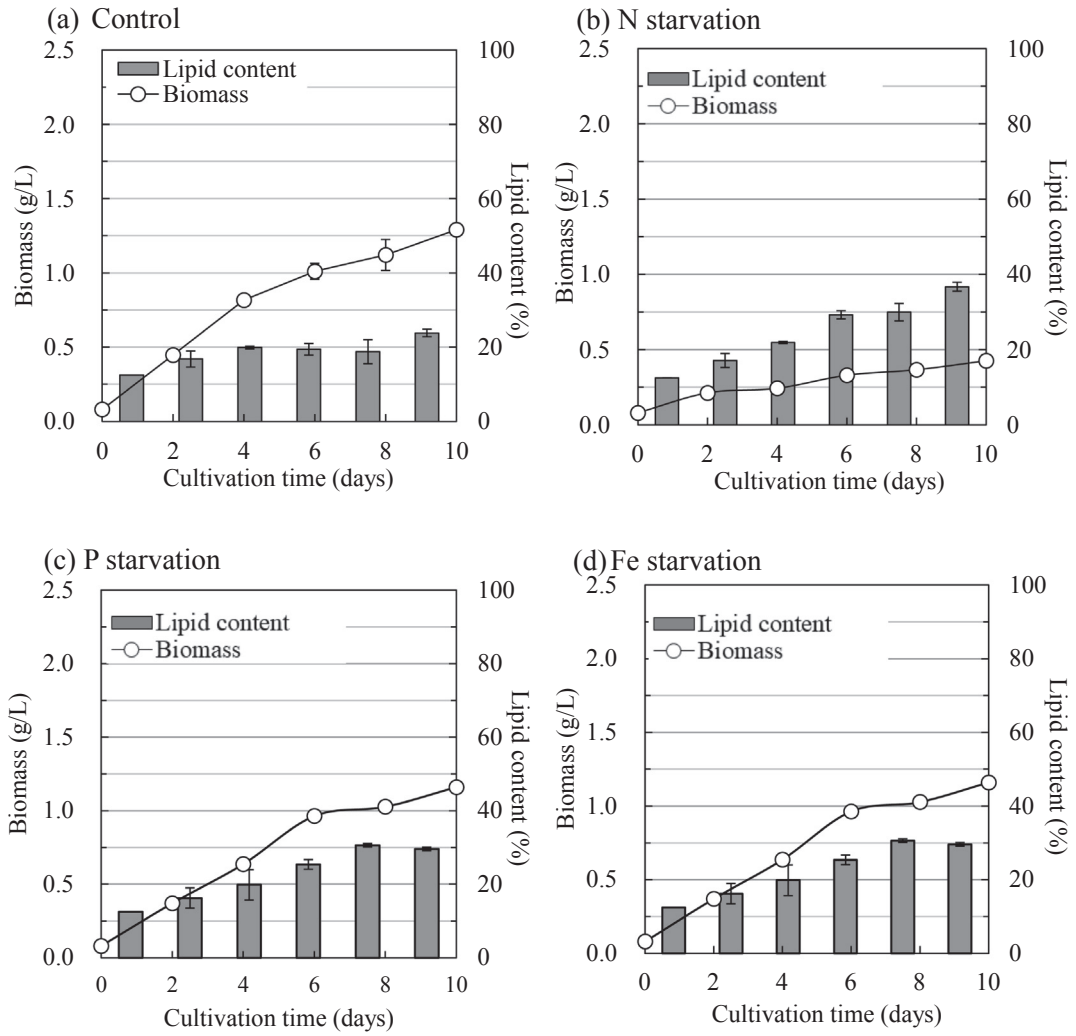

Fig. 2. Cultivation of *M. reisseri* SIT04 under nutrient-rich condition (a, control), N starvation (b), P starvation (c) and Fe starvation (d). Data are means of replicates with standard variation.

3.3. Effects of nutrient starvations on growth and lipid production

The effects of nutrient starvations on growth and lipid production of *M. reisseri* SIT04 and *S. obliquus* SIT06 were evaluated as shown in Figs. 2b–d and 3b-d, respectively. Among three nutrient starvation tests, the P and Fe starvation slightly decreased the biomass while the N starvation drastically decreased the biomass of both strains. Among two strains, *S. obliquus* could survive under nutrient starvations better than *M. reisseri.* The severity of nutrient starvations on their growth are as follows: N starvation > P starvation > Fe starvation. These results confirmed the fact that nitrogen is the important nutrient for the cell proliferation and has significant impacts on cell growth of microalgae [7]. The results in this study were consistent with those previously reported by Karpagam et al. [4,24] who found that the nutrient starvations decreased cell growth and biomass productivity of *Meyerella* sp. N4 and *Chlamydomonas reinhardtii* CC1010. Yeesang and Cheirsilp [6] and Fan et al. [7] also reported that the nutrient starvations had negative effect on the cell growth of *Botryococcus* spp. and *C. pyrenoidosa*, respectively. However, the severity of each nutrient starvation on the cell growth was different depending on the species of the microalgae and also their origins.

In addition to the cell growth, the lipid content also contributes to the lipid productivity. Therefore, both cell growth and lipid content should be considered [25]. The effects of nutrient starvations on lipid contents of both strains are also shown in Figs. 2 and 3. During N starvation, the lipid contents of *S. obliquus* SIT06 and *M. reisseri* SIT04 obviously increased up to 56.87% and 36.72%, respectively. The lipid contents of both strains also increased during P and Fe starvations but with a lower level. The lipid contents of both strains increased up to 40.28% and 30.45% during P starvation and 39.75% and 29.60% during Fe starvation. The relative levels of these effects on lipid content are similar to those on the growth as follows: N starvation > P starvation > Fe starvation. It should be noted that the N starvation was the most effective strategy to increase lipid content of the microalgae.

3.4. Effect of nutrient starvations on fatty acid compositions and biodiesel fuel properties

Fig. 4 shows the fatty acid composition of lipids from two microalgae cultivated under nutrient-rich and nutrient starvation conditions. The two microalgae responded to each nutrient starvation differently. In case of *M. reisseri* SIT04, all types of nutrient starvation increased the content of palmitic acid (C16:0) and decreased the content of oleic acid (C18:1). Therefore, the total

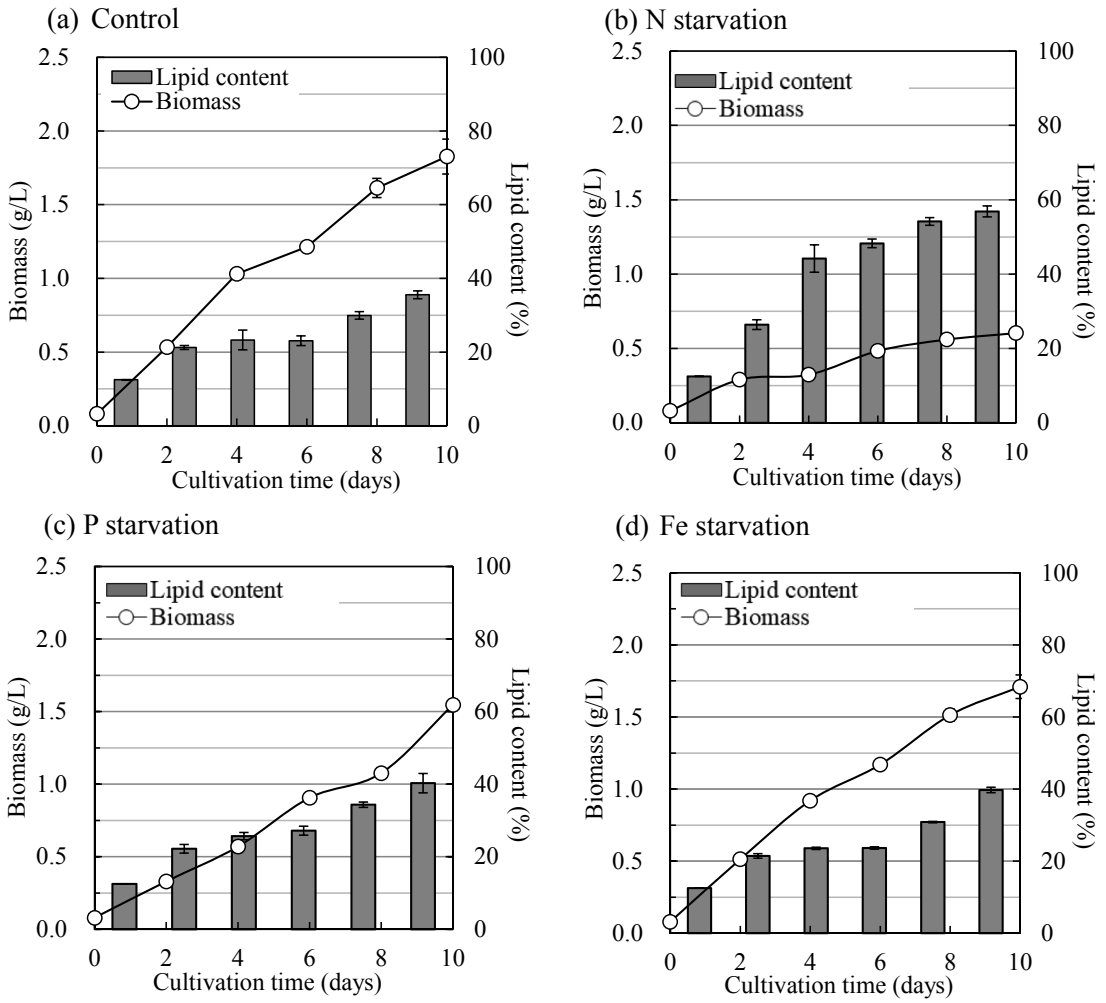

Fig. 3. Cultivation of *S. obliquus* SIT06 under nutrient-rich condition (a, control), N starvation (b), P starvation (c) and Fe starvation (d). Data are means of replicates with standard variation.

saturated fatty acids significantly increased. Usually high content of saturated fatty acids is preferential for increasing fuel quality of biodiesel and oxidative stability. While polyunsaturated fatty acids (PUFAs) showed great cold-flow properties but the biodiesel properties are interior oxidation stability [4,26]. In case of *S. obliquus* SIT06, Fe starvation had no significant effect on the fatty acid compositions while N and P starvations selectively increased the content of palmitic acid (C16:0). It has been reported that the changes in fatty acid composition relate to the activity of enzymes those involve in each fatty acid synthesis. It was therefore possible that the nutrient starvation might positively or negatively affect specific enzyme activities and increase or decrease the corresponding fatty acid content. Sharma et al. [27] have reported that the palmitoyl thioesterase (PT) gene expression in *S. dimorphus* increased during N starvation and resulted in an increased content of palmitic acid. While the decrease of stearic acid could be affected by the decreased activity of stearoyl thioesterase (ST). These results indicate that the nutrient starvations not only increased lipid content but also increased the saturation level of the microalgal lipids.

To assess the suitability of microalgal lipids as biodiesel feedstocks, the fuel properties were calculated from their fatty acid profiles [16,28]. Biodiesel fuel properties including iodine value (IV), saponification value (SV), cetane number (CN), degree of unsaturation (DU), and cold filter plugging point (CFPP) were evaluated under different nutrient starvations as shown in Table 1. The IV of *M. reisseri* SIT04 and *S. obliquus* SIT06 lipids were found to be 0–26.3 and 0–10.3 g I_2/100 g oil, respectively, which meet the European standard (EN-14214) that defines a maximum IV value of 120 g I_2/100 g oil. It should be noted that the N starvation showed the lowest value of IV nearly 0 which indicates the most saturated level and have high stability against oxidation. The SV is the value of KOH (mg) required for the saponification of 1 g of oil. It is a measure of the average molecular weight (namely chain length) of all the fatty acids present. A low SV indicates the presence of long chain fatty acids in the lipids. The SV of *M. reisseri* SIT04 and *S. obliquus* SIT06 were 208.02–214.51 and 210.95–216.73 mg KOH/g oil, respectively, which are close to those of *Micractinium* sp. M-13 (190.6 mg KOH/g oil) and *S. obliquus* (217.5 mg KOH/g oil) [19,25]. The CN is the measure of ignition quality of biodiesel and it increases with increasing the saturation level of fatty acids [24]. The CN for *M. reisseri* SIT04 and *S. obliquus* SIT06 were 66.6–71.9 and 69.8–71.1, respectively. These values ensure high ignition quality of the microalgae biodiesel and meet the minimum values of CN required by the international biofuel standards of EN-14214, ASTM D675140, Australian standard and National Petroleum Agency in Brazil. Cold filter plugging point (CFPP) of both microalgal lipids were found to be appropriate for use in hot regions [19]. DU is an indispensable property as it measures the oxidation stability of biodiesel. Karpagam et al. [4] suggested that the lower value of DU

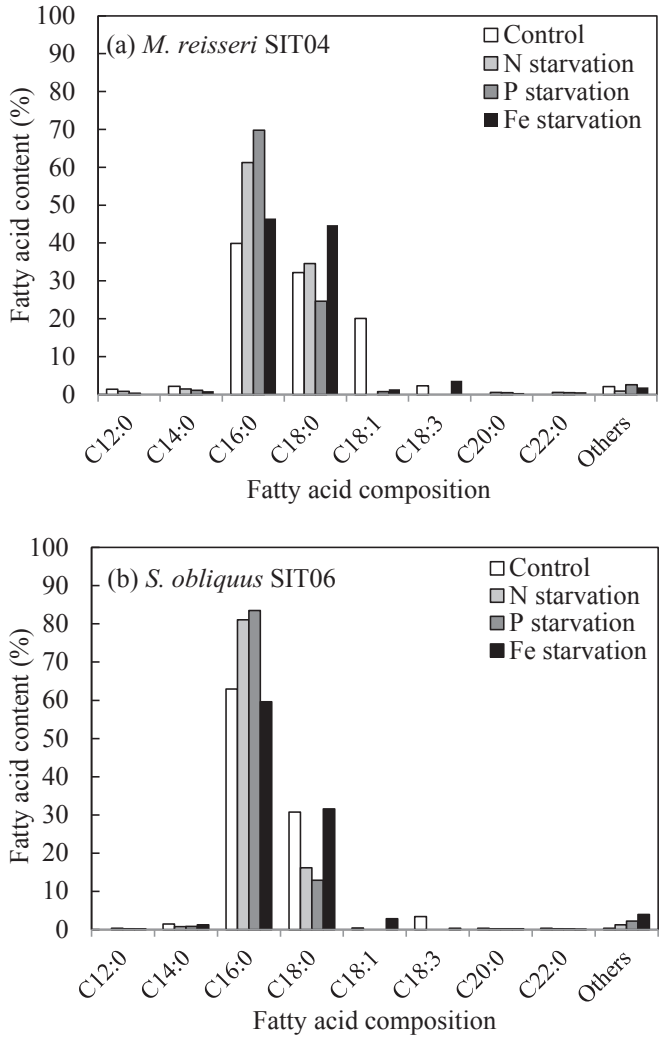

Fig. 4. Fatty acid profiles of two oleaginous microalgae cultivated under different nutrient starvation.

would give higher CN values and higher oxidation stability. The DU of *S. obliquus* SIT06 (0–7.9) was found to be lower than that of *M. reisseri* SIT04 (0–26.7) due to the lower unsaturation level in *S. obliquus* SIT06 lipids. It should be noted that the N starvation did decrease the value of DU to nearly 0%.

