Potential Use of Industrial Wastes for Low-cost Production of Single Cell Oils and Analysis of Techno-Economical Feasibility and Environmental Impact

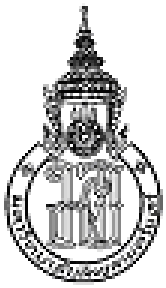
Saithip Sae-ngae

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Environmental Management

Prince of Songkla University

2018



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

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

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ชื่อวิทยานิพนธ์ การเพิ่มศักยภาพการใช้ประโยชน์วัสดุเศษเหลือโรงงานอุตสาหกรรมเพื่อผลิตน้ำมันเซลล์เดียวต้นทุนต่ำ และการวิเคราะห์ความเป็นไปได้เชิงเศรษฐศาสตร์และผลกระทบต่อสิ่งแวดล้อม

ผู้เขียน นางสาวสายทิพย์ แซ่แง่

สาขาวิชา การจัดการสิ่งแวดล้อม

ปีการศึกษา 2561

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาศักยภาพของวัสดุเศษเหลือโรงงานอุตสาหกรรมในการนำมาผลิตเป็นน้ำมันเซลล์เดียวที่มีต้นทุนต่ำ โดยศึกษาวัสดุเศษเหลือที่มีปริมาณมากจากอุตสาหกรรมน้ำอัดลม อุตสาหกรรมการผลิตเบียร์ และอุตสาหกรรมการผลิตไบโอดีเซล น้ำอัดลมหมดอายุเป็นของเสียจากอุตสาหกรรมน้ำอัดลมมีองค์ประกอบหลักของ น้ำตาลฟรักโทส (ร้อยละ 59.80) น้ำตาลกลูโคส (ร้อยละ 27.46) และ น้ำตาลซูโครส (ร้อยละ 12.73) ซึ่งเป็นแหล่งน้ำตาลที่เหมาะสมสำหรับกระเพาะเลี้ยงยีสต์ จากการเปรียบเทียบการเจริญและการผลิตไขมันของยีสต์หลายสายพันธุ์ในน้ำอัดลมหมดอายุ พบว่ายีสต์สายพันธุ์ *Trichosporonoides spathulata* JU4-57, *Yarrowia lipolytica* TISTR 5151 และยีสต์สีแดง *Rhodotorula mucilaginosa* G43 สามารถเจริญและสะสมไขมันได้มากกว่าร้อยละ 30 ของน้ำหนักเซลล์แห้ง สำหรับอุตสาหกรรมผลิตเบียร์มีวัสดุเศษเหลือที่เป็นของแข็ง 2 ชนิด คือ กากเบียร์ที่ได้จากขั้นตอนแรกของการบ่มข้าวบาร์เลย์หรือข้าวมอลต์ และกากยีสต์ที่ได้จากขั้นตอนสุดท้ายของการหมักด้วยยีสต์ ซึ่งทั้งกากเบียร์และกากยีสต์ต้องย่อยด้วยกรดเพื่อเปลี่ยนเป็นน้ำตาลก่อนใช้เป็นแหล่งสารอาหารในการเพาะเลี้ยงยีสต์ โดยน้ำตาลที่ได้จากการย่อยกากเบียร์ประกอบด้วย น้ำตาลไซโลส (ร้อยละ 45.83±1.53) และน้ำตาลอะราบิโนส (ร้อยละ 32.13±2.3) จากส่วนที่เป็นไซแลนของเฮมิเซลลูโลส และน้ำตาลกลูโคส (ร้อยละ 22.0±0.8) จากส่วนของเซลลูโลส ส่วนน้ำตาลที่ได้จากการย่อยกากยีสต์ประกอบด้วย น้ำตาลแมนโนส (ร้อยละ 69.57±1.04) และน้ำตาลกลูโคส (ร้อยละ 31.43±0.38) ผลการทดลองพบว่ายีสต์ทุกสายพันธุ์สามารถเจริญเติบโตได้ดีในน้ำย่อยจากกากเบียร์และกากยีสต์ แต่มีการสะสมไขมันต่ำในช่วงร้อยละ 8-17 เนื่องจากในน้ำย่อยมีปริมาณไนโตรเจนสูง ทำให้ยีสต์ใช้แหล่งคาร์บอนในการเจริญมากกว่าใช้ในการสะสมไขมัน สำหรับอุตสาหกรรมการผลิตไบโอดีเซลมีผลพลอยได้ที่เป็นกลีเซอรอลดิบ เมื่อใช้เพาะเลี้ยงยีสต์ทุกสายพันธุ์ พบว่ามีเพียงยีสต์ *R. mucilaginosa* G43 สามารถเจริญเติบโตและสะสมไขมันได้มากกว่าร้อยละ 50 โดยน้ำหนักเซลล์แห้ง ในขณะที่ยีสต์ *T. spathulata* JU4-57 และ *Y. lipolytica* TISTR 5151 เจริญเติบโตได้น้อยกว่า นอกจากนี้ยังพบว่ายีสต์ *R. mucilaginosa* G43 ยังสามารถผลิตสารสีแดงที่เรียกว่าแคโรทีนอยด์ได้เท่ากับ 3.78±0.29 มิลลิกรัมต่อกรัมเซลล์ และมีโปรตีนเป็นองค์ประกอบร้อย

ละ 16.46±0.17 ดังนั้นยีสต์สายพันธุ์นี้จึงไม่เพียงแต่เป็นน้ำมันเซลล์เดียวได้ แต่ยังสามารถเป็นใช้แหล่งของสารสีและโปรตีนเซลล์เดียวได้อีกด้วย

จากการศึกษาการผลิตน้ำมันเซลล์เดียวจากน้ำอัดลมหมดอายุโดยการเพาะเลี้ยงแบบกึ่งกะได้เพาะเลี้ยงยีสต์สายพันธุ์ *Y. lipolytica* TISTR 5151 และเพิ่มปริมาณน้ำตาลระหว่างการเลี้ยงโดยทำการเติมน้ำอัดลมหมดอายุทุก 24 ชั่วโมง พบว่าการเพาะเลี้ยงแบบกึ่งกะสามารถเพิ่มการสะสมไขมันได้สูงสุดร้อยละ 40-46 ในช่วงเวลา 24-48 ชั่วโมง และจากการศึกษาการผลิตน้ำมันเซลล์เดียวจากกลีเซอรอลดิบโดยการเพาะเลี้ยงแบบกึ่งกะ ได้เพาะเลี้ยงยีสต์ *R. mucilaginosa* G43 และเติมกลีเซอรอลดิบระหว่างการเลี้ยงทุก 36 ชั่วโมง พบว่าการเพาะเลี้ยงแบบกึ่งกะไม่ได้สนับสนุนการเจริญและผลิตไขมันของยีสต์อย่างมีนัยสำคัญ ซึ่งอาจเนื่องมาจากเกิดการสะสมของสารยับยั้งที่อยู่กลีเซอรอลดิบ

ดังนั้นสรุปได้ว่ากลีเซอรอลดิบและยีสต์ *R. mucilaginosa* G43 เป็นแหล่งสารอาหารและสายพันธุ์ยีสต์ที่เหมาะสมที่ให้การผลิตน้ำมันเซลล์เดียวสูงสุด แต่การเพาะเลี้ยงแบบกึ่งกะไม่มีผลการผลิตน้ำมันเพิ่มขึ้น ดังนั้น จึงเลือกการเพาะเลี้ยงแบบกะซ้ำเพื่อขยายขนาดการผลิตในถังหมักปฏิกรณ์ชีวภาพขนาด 2 ลิตร โดยปริมาตรทำงานจริง 1 ลิตร เปลี่ยนถ่ายอาหารเลี้ยงเชื้อร้อยละ 80 ทุก 48 ชั่วโมง ทำการเพาะเลี้ยงซ้ำทั้งหมด 5 ครั้ง รวมเป็นระยะเวลาการเพาะเลี้ยงทั้งสิ้นเท่ากับ 228 ชั่วโมง ผลการทดลองพบว่ายีสต์มีการเจริญเติบโตและสะสมไขมันได้ 4 รอบ และสามารถผลิตไขมันได้สูงสุดเท่ากับ 2.52±0.03 กรัมต่อลิตร นอกจากนี้ยังพบว่าสามารถผลิตสารสีแดงที่เรียกว่าแคโรทีนอยด์ได้สูงสุดในรอบที่ 3 เท่ากับ 21.85±0.58 มิลลิกรัมต่อกรัมเซลล์ และจากการวิเคราะห์องค์ประกอบของน้ำมันเซลล์เดียวพบว่าส่วนใหญ่เป็นกรดไขมันสายยาวที่มีคาร์บอน 16 และ 18 อะตอม ซึ่งคล้ายคลึงกับองค์ประกอบของน้ำมันพืช จึงมีศักยภาพในการนำมาใช้เป็นวัตถุดิบเพื่อผลิตไบโอดีเซลได้

นอกจากนี้งานวิจัยได้ทำการวิเคราะห์ศักยภาพเชิงเทคนิคเศรษฐศาสตร์ และผลกระทบทางด้านสิ่งแวดล้อมของการปลดปล่อยก๊าซเรือนกระจกของกระบวนการผลิตไบโอดีเซลที่ใช้น้ำมันเซลล์เดียวที่เลี้ยงด้วยวัสดุเศษเหลือจากโรงงานอุตสาหกรรมแต่ละประเภท ตั้งแต่ขั้นตอนการเตรียมวัสดุเศษเหลือ การเลี้ยงยีสต์ การเก็บเกี่ยว และการผลิตไบโอดีเซลผ่านกระบวนการทรานสเอสเทอริฟิเคชันโดยตรง ผลการวิเคราะห์พบว่ากลีเซอรอลดิบเป็นวัสดุเศษเหลือที่มีศักยภาพมากที่สุดที่จะนำมาเลี้ยงยีสต์และผลิตเป็นไบโอดีเซล เนื่องจากในการผลิตระดับ 1,000 ลิตร การใช้กลีเซอรอลดิบทำให้สามารถผลิตไบโอดีเซลได้สูงสุดเท่ากับ 2.02 กิโลกรัม รองลงมาคือ กากเบียร์ (1.46 กิโลกรัม), กากยีสต์ (0.68 กิโลกรัม) และน้ำอัดลมหมดอายุ (0.64 กิโลกรัม) เมื่อพิจารณาค่าใช้จ่ายตลอดกระบวนการ พบว่าการผลิตไบโอดีเซลโดยใช้กากยีสต์เป็นแหล่งอาหารในการเพาะเลี้ยงยีสต์มีค่าใช้จ่ายสูงสุดเท่ากับ 9.34 ดอลลาร์ต่อกิโลกรัมไบโอดีเซล รองลงมาคือ กากเบียร์ (4.34 ดอลลาร์ต่อกิโลกรัมไบโอดีเซล) น้ำอัดลมหมดอายุ (4.29 ดอลลาร์ต่อกิโลกรัมไบโอดีเซล) ในขณะที่การผลิตไบโอ

ดีเซลโดยใช้กลีเซอรอลดิบเป็นแหล่งอาหารในการเพาะเลี้ยงยีสต์มีค่าใช้จ่ายตลอดกระบวนการน้อยที่สุดเท่ากับ 1.36 ดอลลาร์ต่อกิโลกรัมไบโอดีเซล และพบว่ากระบวนการผลิตไบโอดีเซลจากกากยีสต์มีค่าการปลดปล่อยก๊าซคาร์บอนไดออกไซด์สูงสุดเท่ากับ 52.84 กิโลกรัมคาร์บอนไดออกไซด์เทียบเท่า รองลงมาคือ กากเบียร์ (24.60 กิโลกรัมคาร์บอนไดออกไซด์เทียบเท่า) น้ำอัดลมหมดอายุ (21.90 กิโลกรัมคาร์บอนไดออกไซด์เทียบเท่า) ในขณะที่กลีเซอรอลดิบมีค่าการปลดปล่อยก๊าซคาร์บอนไดออกไซด์น้อยสุดมีค่าเท่ากับ 6.93 กิโลกรัมคาร์บอนไดออกไซด์เทียบเท่า จะเห็นได้ว่าการปลดปล่อยก๊าซคาร์บอนไดออกไซด์จะขึ้นอยู่ขั้นตอนที่ต้องใช้ในการเตรียมวัตถุดิบและผลผลิตไบโอดีเซลที่ได้ เนื่องจากกากยีสต์และกากเบียร์ต้องผ่านกระบวนการย่อยก่อนนำมาใช้และการผลิตไบโอดีเซลจากกากยีสต์ได้ผลผลิตต่ำ จึงทำให้มีการปลดปล่อยก๊าซคาร์บอนไดออกไซด์ต่อหน่วยการผลิตไบโอดีเซลสูงที่สุด

งานวิจัยนี้ได้แสดงให้เห็นถึงแนวทางในการผลิตเชื้อเพลิงชีวภาพโดยใช้จุลินทรีย์ที่มีต้นทุนต่ำ ซึ่งคาดว่าจะสามารถพัฒนาความยั่งยืนด้านเทคนิค เศรษฐศาสตร์ และผลกระทบต่อสิ่งแวดล้อมให้กับอุตสาหกรรมเชื้อเพลิงชีวภาพและอุตสาหกรรมเกษตรและชีวภาพของประเทศไทย