In summary, *S. obliquus* SIT06 accumulated lipids higher than *M. reisseri* SIT04 and more positively responded to nutrient starvations. The biodiesel fuel qualities of *S. obliquus* SIT06 lipids are also superior to those of *M. reisseri* SIT04. Therefore, *S. obliquus* SIT06 was considered as potential biodiesel feedstocks and then chosen for further study.

3.5. Microalgae harvesting

3.5.1. Screening of bioflocculents

The selected *S. obliquus* SIT06 was cultivated for 10 days and its culture broth added with bioflocculent. It should be noted that as the bioflocculent was added after the cultivation step and at a very small amount, it hardly influenced the fatty acid profiles of the microalgal lipids. Three biopolymers including chitosan, guar gum and starch was compared at the same concentration of 10 ppm with unadjusted final culture pH (around 10) and microalgal cell density of 0.65 g/L. After 60 min of flocculation time, the flocculation efficiency using chitosan (27.31%) was higher than those using other polymers (17–18%). The possible reason would be because chitosan possess the protonated amine groups (NH_3^+) which could effectively induce the flocculation of the microalgae biomass. While the ordered structures of guar gum and starch are destroyed during the solubilization process and resulted in the carboxyl groups (COO^-) [29].

3.5.2. Optimization through response surface methodology (RSM)

Response surface methodology (RSM) has advantages to classical one-variable-a-time optimization including generation of large amounts of information from a small number of experiments and the possibility of evaluating the interaction effect between the variables on the response [30]. In most cases, the flocculation efficiency of the microalgal cells required the optimal levels of the factors including flocculent dosage, pH and cell density. The flocculation efficiency increased with increasing these factors up to the optimal levels and either reached a plateau or decreased when the factors were beyond their optimal levels. These results indicated the curvature response of flocculation efficiency to the factors [29,31–35]. In RSM, a solution to creating a design matrix that permits the estimation of the curvature is suggested to be a three-level factorial design together with a quadratic equation. In addition, the quadratic correlation of the experimental data could be validated by the analysis of variance through the *P*-value, multiple correlation coefficients (R^2) and adjusted R^2 [30].

Based on the results of bioflocculent screening, chitosan was selected and used as bioflocculent in optimization study. Response surface methodology experiment design was used to further determine the optimal combination of bioflocculent dosage, pH and microalgal cell density on flocculation efficiency of microalgae. The experimental results were concerned with flocculation efficiency of *S. obliquus* SIT06 (%; Y_1) as presented in Table 2. The conditions at the center point were: chitosan dosage of 50 ppm, pH 7 and cell density of 0.35 g/L. The estimated response surface model in the form of a second-order regression equation for flocculation efficiency of *S. obliquus* is shown in Eq. (9), respectively.

$$Y_1 = 24.61 + 1.49A - 3.13B + 135.56C - 0.01A^2 - 0.04B^2 - 148.89C^2 - 0.04AB + 0.94AC - 3.08BCE \quad (9)$$

where independent variable *A* is chitosan dosage, *B* is pH and C is cell density. The dependent variables response Y_1 are flocculation efficiency of *S. obliquus*, *A, B, C* are referred as the main effort linear term while AB, AC, BC are the interaction terms, and A^2, B^2, C^2 are the quadratic terms involved in the process.

The statistical significance of the model equation was analyzed by F-test for analysis of variance (ANOVA) (Table 3). The model was highly significant with *P*-value less than 0.0001. The *P*-values of lack of fit, greater than 0.05, for the two responses indicated that the lack of fit of the model was insignificant. The multiple correlation coefficients or R^2 for flocculation efficiency was 0.98. This indicated that up 98% of the variations in response can be explained by the model. These quadratic equations could appropriately describe the relationships between the factor and the responses. The value of the adjust determination coefficients (adjust $R^2 > 0.9$) is quite high, indicating a high significance of the model. Moreover, the coefficient value (C.V. <20%) indicated a high reliability and precision of the experiments. It should be noted that the effect of each variable on the response was a combination of the coefficients and variables as well as the contribution of the joint effect of variables that cannot be observed by traditional optimization methods.

Based on the experiment results (Table 2), the percent of flocculation efficiency ranged from 0 to 90.3%. It was found that Run 15

Table 3
ANOVA for Response Surface Quadratic Model of flocculation efficiency of selected *S. obliquus* SIT06 biomass.

Source	Coefficients	*P*-value
Model intercept	24.61	<0.0001[a]
A- Chitosan concentration	1.49	<0.0001[a]
B- pH	−3.13	<0.0001[a]
C- Initial micoralgal biomass concentration	135.56	0.0002[a]
A^2	−0.01	<0.0001[a]
B^2	−0.04	0.9153
C^2	−148.89	0.0056[a]
AB	−0.04	0.0984
AC	0.94	0.0051[a]
BC	−3.08	0.4526
R^2	0.98	–
Adjust R^2	0.95	–
C·V.	13.91	–
Lack of fit	–	0.1105

C.V. = coefficient value.
[a] Significant level at 95%.

(chitosan dosage of 100 ppm, pH of 4 and cell density of 0.35 g/L) gave the maximum flocculation efficiency of 90.3%. With the same pH, the comparable flocculation efficiency (89.3%) could be obtained when using the cell density was as high as 0.65 g/L with a moderate chitosan dosage of 50 ppm (Run 7). Table 3 showed a linear term of each factor and quadratic terms of chitosan dosage (A^2) and cell density (C^2) were significantly influenced the flocculation efficiency with *P*-value less than 0.05. These indicated that the flocculation efficiency of microalgae *S. obliquus* biomass was affected by all three factors and a suitable chitosan dosage and cell density are required for the highest flocculation efficiency. In addition, the interaction between chitosan dosages vs. cell density significantly affected the flocculation efficiency (*P*-value <0.05).

The three-dimensional response surface and two-dimensional contour plots from the calculated responses in which two variables were kept constant at their center points and the other two variables were varied within their experimental range, are shown in Fig. 5. These graphs were plotted in order to investigate the interactions between independent variables and determine the optimal level of each variable for a desired response. Fig. 5a presented the effect of pH and chitosan dosage on the flocculation efficiency. The presence of chitosan and the acidic pH did increase the flocculation efficiency. This was because under acid condition chitosan having highly positive charge which could effectively bind to negative charge of microalgae cell surface and induced the large

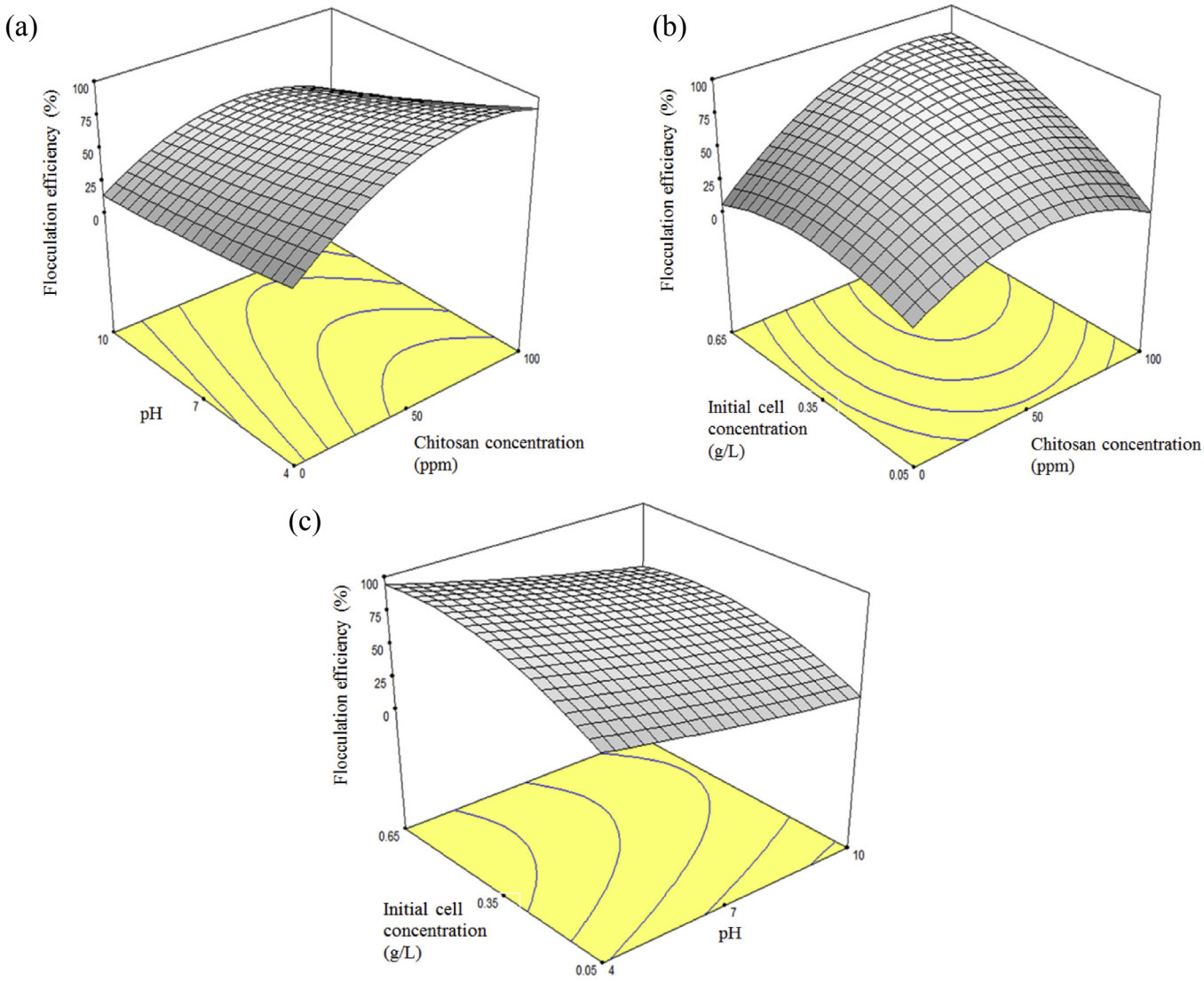

Fig. 5. Contour and 3D of response surface plots of flocculation efficiency of selected *S. obliquus* SIT06 biomass. (a) Effect of chitosan concentration and pH, (b) Effect of chitosan concentration and initial cell concentration and (c) Effect of pH and initial cell concentration.

microalgae particles. Divakaran and Pillai [13] have also reported that the presence of chitosan and the decreasing pH gave higher flocculation efficiency of *Synechocystis* sp. Fig. 5b showed the interaction effect between chitosan dosages and cell density on flocculation efficiency of *S. obliquus*. High chitosan dosage and high cell density did improve the flocculation of microalgae. However, increasing chitosan dosage is costly. Therefore, the strategies to use the interaction effect between factors to increase the flocculation efficiency should be established. Fig. 5c depicted the interaction effect between pH and cell density on flocculation efficiency. When chitosan dosage was set at the center point of 50 ppm, the maximum flocculation efficiency was obtained when using the highest cell density tested and the lowest pH tested. Chen et al. [36] reported that the optimal pH and chitosan dosage for flocculation of *Scenedesmus* sp. biomass were 5 and 80 ppm, respectively. However, the maximum flocculation efficiency was only 80%. Overall, the importance degree of three factors on flocculation efficiency of *S. obliquus* biomass in this study would be: chitosan dosage > cell density > pH and those of the interaction effects would be: chitosan dosage v.s. cell density > chitosan dosage v.s. pH > cell density v.s. pH.

The optimum conditions for maximizing flocculation efficiency of *S. obliquus* biomass, calculated by setting the partial derivatives of Eq. (9) to zero with respect to corresponding variables, were chitosan dosage of 64 ppm, pH of 4 and cell density of 0.65 g/L. The maximum response value for the flocculation efficiency of *S. obliquus* biomass was estimated to be 100%. Three replicates of experiments were performed under the optimal conditions for flocculation of *S. obliquus* of calculated by RSM. The optimal conditions for flocculation of *S. obliquus* were estimated to be as follows: chitosan dosage of 64 ppm, pH of 4 and cell density of 0.65 g/L, which would give the flocculation efficiency as high as 99.50%. The experimental values of all experiments were very similar to the predicted values without significant difference ($P < 0.05$). After optimization, the flocculation efficiency of *S. obliquus* biomass was increased 4.8-fold, comparing with that before optimization.

4. Conclusions

In this study, two locally isolated oleaginous microalgae were characterized as *M. reisseri* SIT04 and *S. obliquus* SIT06, respectively. *S. obliquus* SIT06 showed higher potential to be used as lipid producer due to its higher lipid content and more positive response to the nutrient starvation. Among nutrient starvation tests, N starvation most suppressed the cell growth of both strains but most increased their lipid contents and their saturation levels, especially the content of palmitic acid. The estimated biodiesel fuel properties were in accordance with those of the international standards i.e., EN-14214 and ASTM D675140. The microalgae cells were most effectively harvested using chitosan and the optimal conditions were successfully determined through response surface methodology. After optimization, more than 99% of *S. obliquus* SIT06 biomass could be flocculated.

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Paper V

Photoautotrophic cultivation of oleaginous microalgae and co-pelletization with filamentous fungi for cost-effective harvesting process and improved lipid yield

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Photoautotrophic cultivation of oleaginous microalgae and co-pelletization with filamentous fungi for cost-effective harvesting process and improved lipid yield

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Abstract
Oleaginous microalga *Scenedesmus obliquus* SIT06 was selected as potential biodiesel feedstocks due to its high lipid content and suitable fatty acid composition for production of biodiesel with high oxidative stability and high cetane number. The important factors for cultivating microalgae in photoautotrophic mode were optimized through response surface methodology (RSM). The highest microalgal biomass obtained was 1.99 ± 0.12 g L^{-1} with a high lipid content of $40.86 \pm 0.32\%$. To simplify harvesting process of microalgal cells, pellet-forming filamentous fungi were inoculated into the late log-phase of microalgae culture. Among the fungi tested, *Cunninghamella echinulata* TPU 4652 most effectively harvested the microalgal cells with the highest flocculation efficiency of 92.7%. Moreover, the biomass and lipids of microalgae-fungi pellets were as high as 4.45 ± 0.06 and 1.21 ± 0.08 g L^{-1}, respectively. The extracted lipids were mainly composed of C16:0, C18:0, and C18:1, and their estimated fuel properties meet with the international standards indicating their potential use as biodiesel feedstocks. This study has shown the strategies not only to simplify the harvesting process but also to increase the lipid yield and tailor the lipid composition.