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Author	Miss Saithip Saengae
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ABSTRACT

This study aimed to evaluate the potential use of agro-industrial wastes for low-cost production of single cell oil. Different types of abundant wastes from soft drinks industry, brewery industry and biodiesel industry were compared. Expired soft drinks (ES) is the waste from soft drinks industry. The sugar compositions in the ES were: fructose (59.80 %), glucose (27.46 %) and sucrose (12.73 %), which are good nutrient sources for yeast cultivation. Among oleaginous yeasts tested, *Trichosporonoides spathulata* JU4-57, *Yarrowia lipolytica* TISTR 5151 and a red yeast *Rhodotorula mucilaginosa* G43 could grow on ES and accumulate considerably high lipid content >30%. Brewers' spent grain (BSG) and spent yeast cells (SYC) are solid wastes from malting process and final yeast fermentation, respectively. Both wastes have to be acid hydrolyzed into fermentable sugars before use as nutrient sources for yeast cultivation. The BSG hydrolysate was composed of xylose (45.83±1.53%) and arabinose (32.13±2.3%) from arabinoxylan in hemicellulose and glucose (22.02±0.8%) from cellulose. While main sugars found in the SYC hydrolysate, were mannose (69.57±1.04%) and glucose (31.43±0.38%). All the selected yeasts could grow on BSG and SYC hydrolysate but accumulated low amount of lipids (8-17%) possibly due to the high nitrogen content, which stimulated cell growth rather than lipid accumulation. When cultivated on crude glycerol (CG) from biodiesel industry, only yeast *R. mucilaginosa* G43 grew well on CG and accumulated lipids >50%. While *T. spathulata* JU4-57 and *Y. lipolytica* TISTR 5151 could not grow well and gave relatively low biomass. In addition to lipids, *R. mucilaginosa* G43 also contained red pigment called carotenoids at 3.78±0.29 mg/g-cell and protein at 16.46±0.17 %w/w. It then has high potential not only as single cell oil but also as a source of pigment and protein.

Fed-batch fermentation of ES by *Y. lipolytica* TISTR 5151 was performed. The ES was intermittently added every 24 h to increase the carbon source availability. The lipid content increased up to the maximum level of 40-46% during 24-48 h. Fed-batch fermentation of CG by *R. mucilaginosa* G43 was also attempted by adding CG every 36 h. However, the fed-batch fermentation of CG did not significantly increase yeast cell growth and lipid production possibly due to the accumulation of inhibitors from CG.

From the above experiment, it could be concluded that crude glycerol and *R. mucilaginosa* G43 are suitable nutrient source and yeast strain for production of single cell oil. As fed-batch fermentation did not increase the lipid production by the yeast, the repeated batch fermentation was then chosen for scaling up in 2 L-bioreactor with 1-L working volume and the culture broth was replaced with the fresh medium at 80% replacement rate every 48 h. Five cycles of batch cultivation were repeated and the fermentation proceeded for 228 h. The yeast could grow and accumulate lipids in four cycles and the highest lipid obtained was 2.52±0.03 g/L. It was of interest that the carotenoid content in the cells from the third cycle was highest at 21.85±0.58 mg/g-cell. The fatty acid compositions of single cell oil are long chain fatty acids with 16-18 carbon atoms which are similar to those of plant oils. It then has potential to be used as biodiesel feedstocks.

In addition, this study also performed the techno-economic analysis (TEA) and assessments of environmental impact by greenhouse gas emission (GHG) from bioconversion of each agro-industrial waste into biodiesel. The process included waste preparation, yeast cultivation, harvesting and production of biodiesel via direct transesterification. TEA indicates that CG was the most suitable waste for yeast cultivation and biodiesel production because in 1,000 L production scale CG could give the highest biodiesel 2.02 kg followed by BSG (1.46 kg), SYC (0.68 kg) and ES (0.64 kg). The economic assessment shows the highest production costs involved in the case of SYC (9.34 $/kg-biodiesel) followed by SYG (4.34 $/kg-biodiesel) and ES (4.29 $/kg-biodiesel). While CG requires the lowest production costs of 1.36 $/kg-biodiesel. The highest CO_2 emissions are from the case of SYC (52.84 kg CO_2-eq) followed by BSG (24.60 kg CO_2-eq), ES (21.90 kg CO_2-eq) and CG (6.93 kg CO_2-eq). The CO_2 emissions depend on the steps involved in the process and the yield of biodiesel. As

SYC and BSG needed acid hydrolysis step before use and gave low yield of biodiesel, the CO_2 emission per 1 kg biodiesel was then higher than those from other wastes.

This study has shown the promising approach for cost-effective production of microbial based biofuels and may also contribute to the techno-economic and environmental sustainability of the biofuel industries and the agro-bio industries in Thailand.

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Saithip Sae-ngae

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CHAPTER 1

INTRODUCTION

Single cell oils (SCO) produced by oleaginous microorganisms are considered as potential feedstocks for biodiesel production due to their similar fatty acid compositions to that of plant oils. The cultures of oleaginous microorganisms have advantages over the production of plant oils because they are affected neither by seasons nor by climates, have shorter life cycle, and are easier to scale up (Li *et al.*, 2007). In addition to single cell oils, there are efforts to explore the new alternate such as protein sources as food supplements, primarily to solve the problem of a repeatedly predicted insufficient future protein supply. Several microorganisms are defined as single cell protein (SCP) due to their high protein content and it can be directly used as a source of protein in food and feed (Becker, 2007; Yadav *et al*., 2016).

Among available microorganisms as SCO and SCP, yeasts are more suitable due to their unicellular relatively high growth rate and ability to use low-cost fermentation media such as nutritional residues from agriculture and industry (Meng *et al*., 2009). To reduce the production costs, many studies have focused on using organic wastes instead of high grade substrates for microorganism cultivation (Soccol *et al.*, 2017). Although there are several low-cost industrial wastes that have been explored for cultivation of yeasts, there are still unexplored wastes for this purpose. These include expired soft drinks from the beverage and soft drink industry, spent grain and spent yeast from brewing industry. As soft drinks contain high amounts of sugars such as glucose, fructose and sucrose, when left unsold and expired they cannot be disposed directly into general wastewaters and cannot be treated immediately. The expired soft drinks (ES) having high content of sugars and acids are stored in a storage tank before releasing into the wastewater treatment system. Moreover, the acid content in the expired soft drinks is corrosive to the storage tanks (Arous *et al*., 2016). Brewers' spent grain (BSG) and spent yeast cell (SYC) are the wastes from brewery industry. BSG is the insoluble fraction of wort separated after the mashing phase of beer production. It is the main by-product from brewing process and it is mainly composed of lignocellulosic materials (Vieira *et al.*, 2017; Celus *et al.*, 2006). There are 15-20 kg of

BSG produced per every hectoliter of beer and an annual production of over 30 million ton of BSG worldwide. SYC is the yeast cells separated after beer fermentation. It is abundant, low-cost biomass, rich in carbohydrate, protein and precursors for the food chemical and energy industry (Mussatto *et al.*, 2006; Niemi *et al.*, 2013; Ryu *et al.*, 2013). Another organic waste that has attracted special attention as it is an unavoidable by-product of biodiesel production is crude glycerol. The biodiesel industry generates approximately 10% (w/w) of glycerol with every batch of biodiesel produced (Uprety *et al*., 2017). The utilization of crude glycerol for production of valuable products not only offset the production cost of biodiesel but also recycle the by-product as renewable feedstock and lead to a zero-waste discharge process (Chen *et al*., 2017). In addition, it has been reported that crude glycerol could be directly used by the microorganisms without requirement of purification steps (Qin *et al.*, 2017).

To carry out sustainable biofuel production especially the biodiesel production from microorganism, life-cycle greenhouse gas analysis has been used to compare the energy usage and environmental impact of biofuels and baseline fossil fuels (Wang *et al*., 2011). However, the emission of carbon dioxide from fossil fuel production plants and the consequent effect on the global environment is a major that must concern. To solve the climate problem, methods for managing and reducing these emissions must be found and implemented. The renewable energy source including the source from biomass should use instead the fossil fuels have therefore key role in reducing CO_2 emissions. The combustion from biomass is the main process for energy conversion from this renewable fuel and considered to produce no net CO_2 emissions from this process that this due to the CO_2 emissions was captured by the plants (Huang *et al*., 2013; Thornley *et al*., 2009). The information from economic, GHG emissions and energy analysis could be useful to provide information for policy makers and producers in choosing sustainable management systems or products.

This study aimed to evaluate the potential use of agro-industrial wastes for low-cost production of single cell oil (SCO) and single cell protein (SCP). Potent yeast strains were cultivated on the wastes and screened for their abilities as either SCO or SCP. The process optimization and scale up in bioreactor were attempted to increase the productivity by the selected strains. The biodiesel fuel properties of SCO were evaluated based on their fatty acid compositions. To evaluate the industrial feasibility,

the techno-economic analysis and greenhouse gas emissions of biodiesel production from agro-industrial wastes by oleaginous yeasts were performed. Alternative processing techniques are considered dependently on mass balance of lipid production. An optimized case, which considers the benefits of all the alternative technologies was then proposed.

Objectives of the study

1. To use agro-industrial wastes as low-cost nutrient sources for cultivation of potent yeasts as single cell oils.
2. To increase the cell growth and lipid productivity by process optimization.
3. To scale-up lipid production in bioreactor and operate under optimized conditions.
4. To convert lipids into biodiesel and evaluate its biodiesel fuel properties.
5. To perform technical, economical, and environmental analysis of the proposed processes.

CHAPTER 2

LITERATURE REVIEWS

1. Single cell oils

Single cell oils (SCO) represent oleaginous microorganisms, including yeast, fungi, bacteria, and microalgae with 20% excess lipid content (Meng *et al*., 2009). Most of them can synthesize and accumulate lipids in the form of triacylglycerols (TAGs) in which fatty acid compositions are similar to those of vegetable oils as shown in Table 1 (Li *et al*., 2008). Numerous studies have reported a large number of oleaginous yeasts and microalgae that can grow and accumulate significant amounts of lipids in which the compositions are similar to those of vegetable oils. In recent years, many oleaginous microorganisms have been reported as potent feedstocks for biodiesel production. The amounts of lipid accumulation in microorganisms are determined by their genetic makeup, as maximum obtainable lipid contents greatly differ within species and even among individual strains. In addition to strains, the lipid content and composition are also varied depending on culture conditions including nutrient source, temperature, pH and cultivation time (Papanikolaou *et al*., 2004). For example, *Crptococcus curvatus* can store lipids up to 60% on a dry cell weight basis when grown under limited nitrogen condition. Triacylglycerols makeup 90% w/w of these lipids with 44% of saturated fatty acids (% SFA) – same as several plant seed oils. Molds and oleaginous yeasts store in their cells triacylglycerols particularly rich in polyunsaturated fatty acids (PFA) or compounds with specific structures (Kavadia *et al.*, 2001) containing limited: oleic (18:1) and linoleic (18:2) acids and palmitic (16:0) or palmitoleic acids (C16:1) (Meng *et al*., 2009) (Table 2).

Table 1 Oil content of microorganisms

Microorganisms	Oil content (% dry wt)	Microorganisms	Oil content (% dry wt)
Microalgae		Yeast	
-*Botryococcus braunii*	25–75	- *Candida curvata*	58
- *Cylindrotheca* sp.	16–37	- *Cryptococcus albidus*	65
- *Nitzschia* sp.	45–47	- *Lipomyces starkeyi*	64
- *Schizochytrium* sp.	50–77	- *Rhodotorula glutinis*	72
Bacterium		Fungi	
-*Arthrobacter* sp.	>40	-*Aspergillus oryzae*	57
-*Acinetobacter*	27–38	-*Mortierella isabellina*	86
calcoaceticus	24–25	-*Humicola lanuginosa*	75
-*Rhodococcus opacus*	18–24	-*Mortierella vinacea*	66
-*Bacillus alcalophilus*			

Source: Meng *et al*. (2009)

Table 2 Lipid composition of microorganisms

Microorganisms	Lipid composition (% total lipid)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Microalga	12–21	55–57	1–2	58–60	4–20	14–30
Yeast	11–37	1–6	1–10	28–66	3–24	1–3
Fungi	7–23	1–6	2–6	19–81	8–40	4–42
Bacterium	8–10	10–11	11–12	25–28	14–17	–

Source: Meng *et al.* (2009)

Large amounts of lipids, produced by oleaginous yeasts, consist of C16 to C18 fatty acids that are favorably suitable for use as biodiesel. Thus, the organic wastes utilization by oleaginous yeasts combined with lipid production can be a cost-effective technology to substitute fossil diesel with eco-friendly biodiesel. Cho *et al.* (2018) have studied oleaginous yeasts such as *Yarrowia* spp., *Cryptococcus* spp., *Lipomyces* spp.,

Rhodotorula spp., and *Rhodosporidium* spp. and reported their utilization for microbial lipid production as they have shown exceptional traits to consume various organic substrates and stably produced lipids within a short period of time as shown in Table 3.