Keywords Biodiesel · Filamentous fungi · Flocculation · Lipid · Oleaginous microalgae

Introduction

Fuel security, fossils oil price, and global warming are among the most important concerns of energy government in the world. Recently, there is an increasing need for exploration of

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sustainable energy resources. Biodiesel is one of renewable energy that has been produced from energy crop oils or animal fats. However, producing enough biodiesel from these feedstocks to satisfy existing demand would have a serious impact on food supplies. Among available alternative oil sources, oleaginous microalgae with a lipid content > 20% have been receiving considerable attention because they can fix CO_2 into lipids and also produce high-value products, such as chlorophylls and carotenoids (Jawaharraj et al. 2016). The lipid content in microalgae biomass is highly influenced by microalgae species, medium composition, and culture conditions especially nitrogen concentration and light intensity (Karpagam et al. 2015a).

Nitrogen is an important element that is required in microalgae metabolism for synthesizing nucleic acids and protein. For efficient microalgae growth, the nitrogen source should be provided adequately. However, for lipid accumulation, the microalgae should be cultivated under nitrogen-deficient condition. It has been reported that the microalgae respond to the nitrogen starvation by channeling carbon towards the accumulation of carbon reserve compounds such as lipids (Xin et al. 2010; Yeh and Chang 2011). Factors required for cultivation of microalgae are light, supplied in the form of solar energy, mineral nutrients, CO_2 (the addition of which can be regulated via the pH of the culture), and a suitable means of removing O_2 generated by photosynthesis. In addition, three factors with a strong influence on lipid accumulation are the concentration of nitrogen source, light intensity, and culture pH (Yeesang and Cheirsilp 2011; Parmar et al. 2011; Thawechai et al. 2016). Although there are several reports on the effect of these three factors on growth and lipid accumulation by the microalgae, most of them are the optimization through one-factor-at-a-time. However, as these three factors might also have interaction effects on the performance of the microalgae, to maximize lipid production by the microalgae, these three factors should be simultaneously optimized. Response surface methodology (RSM) is the statistic experimental design, which has been widely used for evaluating and simultaneously optimizing multiple process parameters. They can provide statistical models which help us understand the interactions among parameters at varying levels and to calculate the optimal level of each parameter for a given target (Tongprawhan et al. 2014).

In addition to the culture conditions, the effective harvesting process for microalgae biomass should also be investigated in order to be more sustainable in developing microalgae based biofuel industry. The low concentration and small size of microalgae make the harvesting process difficult. Recently, environmental friendly flocculation techniques using filamentous fungi have been developed (Zhou et al. 2012; Prajapati et al. 2014; Li et al. 2017; Choi et al. 2016). The filamentous fungi represent as attractive bioflocculating agents because of their self-pelletization and possible entrapment of microalgae cells. Generally, the fungal-assisted flocculation could be performed by two techniques. The first technique is the co-cultivation of microalgae with fungal pellets. This technique needs two steps of pelletization of fungal cells and co-cultivation with microalgae which required at least 72 and 48 h, respectively (Wrede et al. 2014; Miranda et al. 2015; Muradov et al. 2015). The second technique is the co-pelletization in which the fungi grow and simultaneously flocculate microalgal cells in their pellets. This technique could reduce the operating steps and increase the overall productivity. Although several researchers have attempted this technique for harvesting of some microalgae (Zhou et al. 2012; Prajapati et al. 2014; Zamalloa et al. 2017), only one research group evaluated the lipid production of the microalgae-fungi pellets through autotrophic cultivation mode (Zhang and Hu 2012). However, the lipid yield in their study was only 0.1 g L^{-1} in 48 h. Therefore, the fast-growing microalgae with high lipid content as well as the

filamentous fungi with the ability to effectively harvest these microalgae should be screened to improve the economics of microalgae based biofuel industry.

In this study, two microalgae strains isolated from Songkhla Lake, Thailand were photoautotrophically cultivated and compared for their growth rate, lipid content, and fatty acid compositions. In this study, BG-11 was used for microalgae cultivation due to its balance of nutrients regarding both their presence and concentrations and the presence of citric acid and ferric ammonium citrate that could solubilize the salts, preventing precipitation and thus increasing their availability to cells (Sunda et al. 2005; Harrison and Berges 2005). The important factors for cultivating microalgae including light intensity, nitrogen source, and culture pH were simultaneously optimized through RSM. To enhance lipid yield and simplify the harvesting process of microalgal cells, several pellet-forming filamentous fungi were tested for their co-agglomeration ability with microalgal cells and their effects on lipid yield and fatty acid compositions were evaluated. The fuel properties of the microalgae-fungi lipids were also evaluated based on the fatty acid compositions.

Materials and methods

Microalgae strain and culture condition

Microalgae were collected from Songkhla lake in southern Thailand using a plankton net (10–12 μm × 7–9 μm in size). The microalgae were isolated using a sterile micropipette washing method (Stein 1973) based on morphological properties (i.e., cell shape and size) under microscope and then were cultured in agar plate. The purity of the culture was ensured by repeated plating and by regular observation under microscope. Two isolated microalgae with high lipid content were selected and identified as *Micractinium reisseri* SIT04 (Accession no. LC153789) and *Scenedesmus obliquus* SIT06 (accession no. LC153788) based on molecular markers. The medium for microalgae cultivation was BG-11 medium consisting of (g L^{-1}): 1.5-g $NaNO_3$, 0.04-g $K_2HPO_4{\cdot}3H_2O$, 0.2-g $H_2PO_4{\cdot}3H_2O$, 0.0005-g EDTA g, 0.005-g Fe ammonium citrate, 0.005-g citric acid, 0.02-g Na_2CO_3, and 1 mL of trace metal solution. One liter of trace metal solution contains 2.85-g H_3BO_3, 1.8-g $MnCl_2{\cdot}4H_2O$, 0.02-g $ZnSO_4{\cdot}7H_2O$, 0.08-g $CuSO_4{\cdot}5H_2O$, 0.08-g $CoCl_2{\cdot}6H_2O$, and 0.05-g $Na_2MoO_4{\cdot}2H_2O$; pH was adjust 7.0 (Cheirsilp and Torpee 2012). Each microalga was inoculated in in 50-mL glass bottle with 400-mL BG-11 medium (initial cell density at 660 nm = 0.2). The cultures were incubated at 28 °C and bubbled with air at a flow rate of 0.01 L min^{-1} under full illumination at 90 μmol m^{-2} s^{-1} using artificial light for 10 days.

Optimization of photoautotrophic culture condition through RSM

Three important growth factors for microalgae cultivation including culture pH, concentration of $NaNO_3$ as nitrogen source, and light intensity, were optimized and their interaction effects were also evaluated. A Box-Benhken Design (Design Expert version 6.0.2) was used in this study and the experiments were designed with 17 runs at three coded level (− 1, 0, + 1) as shown in Table 1. The pH was ranged from 5.0 to 9.0. The nitrogen concentration was ranged from 0.0 to 3.0 g L^{-1}, and the light intensity was ranged from 0 to 180 μmol m^{-2} s^{-1}. The responses Y_1 and Y_2 were biomass concentration (g L^{-1}) and lipid content (%), respectively.

Table 1 Experimental design and results of biomass production and lipid content of selected *S. obliquus* SIT06

Run	Factor A: pH	Factor B: $NaNO_3$ concentration (g/L)	Factor C: light intensity (μmol proton m^{-2} s^{-1})	Biomass concentration (g/L)			Lipid content (%wt)		
				Predicted	Actual	Residual error	Predicted	Actual	Residual error
1	7 (0)	1.5 (0)	90 (0)	2.00	2.09	0.09	36.21	34.94	1.27
2	5 (− 1)	1.5 (0)	0 (− 1)	0.12	0.20	0.09	15.73	15.34	0.39
3	7 (0)	1.5 (0)	90 (0)	2.00	2.08	0.08	36.21	34.34	1.87
4	5 (− 1)	1.5 (0)	180 (+ 1)	0.04	0.10	0.06	8.63	8.87	0.24
5	9 (+ 1)	1.5 (0)	180 (+ 1)	0.29	0.20	0.09	16.48	16.87	0.39
6	7 (0)	0 (− 1)	180 (+ 1)	0.00	0.10	0.10	24.02	21.34	2.68
7	9 (+ 1)	0 (− 1)	90 (0)	1.04	0.92	0.12	45.52	47.81	2.29
8	7 (0)	3 (+ 1)	0 (− 1)	0.64	0.43	0.21	13.32	16.00	2.68
9	9 (+ 1)	3 (+ 1)	90 (0)	2.01	2.28	0.27	28.2	25.76	2.44
10	7 (0)	3 (+ 1)	180 (+ 1)	0.63	0.45	0.18	7.04	9.09	2.05
11	7 (0)	1.5 (0)	90 (0)	2.00	1.93	0.07	36.21	37.29	1.08
12	5 (− 1)	3 (+ 1)	90 (0)	1.47	1.59	0.12	24.77	22.48	2.29
13	7 (0)	1.5 (0)	90 (0)	2.00	2.12	0.12	36.21	36.42	0.21
14	5 (− 1)	0 (− 1)	90 (0)	1.09	0.82	0.27	38.16	40.60	2.44
15	9 (+1)	1.5 (0)	0 (−1)	0.37	0.31	0.06	18.67	18.43	0.24
16	7 (0)	1.5 (0)	90 (0)	2.00	1.80	0.20	36.21	38.04	1.83
17	7 (0)	0 (− 1)	0 (− 1)	0.05	0.23	0.19	27.05	25.00	2.05

Stepwise regression analysis was conducted by generating the following second order polynomial equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{jj} x_j^2 \quad (1)$$

where Y is the response, i and j are the linear and quadratic coefficients, respectively, β is the regression coefficient, and x are the independent variables including culture pH, nitrogen source concentration, and light intensity.

Co-pelletization of microalgae with filamentous fungi

Four filamentous fungi including *Penicillus chrysogenum* TPU 4110, *Fusarium saloni* TPU 4501, *Cunninghamella echinulata* TPU 4652, and *Mortierella alpine* TPU 4880 were cultured in liquid fungal growth agar containing 0.6-g/L peptone, 0.6-g/L KH_2PO_4, 0.001-g/L $ZnSO_4$, 0.4-g/L K_2HPO_4, 0.005-g/L $FeSO_4$, 0.5-g/L $MnSO_4$, 0.5-g/L $MgSO_4$, 20-g/L glucose, and 1.5% agar for 48 h. After spore formation, the spores were isolated and inoculated into the photoautotrophic culture of selected microalgae at 10^9 spores/L. The glucose was added at 20 g/L as a carbon source for the fungi and the pH was adjusted to 5.5. The co-culture was shaken at 120 rpm for 24 h at 30 °C. Each pure culture of fungi and microalgae was also cultivated in the same manner for 24 h. Flocculation efficiency of the microalgal cells with fungi was calculated using the following equation (Mandik et al. 2015):

$$\text{Flocculation efficiency } (\%) = (1\text{–}A/B) \times 100 \quad (2)$$

where A is the OD_{660} of supernatant from half the height of the clarified layer after flocculation and B is the initial OD_{660} of the suspension.

Analytical methods

Cell density was measured by using spectrophotometer at 660 nm. Microalgae biomass was determined as follows: 10 mL of culture sample was centrifuged at 4000 ×*g* for 15 min and to effectively extract the lipids the pellets were dried at 60 °C until constant weight (Viswanathan et al. 2011; Hosseinizand et al. 2017). The lipid content of the dry biomass was determined using solvent extraction with a mixed solution of methanol and chloroform (2:1 *v*/*v*). Dry biomass was mashed and mixed with a mixed solvent solution before sonication for 30 min. The suspension was centrifuged at 4000 ×*g* for 20 min. The supernatant was collected and the precipitate was extracted twice more. After extraction, the solvent solution was evaporated overnight and the extracted lipid was determined gravimetrically (Tongprawhan et al. 2014). Fatty acid methyl esters (FAME) were prepared by transesterification through acid catalysis (Karpagam et al. 2015). The extracted lipid sample (10 mg) was dissolved in 0.5-ml toluene in a screw capped tube to which 1.5 mL of methanol and 50 μl of 35% conc. HCl (final concentration 0.39 M) was added and the contents were incubated at 100 °C for 1 h and 30 min. The reaction mixture was cooled, and 1 mL of hexane was added and vortexed. The hexane layer (FAME) was collected. FAME was analyzed by GC-MS (7890A GC System coupled with a 5975C inert XL EI/CI MSD with a Triple-Axis Detector operated at 70 eV; Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5-ms capillary column (0.25 mm i.d. × 30 m, 0.25-m film thickness; Agilent Technologies) according to the previous publication (Kuwahara et al. 2011).

The microalgae-fungi pellets were filtered and then dried at 60 °C until constant weight to determine the biomass concentration. The lipid content and FAME profiling of dried microalgae-fungi biomass were performed as mentioned above. After lipid extraction, the lipid extracted biomass residues (LEBRs) were determined for their carbohydrate content by the phenol sulfuric acid method using standard curve of glucose and expressed as amount of carbohydrate (Mandik et al. 2015).

The fuel properties of microalgal lipids such as saponification value (SV), iodine value (IV), cetane number (CN), unsaturation degree (DU), long chain saturated factor (LCSF), and cold filter plugging point (CFPP) were calculated based on the fatty acid profiles using the following empirical equations (Karpagam et al. 2015):

$$\mathrm{SV} = \sum[(560 \times F)/\mathrm{MW}] \tag{3}$$

$$\mathrm{IV} = \sum[(254 \times F \times D)/\mathrm{MW}] \tag{4}$$

$$\mathrm{CN} = [46.3 + (5458/\mathrm{SV})] - (0.225 \times \mathrm{IV}) \tag{5}$$

$$\mathrm{DU} = \%\mathrm{MUFA} + (2 \times \%\mathrm{PUFA}) \tag{6}$$

$$\mathrm{LCSF} = (0.1 \times \mathrm{C16}) + (0.5 \times \mathrm{C18}) + (1 \times \mathrm{C20}) + (1.5 \times \mathrm{C22}) + (2 \times \mathrm{C24}) \tag{7}$$

$$\mathrm{CFPP} = (3.1417 \times \mathrm{LCSF}) - 16.477 \tag{8}$$

where D is the number of double bonds, F is the % of each type of fatty acid, and MW is the molecular weight of corresponding fatty acid. MUFA is the weight percentage of the mono-unsaturated fatty acids (wt%), and PUFA is the weight percentage of the polyunsaturated fatty acids (wt%).

All experiments were performed in triplicates. The results are expressed as a mean plus standard deviations. Analysis of variance (ANOVA) was performed to calculate significant differences in treatment means, and the least significant difference was used to separate means, using SPSS software.

Results and discussion

Selection of oleaginous microalgae with high lipid content and suitable fatty acid compositions

Two marine oleaginous microalgae strains, *M. reisseri* SIT04 and *S. obliquus* SIT06 were cultivated in photoautotrophic mode and evaluated for their growth, lipid content, and fatty acid compositions. The biomass production in 10 days of *S. obliquus* SIT06 (2.01 ± 0.05 g L^{-1}) was higher than that of *M. reisseri* SIT04 (1.86 ± 0.02 g L^{-1}) (Fig. 1). The lipid contents in 10 days of *S. obliquus* SIT06 and *M. reisseri* SIT04 increased during cultivation

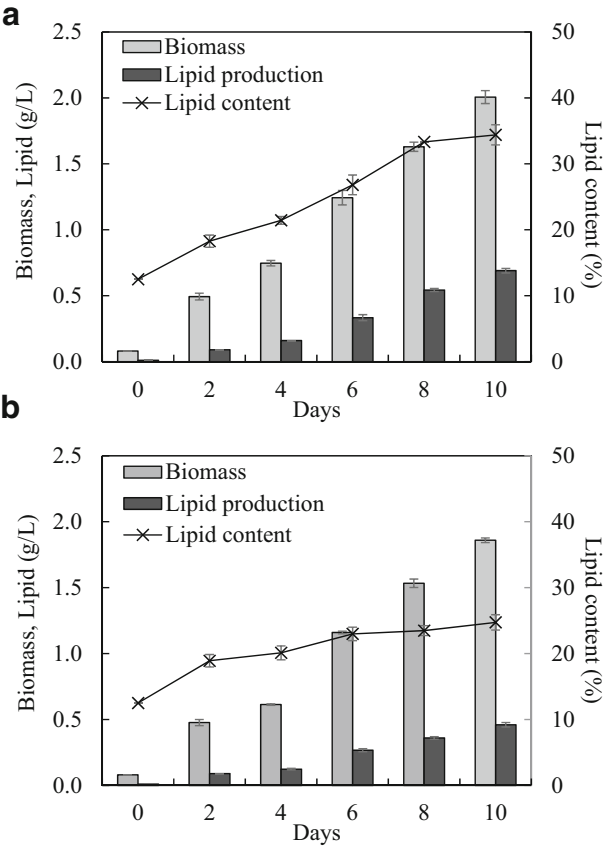

Fig. 1 Growth and lipid accumulation of *S. obliquus* SIT06 (**a**) and *M. reisseri* SIT04 (**b**) cultivated in photoautotrophic mode

and reached the maximum at 34.4 ± 1.53 and 27.7 ± 1.16%, respectively. This was probably due to the decreasing of important nutrients for cell growth such as nitrogen source. As the cell growth is restricted the carbon flow is channeled into lipid accumulation and led to an increase in lipid content (Xin et al. 2010; Yeesang and Cheirsilp 2011; Fan et al. 2014; Karpagam et al. 2015b). Comparing among the two strains, the maximum lipid production by *S. obliquus* SIT06 (0.69 ± 0.02 g L^{-1}) was higher than that of *M. reisseri* SIT04 (0.46 ± 0.02 g L^{-1}) due to its higher biomass production and lipid content.

Based on the analysis of fatty acid compositions of two microalgae, it was found that palmitic acid was the main fatty acid found in *M. reisseri* SIT04 lipids (39.87%) followed by stearic acid (32.18%) and oleic acid (20.10%). While those found in *S. obliquus* SIT06 lipids were palmitic acid (62.94%) and steric acid (30.74%). As the content of saturated fatty acids in *S. obliquus* SIT06 lipids (95.8%) was much higher than that in *M. reisseri* SIT04 (75.6%), *S. obliquus* SIT06 lipids tend to provide better fuel properties such as higher oxidation stability, higher CN, decreased NO_x emissions, and a shorter ignition delay time (Antolin et al. 2002). As *S. obliquus* SIT06 not only gives high biomass and lipid production but also has suitable fatty acid compositions for biodiesel production, it was then selected for further studies.

Optimization of photoautotrophic culture condition through RSM

Experimental design and statistical analysis of the models for biomass and lipid content

Three important factors for cultivating *S. obliquus* SIT06 including culture pH (5–9), light intensity (0–180 μmol proton m^{-2} s^{-1}) and nitrogen source ($NaNO_3$) concentration (0–3 g L^{-1}) were simultaneously optimized through RSM. The experimental design with the observed responses for microalgal biomass concentration and lipid content are presented in Table 1. Based on the experimental data, the biomass concentrations were in the range of 0.1–2.3 g L^{-1} and the lipid contents were in the range of 8–48%. Among the Runs tested, Run 9 (pH of 9, $NaNO_3$ concentration of 3 g/L, and light intensity of 90 μmol proton m^{-2} s^{-1}) gave the highest biomass concentration of 2.28 g/L with a lipid content of 25.76%. The microalgae could not grow and accumulated low lipid content under the condition without light illumination (0 μmol proton m^{-2} s^{-1}) and the condition illuminated with too high light intensity (180 μmol proton m^{-2} s^{-1}) (Runs 2, 4, 5, 6, 8, 10, 15, and 17). The maximum lipid content of 47.81% was obtained under the condition with high pH, low $NaNO_3$ concentration, and moderate light intensity (Run 7).

The ANOVA, regression coefficient, and regression equation from experimental data were analyzed through Design Expert Software (6.0.2 version). Sequential *P* value was used to select the highest order polynomial where the additional terms are significant and the model is not aliased. The response function in the term actual factor to predict biomass concentration and lipid content are given in Eqs. (8) and (9), respectively.

$$\text{Biomass (g/L)} = -4.18 + 1.22A + 0.19B + 0.03C - 0.09A^2 - 0.11B^2 - 0.0002C^2 + 0.05AB - 0.0001AC + 0.0003BC \quad (8)$$

$$\text{Lipid content (\%)} = -12.31 + 10.02A - 2.91B + 0.35C - 0.63A^2 + 0.21B^2 - 0.002C^2 - 0.33AB + 0.01AC - 0.01BC \quad (9)$$

where independent variables *A* is pH, *B* is $NaNO_3$ concentration (g/L), and *C* is light intensity (μmol proton m^{-2} s^{-1}). *A*, *B*, and *C* are referred as the linear terms, while *AB*, *AC*, and *BC* are the interaction terms, and A^2, B^2, and C^2 are the quadratic terms involved in the process.

The statistical significance of the model equations were analyzed by the *F* test for analysis of variance (ANOVA), to the fitted model (Table 2). The models were highly significant with *P* values ≤ 0.0005. In regression, the R^2 coefficient is a statistical measure of how well the regression line approximates the real data points. An R^2 of 1.0 indicates that the regression line perfectly fits the data. The highest determination coefficient (R^2) obtained was 0.9634 for biomass production and 0.9747 for lipid content. This indicated that up to 96.34 and 97.47% of the variations in response can be explained by the models. The value of adjusted determination coefficient (adjust $R^2 > 0.9$) is quite high indicating a high significance of the models. The lack of fit compares the residual error to the pure error from the replicated experimental design points. The *P* values, greater than 0.05, for the two responses indicated that any lack of fit for the model was insignificant. The coefficient values (C.V.) < 25% indicated a high precision and reliability of the experiment. It should be noted that the regression of quadratic term of light intensity (C^2) is the most significant factor for both biomass and lipid production (*P* values < 0.0001), followed by linear term of $NaNO_3$ concentration (*B*) (*P* values < 0.05).

Table 2 ANOVA for response surface quadratic model of microalgae biomass and lipid content of selected *S. obliquus* SIT06

Source	Biomass		Lipid content	
	Coefficients	*P* value	Coefficients	*P* value
Model intercept	− 4.18	0.0003*	− 12.31	< 0.0001*
A—pH	1.22	0.1963	10.02	0.0300*
B—$NaNO_3$ concentration	0.19	0.0065*	− 2.91	0.0001*
C—light intensity	0.03	0.6615	0.35	0.0518
A^2	− 0.09	0.0228*	− 0.63	0.1093
B^2	− 0.11	0.0763	0.21	0.7424
C^2	− 0.0002	< 0.0001*	− 0.002	< 0.0001*
AB	0.05	0.2723	− 0.33	0.5096
AC	− 0.0001	0.9845	0.01	0.4112
BC	0.0003	0.7707	− 0.01	0.5812
R^{2a}	0.9634	–	0.9747	–
Adjust R^{2a}	0.9163	–	0.9422	–
C.V.[c]	23.85	–	10.65	–
Lack of fit	–	0.0521		0.0539

*Significant level at 95%

[a] R^2 = regression coefficient

[b] C.V. = coefficient value

Response surface plots of biomass concentration

Three dimensional response surfaces were generated to visualize the combination effects of three factors on biomass production of *S. obliquus* SIT06 (Fig. 2). When the effects of two factors were plotted, the other factor was set at its center point. Figure 2a shows the interaction effect between pH (*A*) vs. $NaNO_3$ concentration (*B*). The optimal level of $NaNO_3$ concentration for biomass production seems to be pH dependent. At a low pH, the saturation level of $NaNO_3$ was 1.5 g L^{-1}, and above this level, there was no further positive effect on biomass production. At a higher pH, the optimum level of $NaNO_3$ shifted to 2.7–3.0 g L^{-1}, which was close to those for other *Scenedesmus* spp. (Ren et al. 2013; Arumugam et al. 2013). This phenomenon was possibly because the culture pH is one of important factors affecting the solubility and the bioavailability of nutrients and also transport of substrates across the cytoplasmic membrane (Ren et al. 2013). In addition, a lower pH might also cause a loss of carbonate and result in a lower biomass. At a higher pH, the carbonate in the original medium is more stabilized and available for the microalgae. The optimal pH also slightly shifted from 7.0 to 8.0 when $NaNO_3$ concentration was increased from 0 to 3.0 g L^{-1}. It has been reported that this pH range was also the suitable range for other microalgae, especially for *Scenedesmus* spp. (Hodaifa et al. 2010; Ren et al. 2013).

Figure 2b presents the interaction effect between pH (*A*) vs. light intensity (*C*) on biomass production. Light intensity has an important role in the photosynthetic activity of microalgae. The biomass increased with increasing light intensity but sharply decreased when the light intensity was higher than 90 μmol proton m^{-2} s^{-1}. When the light intensity is insufficient, the microalgae growth would be limited but when the light intensity is above the saturation level, the photoinhibition would occur (Jeong et al. 2013). It seems that the optimal pH was not affected by the level of light intensity indicating that there was no interaction effect between these two factors. Figure 2c depicts the interaction effect between $NaNO_3$ concentrations (*B*)

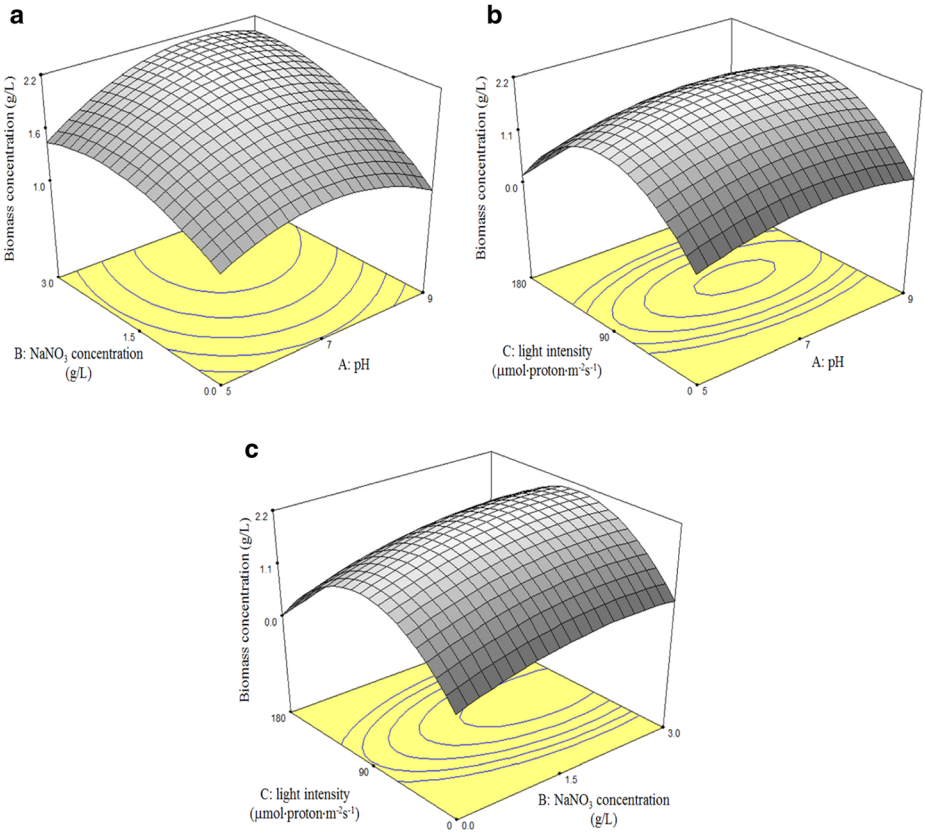

Fig. 2 3D of response surface plots of biomass concentration. **a** Effect of pH and $NaNO_3$ concentration. **b** Effect of pH and light intensity. **c** Effect of $NaNO_3$ concentration and light intensity

vs. light intensity (*C*). The effect of light intensity on biomass production was more obvious than that of $NaNO_3$ concentration. The optimal pH, $NaNO_3$ concentration and light intensity for biomass production were found to be 8.0, 2.7 g L^{-1} and 90 μmol proton m^{-2} s^{-1}, respectively.