Table 3 Lipids production by different oleaginous yeasts

Species	Carbon sources	Lipid content (%)
Cryptococcus curvatus	Glycerol	25
Lipomyces starkeyi	Glucose and xylose	61
Rhodosporidium toruloides Y4	Glucose (Batch culture)	48
Rhodosporidium toruloides Y4	Glucose (Fed-batch culture)	67.5
Rhodotorula glutinis	Monosodium glutamate wastewater	20
Trichosporon fermentans	Glucose	62.4
Yarrowia lipolytica	Industrial glycerol	43

Source: Liang *et al.* (2013)

2. Lipids synthesis and accumulation in oleaginous yeasts

As compared to non-oleaginous yeasts like *Saccharomyces cerevisiae* and *Candida ultilis* which cannot accumulate and store lipids more than 10%, oleaginous yeasts can accumulate lipids as droplets which may account for 70% of their biomass. (Papanikolaou *et al*., 2012). When these yeasts are grown in carbon rich and nitrogen limited medium, the composition of mannans and glucans increases, whereas oleaginous yeasts converts the surplus carbon source into lipids. Reports have shown that, nitrogen depletion in the culture medium triggers the activation of adenosine monophosphate deaminase in oleaginous yeast which catalyzes the deamination of AMP into inosine 5′-monophosphate and ammonium as shown in Figure 1. In addition, the depletion of AMP concentration renders the inactivation of isocitrate dehydrogenase leading to the inhibition of TCA cycle. In the cytoplasm oleaginous

yeast, citrate gets cleaved by ATP: citrate lyases (ACL), and translocate to cytosol through malate/citrate translocase system. Acetyl-Co-A from this reaction gets acted upon by acetyl-Co A carboxylase enzyme to form malonyl-Co A. In the de-novo synthesis of lipids, condensation of both acetyl-Co A and malonyl-Co A result in the formation of fatty acid chains between 14 and 16 carbon long. Interestingly, most of the non-oleaginous microorganisms lack the enzyme ACL, resulting in the synthesis of triacylglycerols as shown in Figure 2. The effect of N-limited condition lets oleaginous yeasts to usually accumulate high lipid content in its cellular compartment (Patel *et al*., 2017).

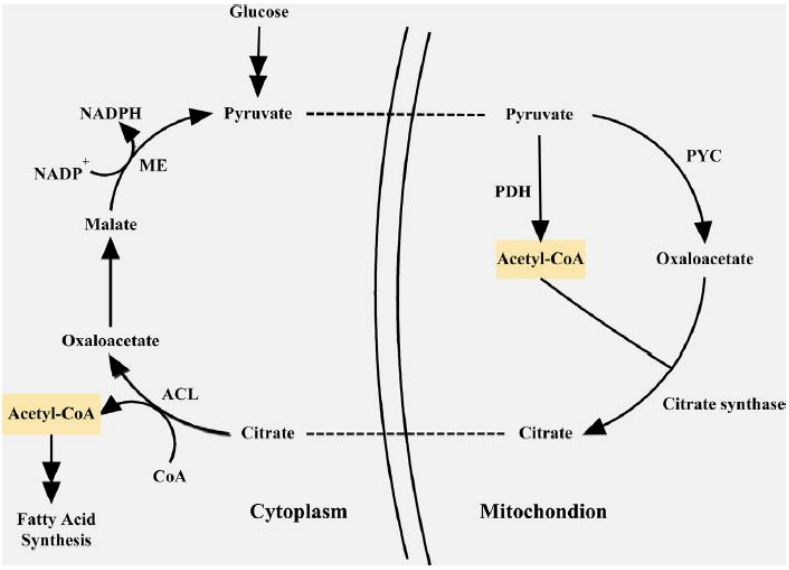

Figure 1 Citrate-pyruvate metabolic shuttle pathway in eukaryotic microbes. This system transfer acetyl groups as citrate to cytoplasm from mitochondrion where they are transported as acetyl-CoA groups for fatty acid biosynthesis. PDH: pyruvate dehydrogenase; PYC: pyruvate carboxylase; ACL: ATP:citrate lyase; ME: Malic enzyme.

Source: Liang *et al*. (2013)

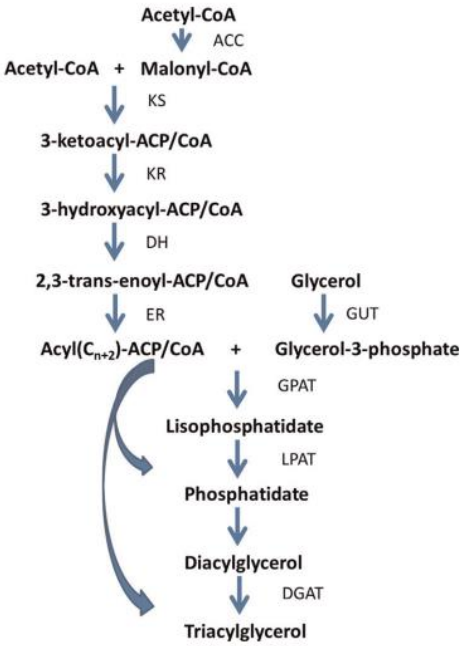

Figure 2 Schematic diagram of fatty acid biosynthetic pathway, fatty acid chain elongation and storage as TAGs in yeast cells. ACC: acetyl-CoA carboxylase; KS: 3-ketoacyl synthase; KR: 3-ketoacyl reductase; DH: enoyl dehydratase; ER: enoyl reductase; GUT: glycerol kinase; GPAT: glycerol-3-phospate acyltransferase; LPAT: lysophosphatidic acid acyltransferase; DGAT: diacylglycerol acyltransferase.

Source: Tang *et al.* (2015)

In most microorganisms, the elongation of fatty acids is catalyzed by the action of two enzyme systems, acetyl-CoA carboxylic enzyme (ACC) and fatty acid synthase (FAS). Figure 3 ACC catalyzes the formation of malonyl-CoA from acetyl-CoA and enables acyl chain elongation. This is followed by the formation form malonyl-acyl-carrier protein (malonyl-ACP) by malonyl-CoA: ACP transacetylase (MAT), a fatty acid synthase (FAS) multi-enzymatic complex subunits. The FAS system uses malonyl-acyl-carrier protein (malonyl-ACP) as a carbon source to synthesize long chain fatty acids, mainly C16 and C18, palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). Other fatty acids like GLA, ARA,

EPA, DHA, are known to be found too. A β-ketoacyl-ACP synthase (KAS) initiates each cycle of C2 addition (Fakas *et al.*, 2009) and involves the condensation of a malonyl-ACP with an acyl acceptor, which will be acted upon b acyl-ACP thioesterase (FAT) on the acyl chain to liberate the fatty acid (Liang *et al.*, 2013)