Response surface plots of lipid content

Three dimensional response surfaces were generated to visualize the combination effects on lipid content of *S. obliquus* SIT06 (Fig. 3). When the effects of two factors were plotted, the other factor was set at its center point. Figure 3a shows the effect of pH (*A*) and $NaNO_3$ concentration (*B*) on the lipid content at a fixed light intensity of 90 μmol proton m^{-2} s^{-1}. The lipid content increased with decreasing $NaNO_3$ concentration. This was likely because the low nitrogen concentration could promote the lipid accumulation (Xin et al. 2010; Yeesang and Cheirsilp 2011). However, the effect of pH on lipid content was insignificant. Similarly, Dayananda et al. (2006) also found that the lipid content in the microalgae was not much affected by the pH of the medium. Figure 3b presents the effect of pH (*A*) and light intensity (*C*) on the lipid content. With a fixed $NaNO_3$ concentration at 1.5 g L^{-1}, obviously the effect of pH on lipid content was less than that of light intensity. The optimal light intensity for lipid

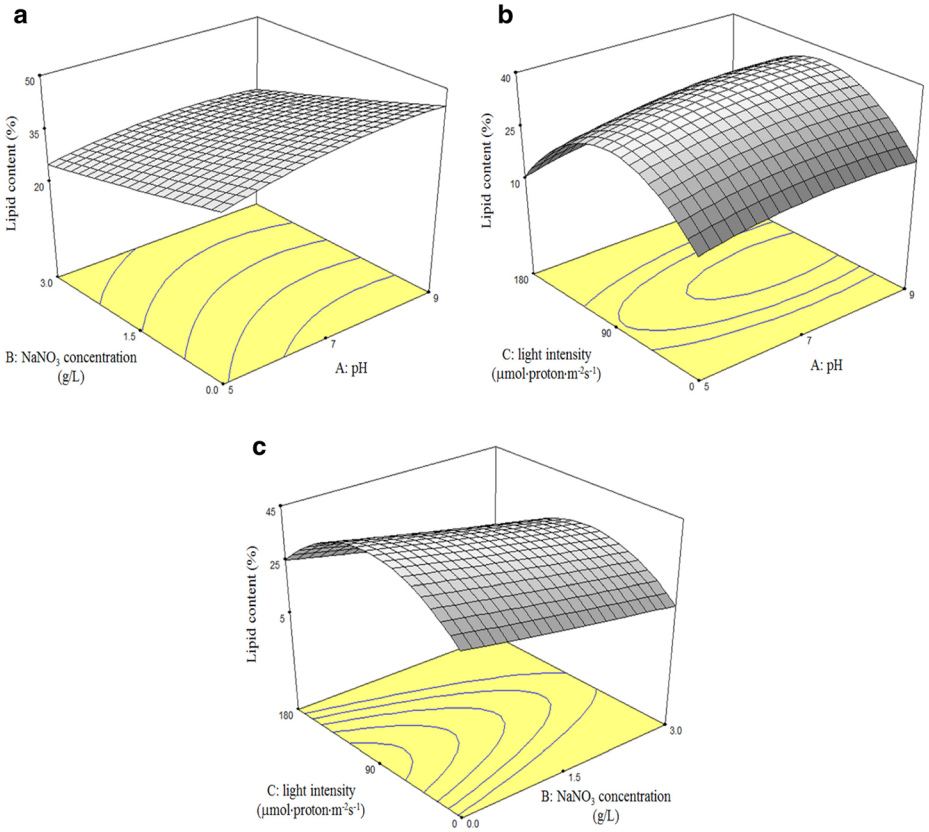

Fig. 3 3D of response surface plots of lipid content. **a** Effect of pH and $NaNO_3$ concentration. **b** Effect of pH and light intensity. **c** Effect of $NaNO_3$ concentration and light intensity

accumulation was close to that for biomass production at 88 μmol proton m^{-2} s^{-1}. However, in several studies, it was found that the optimal light intensities for cell growth and lipid accumulation were different. Cheirsilp and Torpee (2012) found that the cell growth of marine *Chlorella* sp. and *Nannochloropsis* sp. increased with increasing the light intensity up to 135 μmol proton m^{-2} s^{-1} but the lipid content of both strains drastically decreased. While Thawechai et al. (2016) found that the optimal light intensity for the cell growth of *Nannochloropsis* sp. (70 μmol proton m^{-2} s^{-1}) was lower than that for lipid content (80 μmol proton m^{-2} s^{-1}). It could then be concluded that each microalga has a unique requirement for light intensity.

Figure 3c shows the effect of $NaNO_3$ concentration (b) and light intensity (c) on the lipid content at a fixed pH of 7.0. The moderate light intensity at 88 μmol m^{-2} s^{-1} was optimal for lipid accumulation, and there was no interaction effect between the light intensity and $NaNO_3$ concentration. In many microalgae, a decrease in nitrogen concentration resulted in a significant favoring the accumulation of lipid. Although a deficiency of nitrogen favors lipid accumulation, nitrogen is required for microalgae growth. Yeesang and Cheirsilp (2011) compared the lipid content in *Botryococcus* sp. isolated from freshwater source in Thailand under nitrogen-rich and nitrogen-deficiency conditions. They found that the highest lipid content was obtained under nitrogen-deficiency conditions. Similarly, the microalgae

S. quadricauda tended to accumulate high lipids under nitrogen-deficient conditions (Ahlgren and Hyenstrand 2003). The optimal conditions for lipid content of *S. obliquus* SIT06 were found to be pH of 8.0 without the addition of $NaNO_3$ concentration and moderate light intensity of 88 μmol proton m^{-2} s^{-1}. The maximum lipid content obtained would be 45.7% based on its dried biomass.

Numerical optimization and verification of the model

From the previous section, the optimal pH for biomass and lipid content were the same at pH 8.0, and the optimal light intensity for both responses was 90 and 88 μmol proton m^{-2} s^{-1}, respectively. Although 2.7 g L^{-1} of $NaNO_3$ is needed for biomass production, there is no need to add $NaNO_3$ for lipid accumulation. Therefore, $NaNO_3$ should be added at initial and depleted during cultivation. To optimize multiple responses, the desired goals for each response are combined into an overall desirability function (Islam et al. 2010). By seeking from several starting points in the response surface changes, the best conditions for compromising between biomass production and lipid accumulation were as follows: pH of 8.0, $NaNO_3$ concentration of 1.1 g L^{-1}, and light intensity of 87 μmol proton m^{-2} s^{-1}. The predicted biomass and lipid content were 1.91 g L^{-1} and 38.7%, respectively. Experiment validation for the optimal condition was done. The experimental biomass and lipid content were 1.99 ± 0.12 g L^{-1} and 40.86 ± 0.32%, respectively. With these conditions, the maximum lipid production of 0.81 ± 0.06 g L^{-1} was obtained.

Harvesting of microalgal cells by co-pelletization with filamentous fungi

Filamentous fungi have ability to form pellet and easily being harvested by sieving method. In this study, four filamentous fungi including *P. chrysogenum* TPU 4110, *F. saloni* TPU 4501, *C. echinulata* TPU 4652, and *M. alpine* TPU 4880 were screened for their abilities to form pellets and accumulate lipids in the medium for microalgae added with 2% glucose. All fungi could form pellets within 24 h. The pellet sizes of *P. chrysogenum* TPU 4110 was smallest < 1.0 mm, while those of *F. saloni* TPU 4501, *C. echinulata* TPU 4652, and *M. alpine* TPU 4880 were in the same range of 1.0–3.5 mm. Figure 4 shows the biomass and lipid production by pure culture of filamentous fungi during 24-h cultivation. At initial, the fungi biomass was

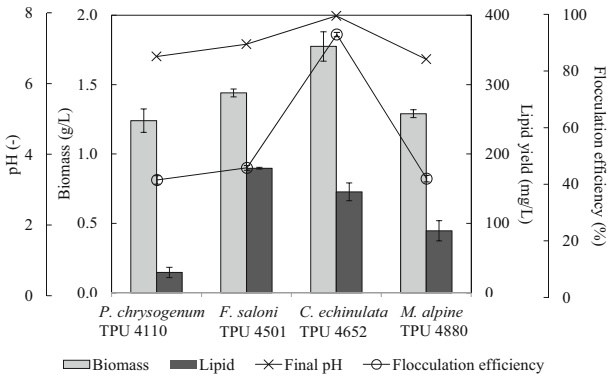

Fig. 4 Biomass and lipid production by the pure culture of filamentous fungi and their use for harvesting of microalgal cells

approximately 0.1 g/L. *C. echinulata* TPU 4652 grew well and gave the highest biomass with the final pH increased higher than other fungi. The lipid yields of *F. saloni* TPU 4501 and *C. echinulata* TPU 4652 were obviously higher than those of other two fungi but less than those of the microalgae (Fig. 1).

To harvest the microalgal cells by co-pelletization with filamentous fungi, the spores of fungi were added into the 10 days-cultured microalgae and the co-cultivation was conducted for 24 h. The microalgae-fungi pellets were rapidly formed within 24 h through co-pelletization process. The flocculation efficiency after 24 h was determined. The maximum flocculation efficiency of 92.65% was obtained when using *C. echinulata* TPU 4652 (the final pH was 7.96), followed by *F. saloni* TPU 4501 (45.53%, final pH of 7.15), *P. chrysogenum* TPU 4110 (41.25%, final pH of 6.8), and *M. alpine* TPU 4880 (40.90%, final pH of 6.82). There are several factors affecting the co-agglomeration between filamentous fungi and microalgae, such as the pH of the medium, pellet sizes, agitation speed, and carbon source addition (Zhou et al. 2012; Xie et al. 2013). It was possible that fungal hyphae and mycelia containing the positively charged polysaccharide and hydrophobic proteins can potentially neutralize the negative charges on the microalgae surface resulting attachment to the fungi cells (Prajapati et al. 2014; Gultom and Hu 2013). Moreover, Muradov et al. (2015) have found that within fungi-pellets, microalgae cells did not just get entrapped within fungal filaments but also got attached to them.

In this study, as *C. echinulata* TPU 4652 was the most suitable filamentous fungi for harvesting of microalgal cells, their microalgae-fungi pellets were analyzed for total biomass and lipid yield. It was found that the fungi not only co-agglomerated with the microalgal cells but also increased the overall biomass and lipid yield. The overall biomass and lipid yield were 4.45 ± 0.06 and 1.21 ± 0.08 g L^{-1}, which were 2.24 and 1.49 folds of the pure microalgae, respectively. This could be the effect of CO_2 addition by fungi to the microalgae. It is well-known that some algae live in symbiotic association with fungi, so called lichens. In the majority of lichens, the algae are entrapped in the fungal hyphae. It is possible that the microalgae could act as an oxygen generator for the fungi, while the fungi could provide CO_2 to the microalgae and together they would be able to carry out biomass and lipid production inside the pellets (Kitcha and Cheirsilp 2014). As two metabolic reactions of CO_2 release and uptake were combined and complementary, this technique not only effectively harvested the microalgal cells but also contributed to the increase in lipid production. Since the production of lipid from microorganisms is subjected to the constraints of high operation costs, even a small improvement in the culture and harvesting techniques could result in substantial savings in the microbial lipid production.

Table 3 shows the earlier studies on fungal-assisted microalgae flocculation through co-pelletization technique. Although there are several studies on the fungal-assisted flocculation through this technique, there is only one study evaluated the lipid yield of the microalgae-fungi pellets (Zhang and Hu 2012). However, the lipid yield in their study was only 0.1 g L^{-1}, which was much lower than the lipid yield in this study (1.21 g L^{-1}). The time required for flocculation in this study was also shorter than that in the study of Zhang and Hu (2012). Therefore, the harvesting process of microalgae by the pellet-forming filamentous fungi in this study may contribute greatly to the industrialization of microorganism based biofuel production. Moreover, it has been reported that the microalgae-fungi pellets could perform higher nutrient removal rate than the free cells (Zhou et al. 2012). As the microalgae can grow both with light (autotrophic mode) and without light (heterotrophic mode) by using energy source from organic carbon source, the microalgae inside the pellets would grow with heterotrophic mode and use organic nutrients. Recently, microalgal

Table 3 Summary of the earlier studies on fungal-assisted microalgae flocculation through co-pelletization technique

Microalgae	Fungi	Conditions	Flocculation efficiency	Pellet sizes (mm)	Lipid yield (g/L)	References
C. vulgaris UMN235	*Aspergillus* sp.	pH 6.2, 100 rpm	100% in 60 h	2–5	–[a]	Zhou et al. (2012)
Chroococcus sp.	*A. lentulus*	pH 7.82, 150 rpm	100% in 24 h	0.5–0.7	–	Prajapati et al. (2014)
C. sorokiniana	*Isaria fumosorosea*	pH 7, 75 rpm	100% in 48 h	1–2	–	
C. vulgaris	*A. niger*	pH 7, 150 rpm	96% in 72 h	3.5	–	Zamalloa et al. (2017)
C. vulgaris	*A. niger*	pH 5.0, 150 rpm	98.1% in 48 h	–	0.1	Zhang and Hu (2012)
S. obliquus SIT06	*C. echinulata* TPU4652	pH 5.5, 120 rpm	92.7% in 24 h	3.5	1.21	Present study

[a] Not available

bacterial flocs (MAB flocs) formed in raceways of nutrient-enriched effluent from aquaculture facilities have been reported and the use of MAB flocs drastically reduced harvesting costs in sequencing batch reactors (Van Den Hende et al. 2014). Therefore, the microalgae-fungi pellets in this study would be useful as immobilized cells in wastewater treatment prior to lipid extraction and also can reduce the harvesting costs.

Fatty acid composition of lipids and the assessment for their fuel properties

Based on the results from the above experiments, *C. echinulata* TPU 4652 was the most suitable fungi strain for being used for harvesting of microalgal biomass and enhancing biomass and lipid yields. The extracted lipids from the microalgae-fungi pellets were analyzed for their fatty acid compositions and fuel properties. The microalgae-fungi lipids were mainly composed of long-chain fatty acids of 16 and 18 carbon atoms, and the major fatty acids were palmitic acid (C16:0, 52%) and stearic acid (C18:0, 35%). It should be noted that these found in pure *S. obliquus* SIT06 lipids were also palmitic acid (62.94%) and steric acid (30.74%). It is well-known that the proportions of saturated and unsaturated fatty acids greatly influence the biodiesel properties, such as CN and oxidative stability (Karpagam et al. 2014; Jawaharraj et al. 2016). In this study, biodiesel fuel parameters including SV, IV, CN, DU, LCSF, and CFPP were calculated based on the fatty acid compositions.

The SV is a measure of the average molecular weight of total fatty acids. The SV of microalgae-fungi was found to be 209.5 mg KOH g^{-1} of oil, which is the amount of KOH (in milligrams) required to saponify 1 g of oil. The CN was found to be 69.8 that indicating high combustion quality of the biodiesel. As per the standards, the minimum limit of CN is 47, which ensures the ignition properties and good engine performance as well as the reduction of white smoke formation from the engine. The IV was found to be 11.4-g iodine/100 g of oil. The maximum limit of IV was 120-g iodine/100 g of oil. IV biodiesel positively correlates with the content of UFA. The DU is the summation of the mass of MUFAs and PUFAs and is one of the key properties that would highly influence the oxidative stability of biodiesel (Karpagam et al. 2014). The low DU greatly increases the oxidative stability of the produced biodiesel for longer storage in tropical regions (Talebi et al. 2013). The DU was found to be 12.6%. Further, cold

flow properties like LCSF and CFPP of fungi-algae were in accordance with the international standard revealing the good flow performance of biodiesel at low temperature. It should be noted that the fuel properties of biodiesel obtained from fungi *C. echinulata*-microalga *S. obliquus* were found to match with the biodiesel fuel specifications given by the regulatory international standard, ASTM D6751, and EN 14214. Thus, fungi *C. echinulata*-microalga *S. obliquus* pellets can be a promising alternative feedstock for biodiesel.

Conclusions

This study has revealed the optimal conditions for cultivating oleaginous microalgae in photoautotrophic mode for production of biomass with high lipid content and the strategy to harvest the microalgal cells using pellet-forming filamentous fungi. The filamentous fungi not only effectively flocculated the microalgal cells but also increased the overall lipid yield. The fatty acid composition of microalgae-fungi lipids and their estimated fuel properties indicated their potential use as biodiesel feedstocks. The harvested microalgae-fungi pellets can be further used for wastewater treatment which have higher efficiency than the free cells and may offer a sustainable and efficient way to produce biofuels and simultaneously treat the wastewater. This study may contribute greatly to the industrialization of microbial based biofuel production.

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Compliance with ethical standards

Ethical statement I testify on behalf of all co-authors that the manuscript has not been published in whole or in part elsewhere. The manuscript is not currently being considered for publication in another journal. All authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

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Paper VI

A rapid method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi and the application in phytoremediation of secondary effluent

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A rapid method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi and the application in phytoremediation of secondary effluent

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ABSTRACT

A rapid method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi was developed. The suitable conditions for pellet formation by filamentous fungi were determined. Among the strains tested, *Trichoderma reesei* QM 9414 showed superior pellet forming ability. Its pellets were used to harvest oleaginous microalga *Scenedesmus* sp. With increasing volume ratio of fungal pellets to microalgae culture up to 1:2, >94% of microalgal cells were rapidly harvested within 10 min. The ratio of fungal pellets could manipulate both harvesting time and initial concentration of microalgal cells in the pellets. The microalgae–fungal pellets were successfully used as immobilized cells for effective phytoremediation of secondary effluent from seafood processing plants under nonsterile condition. The chemical oxygen demand, total nitrogen, and total phosphorus removal were >74%, >44%, and >93%, respectively. The scanning electron microscopy showed that the microalgal cells were not only entrapped in the pellets but also got attached to the fungal hyphae with sticky exopolysaccharides, possibly secreted by the fungi. The extracted lipids from the pellets were mainly composed of C16–C18 (>83%) with their suitability as biodiesel feedstocks. This study has shown the promising strategy to rapidly harvest and immobilize microalgal cells and the possible application in phytoremediation of industrial effluent.

KEYWORDS

filamentous fungi; harvesting; immobilized cells; oleaginous microalgae; phytoremediation

Highlights

- *Trichoderma reesei* rapidly harvested *Scenedesmus* sp. cells within 10 min.
- Microalgae–fungi pellets were used in phytoremediation of secondary effluent.
- Pollutants in effluent were effectively removed by the microalgae–fungi pellets.
- Lipids extracted from microalgae–fungi pellets are suitable as biodiesel feedstocks.

1. Introduction

Microalgae are considered as renewable sources for production of biofuels and valuable chemicals. Oleaginous microalgae *Scenedesmus* spp. have been selected as potential biodiesel feedstocks due to their high lipid content and suitable fatty acid composition for production of biodiesel (Ren *et al.* 2013; Srinuanpan *et al.* 2017). In addition to cultivation step, the harvesting step is also important step whose cost would be as high as 20–30% of the total cost of biofuel production (Grima *et al.* 2003). Several methods including gravity sedimentation, filtration, centrifugation, flocculation, or combination of these have been implemented to harvest microalgal cells. However, these methods require considerably large amounts of energy and/or auxiliary materials and chemicals. Recently, low energy requiring and environmental friendly harvesting methods have been developed. Several researches have shown the possible use of pellet-forming filamentous fungi for harvesting of microalgal cells (Zhou *et al.* 2013; Talukder *et al.* 2014; Bhattacharya *et al.* 2017) and some of them attempted this method as immobilization technique (Wrede *et al.* 2014; Miranda *et al.* 2015). Filamentous fungi can grow in different morphological forms including suspended mycelia, clumps, and pellets. The pellet-forming ability is influenced by culture conditions such as medium composition and viscosity, pH, inoculums, and shaking speed. The pellet form of the fungi is favorable since it can improve the culture rheology and make the repeated use of fungal cells possible and easily be harvested by a simple sieving method (Papagianni, 2004; Zhou *et al.* 2012; Zamalloa *et al.* 2017). Several filamentous fungi such as *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Isaria fumosorosea*, *Cunninghamella echinulata*, and *Mucor circinelloides*, have been reported for their capability of forming large pellets, which may provide an alternative cost effective way of harvesting microalgae biomass (Zhou *et al.* 2013; Wrede *et al.* 2014; Mackay *et al.* 2015).

Phytoremediation using microalgae have been of growing interest because the microalgae can utilize low-quality water,

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such as agricultural runoff or municipal, industrial or agricultural wastewaters, as a source of water for the growth medium as well as a source of nitrogen, phosphorus, and minor nutrients. These nutrients are directly the cause for eutrophication of rivers, lakes, and seas if they are present at high concentrations. Microalgae are highly effective in phytoremediation of wastewater for two reasons. Firstly, they can effectively remove inorganic nitrogen and phosphorus from the wastewater, and secondly they also purify the wastewater by producing oxygen (An *et al.* 2003; Pizarro *et al.* 2006). Nitrogen and phosphorous removal efficiencies by the microalgae could be in the range of 20–100% (Wang *et al.* 2010; Hongyang *et al.* 2011). It has been reported that apart from the nitrogen and phosphorus, the organic carbon sources could also be removed by the microalgae through mixotrophic metabolism and this contributed to the chemical oxygen demand (COD) removal (Devi *et al.* 2013). Moreover, many researchers have studied the phytoremediation of industrial wastewater by using immobilized microalgae (Lam and Lee 2012; Ruiz-Marin *et al.* 2012). The use of immobilize technology in microalgae cultivation is an interesting technique to reduce the operational costs, allow the continuous process using high cell density of microalgal cells, and require lower energy input in harvesting process than the free cells.

This study aimed to use pellet-forming filamentous fungi for harvesting and immobilizing oleaginous microalgae for phytoremediation and lipid production. There are two strategies for filamentous fungi to entrap the microalgal cells: (1) the copelletization in which the microalgal cells are entrapped during the fungal pelletization and (2) the cocultivation of microalgae with the fungal pellets, in which the microalgal cells are absorbed in the fungal pellets during cocultivation. In the present study, several filamentous fungi were tested for their ability to harvest oleaginous microalga *Scenedesmus* sp. through two techniques. The suitable conditions for pellet formation by the fungi and those for harvesting of microalgal cells were determined. The harvested microalgae in the fungal pellets were also used as immobilized cells in phytoremediation of sterilized and nonsterilized secondary effluent from seafood processing plants. After cultivation, the microalgae–fungi pellets were harvested by a simple sieving method and their lipids were extracted. The biodiesel properties of the mixed microalgae–fungal lipids were evaluated based on their fatty acid profiling.

2. Materials and methods

2.1. Cultivation of microalgae

Oleaginous microalga *Scenedesmus* sp. was obtained from the National Institute of Coastal Aquaculture in Southern region of Thailand. The modified Chu13 medium used as the basic medium in this study, contained 0.2 g KNO_3 as a nitrogen source, 0.04 g K_2HPO_4 as a phosphorus source, 0.1 g citric acid, 0.01 g Fe citrate, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.036 g $NaHCO_3$, and 1 mL of trace metal solution per 1 L, pH was 6.8. The trace metal solution consisted of 2.85 g H_3BO_3, 1.8 g $MnCl_2{\cdot}4H_2O$, 0.02 g $ZnSO_4{\cdot}7H_2O$, 0.08 g $CuSO_4{\cdot}5H_2O$, 0.08 g $CoCl_2{\cdot}6H_2O$, and 0.05 g $Na_2MoO_4{\cdot}2H_2O$ per 1 L (Srinuanpan *et al.* 2017). The microalga was cultured in 400 mL Chu 13 medium and air aerated at 0.025 volume air per volume medium per minute under 3500 lux light intensity and 24:0 h light photoperiod at 30±2°C for 5 days until Optical Density at 680 nm [OD_{680}] reached 2, corresponding to approximately 1 g/L of microalgal biomass.

2.2. Screening of filamentous fungi

Filamentous fungi including *Aspergillus niger* ATCC 6375, *Trichoderma reesei* QM 9414, and *Aspergillus oryzae* were screened for their ability to individually form pellets and copelletize with microalgal cells. Fungal culture was maintained on sterile potato dextrose agar (PDA) slants at 30±2°C. Fungal spore suspension prepared with 0.5% Tween-20 (≈10^4 spores/mL) was inoculated in 50 mL potato dextrose broth and incubated for 24 h at 30±2°C. The effect of shaking speed on the pellet formation by each filamentous fungus was investigated. The quantity and diameter of the pellets were analysed.

2.3. Harvesting of microalgal cells using filamentous fungi

The harvesting experiments were carried out in 250 ml conical flasks. The precultured fungi were directly added into the microalgae culture and shaken during cultivation. The copelletization of microalgae with fungi was evaluated. In another technique, the fungi were pelletized prior to cocultivation with microalgae. A suitable technique was selected and the effect of fungi to microalgae culture ratio was investigated. To evaluate the harvesting efficiency, the liquid samples were taken every 10 min and the optical density (OD) was measured at 680 nm. The harvesting efficiency was calculated as followed (Bhattacharya *et al.* 2017):

$$\text{Harvesting efficiency} = [(OD_0 - OD_t)/OD_0] \times 100 \quad (1)$$

where OD_t = optical density at time t and OD_0 = initial optical density.

2.4. Cultivation of microalgae–fungal pellets as immobilized cells

The microalgae–fungal pellets were applied in phytoremediation of secondary effluent from seafood processing plant. The effluent was filtered to remove suspended solids and characterized for pH, COD, total nitrogen (TN), and total phosphorus (TP). Those values were 7.7, 1,239 mg/L, 144 mg/L, and 18.6 mg/L, respectively. The microalgae–fungal pellets were inoculated in 100 mL of sterilized and nonsterilized effluent in a 250 mL conical flask. The cultures were cultivated under 3,500 lux light intensity, 24:0 h light photoperiod and shaking speed at 100 rpm, 30±2°C for 7 days. The microalgae–fungal pellets were harvested through a simple sieving method using a mesh. The pollutant removals by the microalgae–fungal pellets were evaluated.

2.5. Analytical methods

The morphology of the fungi pellets and immobilized microalgae was observed using microscopic and scanning electron microscopy (SEM) analysis. The harvested pellets were washed

with the same volume of distilled water three times to remove the residual nutrients and then dried at 60°C to constant weight. The total biomass was weighted and the lipids were extracted from dried biomass using a mixture of chloroform: methanol (2:1, v/v) for 1 h (Bligh and Dyer 1959). The extracted lipids were centrifuged to obtain a clear supernatant and the solvent was removed by evaporation under vacuum. The extracted lipids were converted to fatty acid methyl esters (FAMEs) by hydrolysis and esterification reactions (Jham *et al.* 1982). The fatty acid compositions of the FAMEs were analysed using a HP6850 Gas Chromatography equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mmID, 0.25 mm film thickness) and a flame ionization detector. Operating conditions were as follows: inlet temperature 290°C; oven temperature initial 210°C held for 12 min; then ramped to 250°C at 20°C/min; held for 8 min and the detector temperature was 300°C. Fatty acids were identified by comparing their retention times with known pure standards. The fuel properties were evaluated from their fatty acid composition using equations previously reported (Karpagam *et al.* 2015). These properties include saponification value (SV), iodine value (IV), cetane number (CN), unsaturation degree (DU), long chain saturation factor (LCSF), and cold filter plugging point (CFPP).

All experiments were performed at least in triplicates. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference ($p \leq 0.05$) was used to separate means, using the SPSS software.

3. Results and discussion

3.1. Screening of filamentous fungi

In this study, the fungal strains including *A. niger* ATCC 6375, *T. reesei* QM 9414, and *A. oryzae* were investigated for pellets forming ability by inoculating their spores in potato dextrose broth and shaking. The effect of shaking speeds on the pellet-forming ability was evaluated from the formation of firm pellets (Figure 1) and the size of the pellets (Table 1). *A. niger* ATCC 6375 formed loose and fluffy-like irregular aggregates rather than the pellets; nevertheless, the shaking speed was increased up to 150 rpm. While other two fungi formed the firm pellets at different sizes (0.25–8 mm in diameter) depending on the shaking speeds. A low shaking speed (80 rpm) was more suitable for the fungi to form large pellets. With increasing the shaking speed, the size of the pellets decreased. This was likely because high shaking speeds could cause high shear stress and the fungi could form large pellets (Purwanto *et al.* 2009). It should be noted that the sizes of the pellets formed and their distributions were mainly affected by both shaking speeds and types of the fungi. In addition to the pellet-forming ability, the cell growth and lipid production by the fungi were also compared (Figure 2). Among the strains tested, *A. niger* ATCC 6375 gave the highest biomass of 6.78 g/L and lipid production of 1.62 g/L after 24 h of cultivation followed by *T. reesei* QM 9414 and *A. oryzae*. However, as *A. niger* ATCC 6375 could not form firm pellets it was not selected. On the contrast, *T. reesei* QM 9414 which could form firm and adequate pellets and also had comparable high biomass and lipid yield was selected instead.