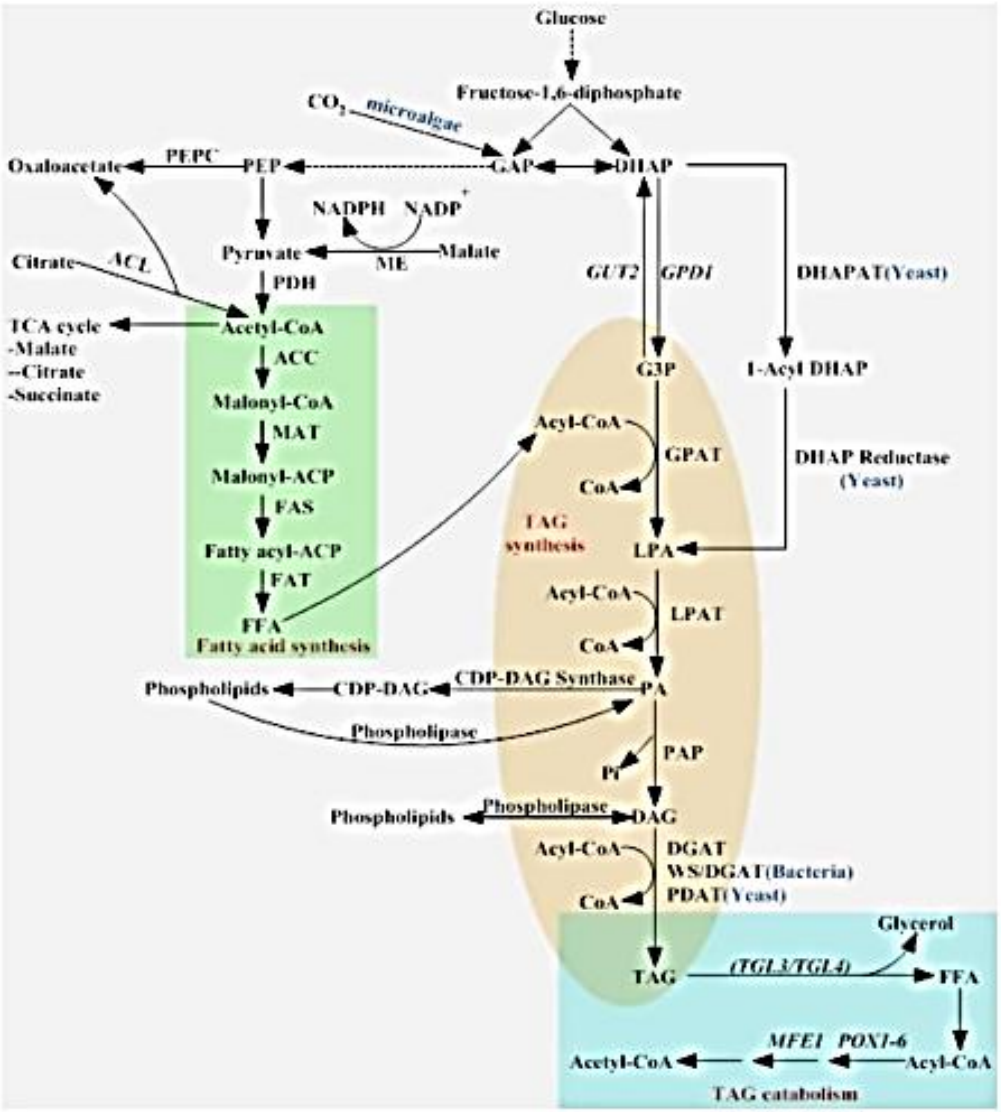

Figure 3 The fatty acid and TAG biosynthesis pathway in microorganisms
Source: Liang *et al*. (2013)

Most of microorganisms can efficiently use some common sugars especially glucose, as the substrate for growth and production of various metabolites and enzymes. Oleaginous yeasts utilize sugar based media such as mono and disaccharides (glucose, fructose xylose lactose and sucrose) as substrate for de novo lipid synthesis. Hydrophobic carbon source such as plant oil, fatty acid by product or wastes of fatty ester have been considered as substrate for ex novo lipid synthesis (Xie, 2017) (Figure 4). Oleaginous yeasts also assimilate various sugar substrates derived from renewable resource including C6 sugars (glucose, fructose, mannose, and galactose) but there may be some difference in the specific growth rate and consumption rate for each sugar substrate. Flores et al. (2000) reported that oleaginous yeast Y. lipolytica could use monomers of sugar such as C5 and C6 sugars in industrial after pretreated. The general and popular carbon source is glucose for industrial fermentation. As glucose is one of the major cost contributors the total cost can be significantly reduced and the oleaginous yeast can directly use the material of polysaccharide (Xie, 2017). When cultivated Y. lipolytica on mix carbon source (glucose, fructose and sucrose) it was found that glucose is more preferred than fructose and sucrose (Lazar et al., 2011). The carbon source of sugar could be cleaved by an extracellular invertase into glucose and fructose before used by the oleaginous yeasts.

For the hydrophobic substrate, the first step of substrate utilization is the breakdown of lipid often exists in the form of triacylglycerol (TAG) into fatty acids and glycerol by the catalysis of extracellular lipases so that they can be utilized (Thevenieau et al., 2010). The intracellular fatty acids can be degraded via both β-oxidation and ω-oxidation pathways (Figure 4). The fatty acid, one carbon lost from the fatty acid backbone and another one is generated by acetyl-CoA (Thevenieau et al., 2010).

Since glucose generally represses the uptake of other carbon sources, glycerol is the one of carbon source that may become a preferred for fermentation (Mori et al., 2013). Glycerol can be redirecting toward lipid synthesis and preventing its conversion to DHAP by deletion of G3P dehydrogenase (GUT2) (Beopoulos et al., 2008) and that can significantly increase the production of biomass and lipids accumulation from glycerol in the fermentation medium (Celinska and Grajek, 2013) (Figure 4). For high lipid accumulation, FFAs in cytosol should be converted into TAGs and stored in lipid

bodies. They are transported into peroxisome, become activated, and then are degraded into acetyl-CoAs through β-oxidation (Xie, 2017).

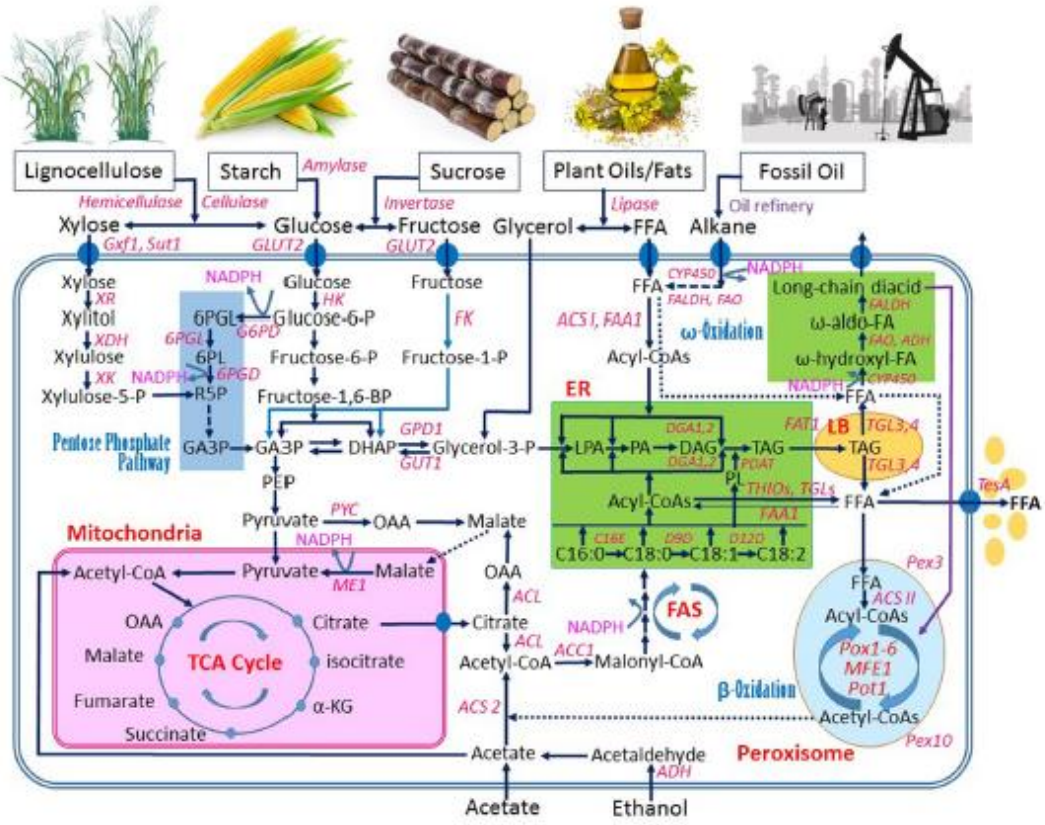

Figure 4 An overview of metabolic pathways in *Yarrowia lipolytica* for synthesis of fatty acids from various substrates.

Source: Xie (2017)

3. Low-cost substrates for oleaginous yeasts

The utilization of cheap substrates for cultivation of oleaginous yeasts could contribute incredibly in economical production of lipids and simultaneously freeing the environment from highly polluted wastes. A wide variety of cheap raw materials were examined as feedstocks for the sustainable production of biodiesel. Generally, the substrates used by oleaginous microorganisms for lipid production can be divided into hydrophilic and hydrophobic types. Depending on the substrate type, two pathways exist in oleaginous microorganisms - the ‘‘*de novo*” lipid accumulation carried out on hydrophilic materials requires limited nitrogen conditions, whereas ‘‘*ex novo*” synthetic pathway makes use of the fermentation of hydrophobic materials for the production of lipids (Papanikolaou and Aggelis, 2011; Qin *et al*., 2017).

Although there are several low-cost industrial wastes that have been explored for cultivation of yeasts, there are still unexplored wastes for this purpose. These include expired soft drinks from the beverage and soft drink industry, spent grain and spent yeast from brewing industry. Another organic by-product, from biodiesel production, crude glycerol has also attained exclusive consideration.

3.1 Expired soft drinks

The effluents from soft drinks industries, comprising products rejected in the quality checks during the bottling process or that are returned from the consumer markets from various reasons such as lack of gas or expiration of shelf life, are generated in huge quantities relative to the high production volumes of these beverages (Isla *et al*., 2013). The soft drinks consist primarily of water along with approximately 10–12% w/v dissolved carbon. Specifically, expired soft drinks contain high reducing and total sugar concentration as shown in Table 4. The beverage product waste disposal waste is usually carried out by dumping into municipal wastewater streams, in some cases onsite treatment, or others by land spreading. These methods result in the loss of potential recoverable energy and may cause environmental pollution, despite the awareness of the sheer waste volume and its potential use as a substrate for lipid production (Wickham *et al*., 2018). Utilization of these expired carbonated beverages is advantageous in terms of their huge mass and high sugar content like glucose,

approximately 110 g/L of carbohydrates in most of them. Besides, these sugars are mainly in the form of glucose (45%), fructose (55%) and sucrose, can be used for fermentation by a large number of strains used for biofuel production, without involving hydrolysis (Zhang. *et al*., 2009; Qureshi *et al*., 2007; Dwidar *et al*., 2012). Arous *et al.* (2016) studied the potential utilization of agro-industrial wastewaters for lipid production by *Debaryomyces etchellsii* by applying the mixture medium optimization using three media components consisting of expired soft drinks, wastewaters from olive mill (25%, v/v in water) and deproteinized cheese whey. It was reported that the mixture of expired soft drinks and olive mill wastewater (25% in water, v/v) was used as at a percentages of 37.6% and 62.4%, respectively, the maximum lipid content, lipid yield and biomass values obtained were 28.1% wt/wt, 1.2 g/L and 4 g/L, respectively. Whereas, another set of medium with cheese whey and expired soft drinks at 50:50 ratios (v/v) yielded high biomass of 7.9 g/L but resulted in low lipid content of 15.1% and the same lipid yield of 1.2 g/L.

Table 4 Characteristics of expired soft drinks

Characteristics	Expired soft drinks
pH	1.10
COD (g/L)	136.49 ± 7.12
Total Nitrogen (g/L)	0
Total sugars (g/L)	135.41 ± 0.11
Reducing sugars (g/L)	100.80 ± 0.64
Glucose (g/L)	37.84 ± 0.21
Lipids (%, w/w)	0

Source: Arous *et al.* (2016)

3.2 Brewers' spent grain and spent yeast cell

The manufacturing of beer results in the generation of several types of residues and by-products. The most prevalent ones are brewers' spent grains (BSG) and spent yeast cell (SYC), which are generated as the by-products from the chief raw materials (Mussatto, 2006). However, as vast majority of these are agricultural products, they can be readily recycled and reused. Therefore, brewing industry is credibly seen as an eco-friendly production unit when compared to the other industries. (Mussatto *et al*., 2006).

Brewers' spent grain (BSG), composed mainly of lignocellulosic material and proteins, is one the most abundant by-products generated from the beer-brewing process after the mashing process (Vieira *et al.,* 2017). BSG is basically a lignocellulosic material containing cellulose, hemicelluloses and lignin with high protein content (approximately 20%). These components varies depending on the raw materials and methods used in the process as presented in Table 5.