3.2. Harvesting of microalgal cells using filamentous fungi

The selected filamentous fungus *T. reesei* QM 9414 was added directly into the microalgae culture to observe the possibility of copelletization with microalgal cells. Although the presence of organic carbon source and the pH of the medium have been reported as primary factors for pellet formation by the fungi (Zhou *et al.* 2012), in the medium for microalgae *T. reesei* QM 9414 could not form the pellets neither with nor without the addition of glucose as organic carbon source and at any pH tested (5–8) (data not shown). It was possible that the fungi needed other factors for their pellet formation. Therefore, the fungal pellets were firstly formed in PDA medium prior to their

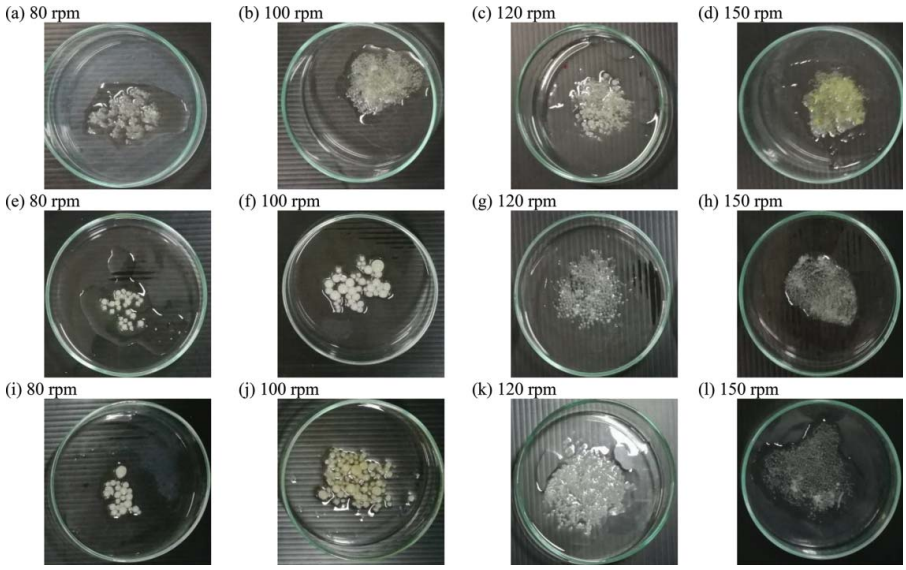

Figure 1. Effect of shaking speed on pellet forming abilities of three filamentous fungi including *A. niger* ATCC6375 (a–d), *A. oryzae* (e–h), and *T. reesei* QM9414 (i–l) after 24 h of cultivation in PDB.

Table 1. Pellet-forming ability and pellet size of filamentous fungal strains.

	A. niger ATCC 6375		*A. oryzae*		*T. reesei* QM 9414	
Shaking speed (rpm)	Pellet- forming ability	Size (mm)	Pellet- forming ability	Size (mm)	Pellet- forming ability	Size (mm)
80	+	1–2	+	3–4	+	4–7
100	+	1–2.5	+	2–8	+	3–5
120	+	1–1.5	+	1–3.5	+	0.5–2.5
150	+	0.25–0.5	+	0.25–1	+	0.5–2

Note: (+) pellet forming; (−) nonpellet forming.

use for harvesting of microalgal cells. With this method, there was no need to adjust the pH of the microalgae culture which was at 7.8–8.0. Figure 3 shows the fungal pellets before and after harvesting of microalgal cells. It seems that the fungal spores germinated into hyphae and intertwined into pellets. After cocultivation of fungal pellets with microalgae culture, it was obvious that the microalgal cells were entrapped in the hyphae of *T. reesei* QM 9414. Muradov *et al.* (2015) suggested that as the microalgae have a negative surface charge due to the presence of proton-active carboxylic, phosphoric, phosphodiester, hydroxyl, and amine functional groups and the fungal hyphae and mycelia contain polysaccharides that have positive charge, the fungi may potentially neutralize the negative charges on the microalgal surface, enabling the attachment of microalgal cells to the fungal cell wall. As it has been reported that the fungal cells can secrete concentrated exopolysaccharides during interaction with other microorganisms (Selbmann *et al.* 2003; Miranda *et al.* 2015), it was also possible that the microalgal cells might not only get entrapped within fungal pellets but also get attached with the sticky exopolysaccharides secreted by the fungi.

In this study, three volume ratios of fungal pellets to microalgae culture (F/M ratio) at 1:10, 1:5, and 1:2 were tested to evaluate their effects on harvesting efficiency. Figure 4 shows the effects of harvesting time and F/M ratio on the harvesting efficiency of the microalgae. The harvesting efficiency increased with increasing the harvesting time. When using 1:10 F/M ratio, the harvesting efficiency continuously increased up to 58.4% during first 2 h of harvesting time and slowly reached 69.3% at 4 h. It was possible that there would be a limit of absorbing surfaces for the microalgal cells so that not all of the microalgal cells could be harvested. The immobilization matrixes are generally preferred as high surface area which could permit a higher cells loading and the immobilized cells receive better protection from the environment. When the F/M ratio was increased up to 1:5, the harvesting efficiency rapidly increased up to 83.6% within 1.5 h and saturated at this level. Interestingly, when using 1:2 F/M ratio the harvesting efficiency reached 94.5% within 10 min and almost all microalgal cells were entrapped in the fungal pellets within 30 min. The total biomass and lipid yield of the fungal pellets after harvesting of microalgal cells were 2.17±0.35 g/L and 0.60±0.09 g/L, respectively, when using 1:10 F/M ratio. With increasing the F/M ratio up to 1:5, the total biomass and lipid yield were increased up to 3.78±0.21 g/L and 0.75±0.19 g/L, respectively. At the highest F/M ratio of 1:2, the total biomass and lipid yield were 6.64±0.65 g/L and 1.70±0.09 g/L, respectively. It should also be noted that this study has shown the superior harvesting efficiency of the oleaginous microalgae than other studies. Zhang and Hu (2012) have evaluated the harvesting of *Chlorella vulgaris* using pellet forming fungi. However, in their study the flocculation efficiency was only about 60% and the total fatty acid was <0.3 g/L. Xie *et al.* (2013) also found that when using the F/M ratio of 1:2, nearly 99% of the *C. vulgaris* cells were removed from the liquid medium by cocultivation with fungus *Cunninghamella echinulata* but the harvesting time required was as long as 2 days. The total lipid yields in their study were in the range of 0.75–1.0 g/L. Bhattacharya *et al.* (2017) found that when using 1:5 F/M ratio 99% of *Chlorella pyrenoidosa* could be harvested by the fungus *Aspergillus fumigatus* within 3 h but there is no available information regarding the lipid yield.

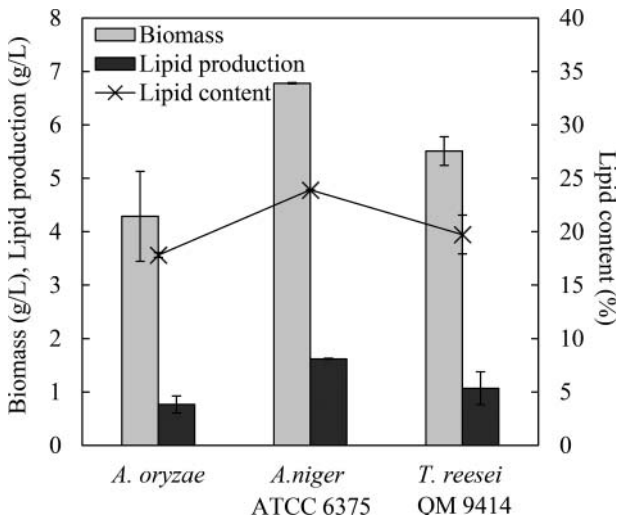

Figure 2. Biomass, lipid production, and lipid content of three filamentous fungi after 24 h of cultivation in PDB medium under agitation rate at 100 rpm. Data are means of triplicates.

As this method requires low energy inputs and no addition of chemicals, it is therefore suitable as a new insight for immobilization of microalgal cells. According to the harvesting efficiency and initial microalgal cells in the culture (1 g/L), at 1:10 F/M ratio 0.693 g (693 mg) of microalgal cells would be entrapped in 100 mL of fungal pellet volume and this corresponded to 6.93 mg/mL microalgal cells in the pellets. This high concentration of microalgal cells could also be observed from the dark green color of the pellets. The microscopic photo also showed the high cell density of the microalgae inside the pellets. When using higher amount of fungal pellets at 1:5 F/M ratio, the color of pellets was lighter green and had less microalgal cells inside the pellets. At this F/M ratio, the approximate microalgal cells inside the pellets was (836 mg/200 mL =) 4.18 mg/mL. When using F/M ratio at 1:2, the green color of

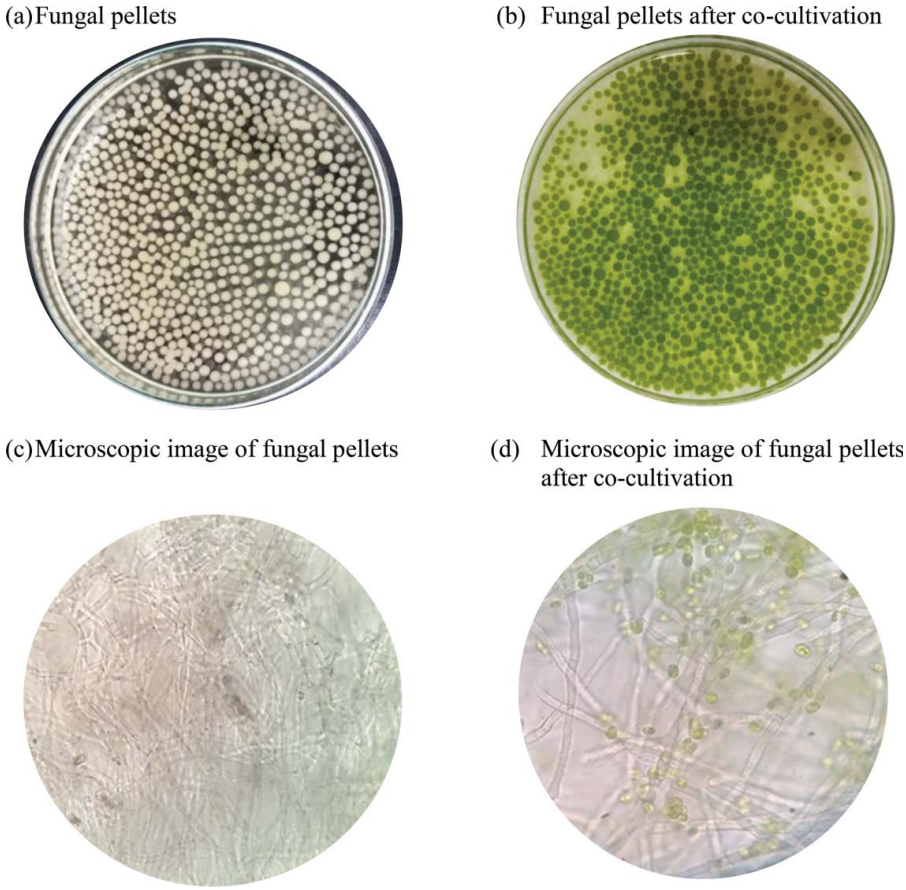

Figure 3. Photograph and microscopic image of fungal pellets before and after cocultivation with microalgae.

the pellets became much lighter and the approximate microalgal cells inside the pellets was (945 mg/500 mL =) 1.89 mg/mL. Therefore, the harvesting efficiency, harvesting time, and the density of microalgal cells inside the pellets could be manipulated by varying the volume ratio of fungal pellets to microalgae culture.

3.3. Phytoremediation of secondary effluent by immobilized cells

After harvesting of microalgal cells in the fungal pellets, the microalgae–fungal pellets were used as immobilized cells and applied for phytoremediation of effluent from seafood processing plants. The initial pH, COD, TN, and TP were 7.7, 1,239 mg/L,

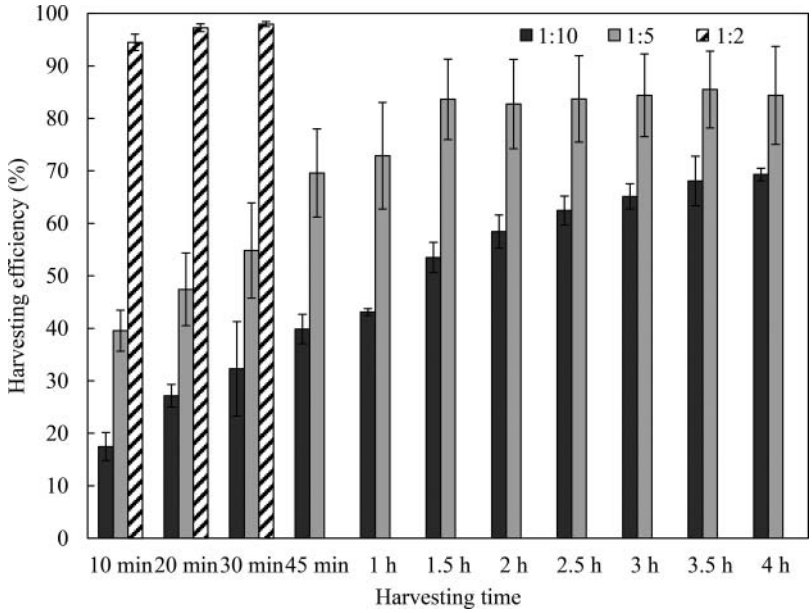

Figure 4. Effect of fungal pellets microalgae ratio on flocculation efficiency of *Scenedesmus* sp. by *T. reesei* QM 9414. Data are means of triplicates.

144 mg/L, and 18.6 mg/L, respectively. The microalgae–fungal pellets were inoculated in sterilized and nonsterilized effluent. Although it was not possible to evaluate the quantitative change of the microalgal cells inside the pellets, the pollutant removal and pH change during the cultivation of microalgae–fungal pellets were observed (Figure 5). As the concentration of COD was much higher than those of total nitrogen and total phosphorus, the removal efficiency for COD was monitored in detail and the removal efficiency of TN and TP were determined after cultivation for 7 days. The pH increased from 7.4 to above 8.5 and there was no significant difference between those of sterilized and nonsterilized effluent. The biomass of the immobilized cells slightly increased during cultivation, indicating that the immobilized cells could grow and use the nutrients in the effluents. These results were consistent with those studied by Wrede *et al.* (2014) and Miranda *et al.* (2015) who reported that the industrial wastewaters are suitable as low-cost media for cultivation of the microalgae–fungal pellets. The immobilized cells could reduce the COD better when using nonsterilized effluent (>74%) than sterilized effluent (>54%). The reduction of total nitrogen and total phosphorus were 63 mg/L and 18.6 mg/L, respectively. These corresponded to the removal efficiencies of 44% and 93%. The better removal efficiency of TP resulted from the much lower initial concentration of phosphorus (18.6 mg/L) compared to that of nitrogen (144 mg/L). It was possible that with nonsterilized effluent, other contaminated microorganisms might also use the nutrients in the effluent and contribute to a better pollutant removal. As the ratio of microalgae and fungi could not be quantitatively determined, the SEM analysis was performed to qualitatively determine the presence of microalgae and fungi in the pellets. Figure 6 shows the SEM images of the microalgae–fungi pellets cultivated in secondary effluent for 7 days. The SEM images show that the microalgal cells not only get entrapped within the fungal pellets but also get attached to the fungal hyphae with sticky exopolysaccharides possibly secreted by the fungi. Interestingly, the indigenous bacteria from the effluent also got attached to the fungal hyphae. In this consortia system, a symbiosis between microalgae and aerobic microorganisms including fungi and bacteria would occur. As the microalgae could generate oxygen through their photosynthesis, this could satisfy the oxygen requirement by aerobic microorganisms. While the aerobic microorganisms could produce CO_2 and serve as carbon source for microalgae growth (Jia and Yuan 2016). Similarly Cao *et al.* (2017) and Wang *et al.* (2017) also found that the immobilized microalgal cells in fungal pellets could remove pollutants from the industrial effluents but there was no report on their lipid production. Wrede *et al.* (2014) and Miranda *et al.* (2015) studied the entrapment of microalgal cells by *Aspergillus fumigatus* and the further use of microalgae–fungi pellets for removal of nitrogen and phosphorus in swine wastewater. However, the lipid yield in their study was less than 0.3 g/L.

3.4. Fatty acid composition of microalgae–fungal lipids and their biodiesel properties

The lipids extracted from the microalgae–fungal lipids cultivated under nonsterilized effluent were converted to fatty acid methyl ester (FAME) and their fatty acid compositions are shown in Table 2. The fatty acids profiles are composed mostly of palmitic acid (C16:0, 32.06%), oleic acid (C18:1, 24.51%), linolenic acid (C18:3, 14.16%), and arachidic acid (C20:0, 10.57%). These fatty acids have been reported to be favorable as biodiesel feedstocks (Knothe 2008; Srinuanpan *et al.* 2017). Similar compositions of FAMEs in the fungi–microalgae lipids have been reported (Mackey *et al.* 2015; Wrede *et al.* 2014; Miranda *et al.* 2015). The fatty acids found in those studies were also palmitic acid and oleic acid. The percentages of saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) observed in the microalgae–fungi lipids were 55.34 and 44.66, respectively. It is well-known that higher degrees of saturation would result in greater density and viscosity values of the subsequent fuel.

The important biodiesel properties such as IV, SV, CN, DU, LCSF, and CFPP that determine the quality of biodiesel are estimated based on the fatty acid compositions (Table 2). The SV is the value of KOH (mg) required for the saponification of 1 g of oil indicating the average molecular weight of fatty acids. The SV of the microalgae–fungi pellets was found to be 204.36 mg KOH/g oil. The IV is a measure of the total unsaturation of a biodiesel relating to its oxidative stability. According to the European biodiesel standard, IV is limited to 120 g I_2/100 g oil. The IV in this study was 71.64 g I_2/100 g oil which indicates the high stability against oxidation. The CN value refers to the ignition time delay of an engine and combustion quality. The

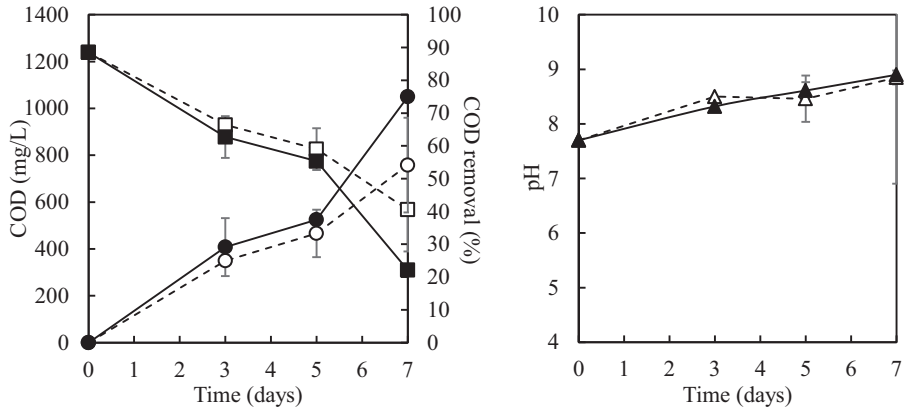

Figure 5. The COD removal by the microalgae–fungi pellets as immobilized cells for phytoremediation of sterilized and nonsterilized effluent. Data are means of triplicates.

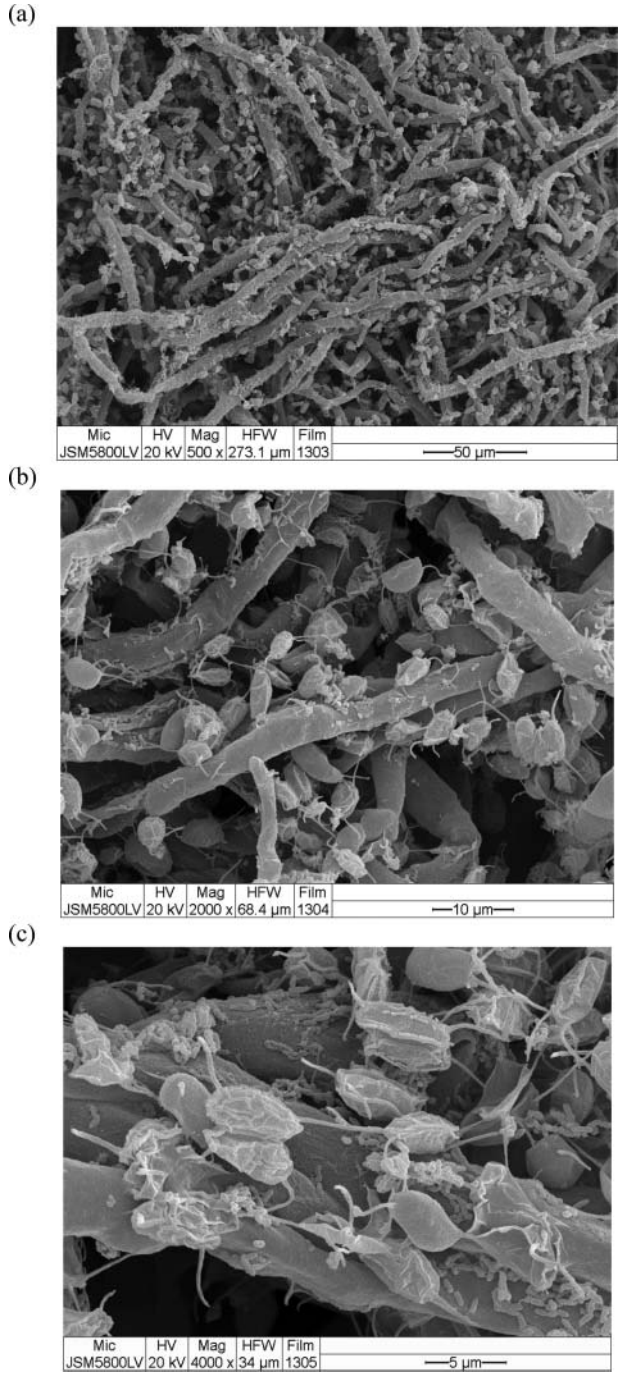

Figure 6. Scanning electron microscopy (SEM) image of microalgae–fungi pellets after 7 days of cultivation in nonsterilized effluent. (a) 500 × magnitude, (b) 2000 × magnitude and (c) 4000 × magnitude.

minimum value of CN for a biodiesel as per the standards is 47. The CN of this study was 56.89 which ensures better ignition properties and gives better engine performance as well as the reduction of white smoke formation from the engine (Karpagam *et al.* 2015). The DU value indicates the oxidative stability of a biodiesel pertaining to the stability of its long-term storage. The DU was found to be 64.84 which ensures more oxidatively stable in long-term storage (Talebi *et al.* 2013). LCSF and CFPP are another important biodiesel quality parameter typically used to predict the flow performance of a biodiesel at low temperatures. CFPP and LCSF were correlated with SFAs. The

Table 2. Fatty acid profiling and estimated fuel properties of fungi-microalgal lipids.

Fatty acids	Relative content (%)
Undecylic acid (C11:0)	0.15
Lauric acid (C12:0)	0.21
Tridecylic acid (C13:0)	1.57
Myristic acid (C14:0)	1.33
Pentadecylic acid (C15:0)	0.41
Palmitic acid (C16:0)	32.06
Heptadecanoic acid (C17:0)	2.19
Stearic acid (C18:0)	4.18
Oleic acid (C18:1)	24.51
Linoleic acid (C18:2)	5.99
Linolenic acid (C18:3)	14.16
Arachidic acid (C20:0)	10.57
Behenic acid (C22:0)	1.66
Lignoceric acid (C24:0)	1.01
C16–C18	83.09
Saturated fatty acid (SFA)	55.34
Unsaturated fatty acid (UFA)	44.66
Monounsaturated fatty acid (MUFA)	24.51
Polyunsaturated fatty acid (PUFA)	20.15
SFA/UFA	1.24
Estimated fuel properties	Values
Saponification value (SV)	204.36
Iodine value (IV)	71.64
Cetane number (CN)	56.89
Degree of unsaturation (DU)	64.81
Long chain saturation factor (LCSF)	20.38
Cold filter plugging point (CFPP)	47.54

SFAs of esters like stearic acid and palmitic acid are the first components to precipitate, and therefore biodiesel rich in SFAs attains high CFPP. Higher CFPP and LCSF indicate poor performance in low-temperature zones as the oil precipitates, blocking the filter (Karpagam *et al.* 2015). The CFPP and LCSF were found to be 20.38 and 47.54°C, respectively, which indicates poor performance in cold countries, but can be readily used in tropical regions.

4. Conclusions

This study has shown the effective method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi. With the suitable conditions, the microalgal cells could be harvested within 10 min. As this method requires low energy inputs and no use of chemicals, it is, therefore, also suitable for rapid immobilization of microalgal cells. The microalgae–fungal pellets have been proven to be effective in phytoremediation of nonsterile secondary effluent and easily recovered by a simple sieving method. The SEM images did show that the microalgae were not only entrapped in the pellets but also got attached to the fungal hyphae by exopolysaccharide. The extracted lipids from the microalgae–fungal pellets have similar fatty acid compositions with those of plant oil and could be used as biodiesel feedstocks. This study may contribute greatly to the industrialized microalgae based biofuel production.

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- Research and Researchers for Industries (RRI), the Thailand Research Fund (February 2015 – January 2019)

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- Visiting Research Scholar, Wageningen University and Research (Supported by the Thailand Research Fund) (April – December 2017)

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- Energy Conservation Promotion Fund, Ministry of Energy, Thailand.

Training

- The Classification of Algae and Plankton Training Course for 3 days during 25th – 27th June, 2014 at at Kasetsart University, Bangkok, Thailand.

- How to Write the Patent and Pretty Patent Course for 2 days during $10^{th} - 11^{th}$ May, 2018 at Prince of Songkla University, Songkhla, Thailand.
- The Searching Patent and Analysis of Technology Trend using Derwent Innovation Course for 2 days during $5^{th} - 6^{th}$ November, 2018 at Prince of Songkla University, Songkhla, Thailand.

Invited Speaker

-The Innovations of Microalgae Cultivation by Fermenter Training Course at Gibthai Training Center, Songkhla, Thailand on May 15, 2018.

Book Chapters

Srinuanpan, S., Cheirsilp, B. Integrated microalgae cultivation with biogas upgrading, production of biodiesel feedstock and phytoremediation of wastewater. In: Yousuf, A. (Ed). Microalgae Cultivation for Biofuels Production, Elsevier, London. (Submitted)

Mandik, Y.I.*, **Srinuanpan, S.***, Cheirsilp, B. Efficient harvesting of microalgae biomass and direct conversion of microalgal lipid into biodiesel. In: Yousuf, A. (Ed). Microalgae Cultivation for Biofuels Production, Elsevier, London. (Submitted; *Both authors contributed equally)

Publications

Tongprawhan, W., **Srinuanpan, S.**, Cheirsilp, B. 2014. Biocapture of CO_2 from biogas by oleaginous microalgae for improving methane content and simultaneously producing lipid. Bioresour. Technol. 170: 90-99.

Srinuanpan, S., Cheirsilp, B., Kitcha, W., Prasertsan, P. 2017. Strategies to improve methane content in biogas by cultivation of oleaginous microalgae and the evaluation of fuel properties of the microalgal lipids. Renew. Energy. 113: 1229-1241.

Srinuanpan, S., Cheirsilp, B., Prasertsan, P., Kato, Y., Asano, Y. 2018. Strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks: nutrient starvations and cost-effective harvesting process. Renew. Energy. 122: 507-516.

Srinuanpan, S., Cheirsilp, B., Prasertsan, P. 2018. Effective biogas upgrading and production of biodiesel feedstocks by strategic cultivation of oleaginous microalgae. Energy. 148: 766-774.

Srinuanpan, S., Chawpraknoi, A., Chantarit, S., Cheirsilp, B., Prasertsan, P. 2018. A rapid method for harvesting and immobilization of oleaginous microalgae using pellet-forming filamentous fungi and the application in phytoremediation of secondary effluent. Int. J. Phytoremediat. 20: 1017-1024.

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Srinuanpan, S., Cheirsilp, B., Boonsawang, P., Prasertsan, P. Immobilized oleaginous microalgae as effective two-phase purify unit for biogas and palm oil mill effluent and production of biodiesel feedstock. (Manuscript)

Conferences / Seminars

Tongprawhan, W., **Srinuanpan, S.**, Cheirsilp, B. 2014. CO_2 Capture in biogas by microalgae for lipid production. TRF-Master Research Congress VIII and RRI-MAG Congress I. The Twin Tower Hotel, Bangkok, Thailand. April 3-5, 2014. (Oral presentation)

Srinuanpan, S., Cheirsilp, B. 2015. Optimization of CO_2 removal from biogas coupling with lipid production by oleaginous microalgae cultivation. The 6th International Conference on Fermentation Technology for Value Added Agricultural Products. Centara Hotel & Convention Center, Khon Kaen, Thailand. July 29-31, 2015. (Oral presentation)

Srinuanpan, S., Cheirsilp, B. 2015. Response surface methodological approach to optimize operating conditions for CO_2 removal from biogas and simultaneously lipid production by oleaginous microalgae. The 2nd Biotech, UPM-PSU joint seminar 2015. Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. August 12-14, 2015. (Oral presentation)

Srinuanpan, S., Cheirsilp, B. 2016. Process development of CO_2 removal from biogas coupling with lipid production by microalgae cultivation. RRi WU Forum 2016. Walailak University, Nakhon Si Thammarat, Thailand. November 27, 2016. (Oral presentation)

Srinuanpan, S., Cheirsilp, B., Boonsawang, P., Prasertsan, P. 2018. Biogas upgrading and phytoremediation of palm oil mill effluent after biogas digester by immobilized oleaginous microalgae. The Water and Environment Technology Conference 2018 (WET2018). Ehime University, Matsuyama, Japan. July 14-15, 2018. (Oral and poster presentation)