Spent yeast cell (SYC) is the yeast cells separated after beer fermentation (Figure 5), is an abundant, low-cost biomass, rich in carbohydrates, proteins, and a precursor for the food, chemical and energy industry. (Mussatto *et al*., 2006; Niemi *et al*., 2013; Ryu *et al*., 2013). BSG and SYC wastes could be about 85% of the total by-products obtained. BSG present as an insoluble solution of the barley grain with the soluble liquid wort. A 100 L of brewed beer generates around 20 kg of wet BSG (Lynch *et al*., 2016). SYC contains 30–50% cell wall, 10–15% protein along with other soluble components (Feofilova, 2010; Yang *et al*., 2014). The cell walls of yeasts are made up of up to 90% polysaccharides, mainly glucans and mannans and a small amounts of chitin. The hydrolysis of spent yeast cell releases sugars and other nutrients and the hydrolysates could straightaway be used for the cultivation of microorganisms (Yang *et al*., 2014). The oleaginous yeast *R. toruloides* Y4 when cultivated in SYC hydrolysates as nutrient sources without any additional nutrients incorporation has consumed both glucose and mannose simultaneously – a property may be of a general interest for microbial conversion of organic wastes and special resources. Furthermore, this provides an insight in developing a consolidated process by recycling spent yeast cell with reducing the production cost of microbial lipids (Cescutti *et al*., 2002; Yang *et al*., 2014).

Table 5 Chemical composition of brewers' spent grain (BSG)

Component	Hemicellulose	Cellulose	Protein	Lignin	Lipids
Jay *et al*. (2008)	n.d.	31–33	15–17	20–22	6–8
Xiros *et al.* (2008)	40	12	14.2	11.5	13
Robertson *et al.*(2010)	22–29	n.d.	20–24	13–17	n.d.
Waters *et al.* (2012)	22.2	26.0	22.1	n.d.	-
Meneses *et al.* (2013)	19.2	21.7	24.7	19.4	-

All values expressed in g per 100 g dry material (% w/w); n.d., not determined

Source: Lynch *et al.* (2016)

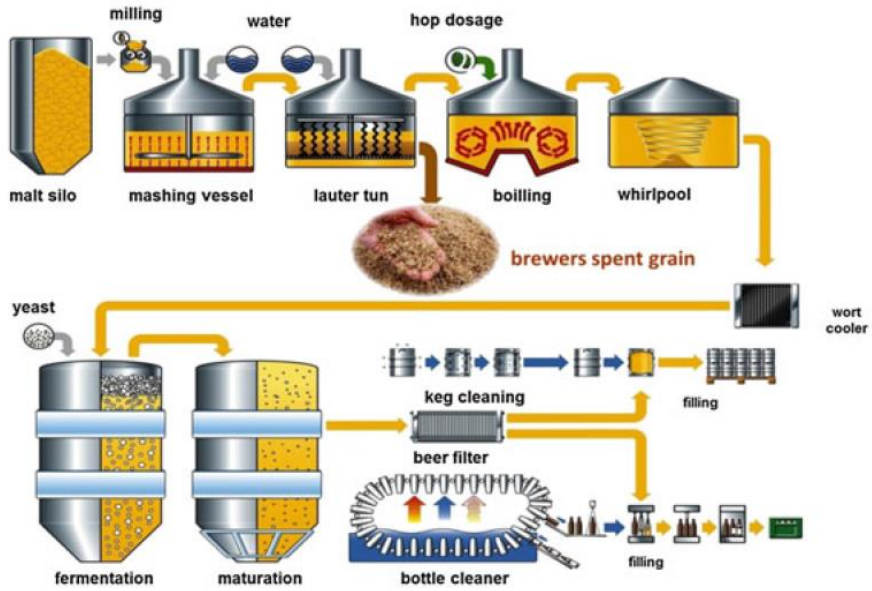

Figure 5 Overview of the brewing process

Source: Lynch *et al*. (2016)

Ryu *et al*. (2013) studied the lipid production in spent yeast cell wastes from brewery industry as a sole source of growth by oleaginous yeast *Cryptococcus curvatus. C. curvatus* was grown in a baffled hybrid flask (250 mL) with an air vent and 100 mL of working volume. All the batch cultivations were performed on a rotary shaker at 150 rpm at 28ºC. Without additional nutrients and any pretreatment, the biomass productivity obtained was approximately 7 g/L/d when using only spent yeast cell (30 g/L). In a batch culture operating mode and the highest biomass of 50.4 g/L and a lipid content of 37.7% were attained at 35:1 of C/N ratio.

Yang *et al.* (2014) studied the utilization of spent yeast cell waste for lipid production by *Lipomyces starkeyi.* The spent yeast cell wastes was acid hydrolyzed using 1.0 M H_2SO_4 at a solid/liquid ratio of 1/10 (v/w) and 120 ºC for 1.5 h. The hydrolysate containing glucose and mannose was used as nutrient sources for cultivation of oleaginous yeast *Lipomyces starkeyi*. It was found that the yeast consumed sugars with a rate of 0.58 g/L/h and gave the lipid yield of 0.18 g lipid/g sugar.

3.3 Crude glycerol

Crude glycerol, a familiar by-product from biodiesel production, alcoholic beverage industries, and saponification of fats and oils, is considered as one of the most interesting carbon sources, due to its cheap and abundance (Figure 6) (André *et al*., 2009; Leiva-Candia *et al.*, 2014). Crude glycerol comprised of approximately 80% glycerol, 10% water, 7% ash and less than 1% methanol. The manufacturing process of 10 kg biodiesel generates1 kg of glycerol as by-product (Bauer and Hulteberg, 2013).

A wide range of yeast species can convert glycerol into lipids in a short periods of time through *de novo* process. In yeast cells, glycerol is phosphorylated to dihydroxyacetone phosphate by glycerolphosphate oxidase. Unlike crude glycerol, the utilization of pure glycerol for cultivation of yeast in large-scale is expensive and impractical. In addition, crude glycerol from biodiesel manufacturing contains macro elements that are great source for the growth of microorganisms. The reuse of biodiesel-derived glycerol utilization with lipid production by yeasts, not only reduce the cultivation costs but also provides a favorable opportunity to recycle the glycerol waste stream from biodiesel production (Leiva-Candia *et al.,* 2014). The utilization of crude

glycerol without requirement of any purification has advantages in microbial fermentation, and it also reduces glucose repression present in case of glucose utilization. Several yeasts have been identified that can utilize crude glycerol and are deeply evaluated in the genera including *Candida*, *Cryptococcus, Lipomyces, Rhodosporidium*, *Rhodotorula, Schizosaccharomyces, Trichosporonoides* and *Yarrowia* (Liang *et al.*, 2010; Signori *et al*., 2016). Recent literature on the use of oleaginous yeasts for the bioconversion of industry-derived crude glycerol to lipids is shown in Table 6.

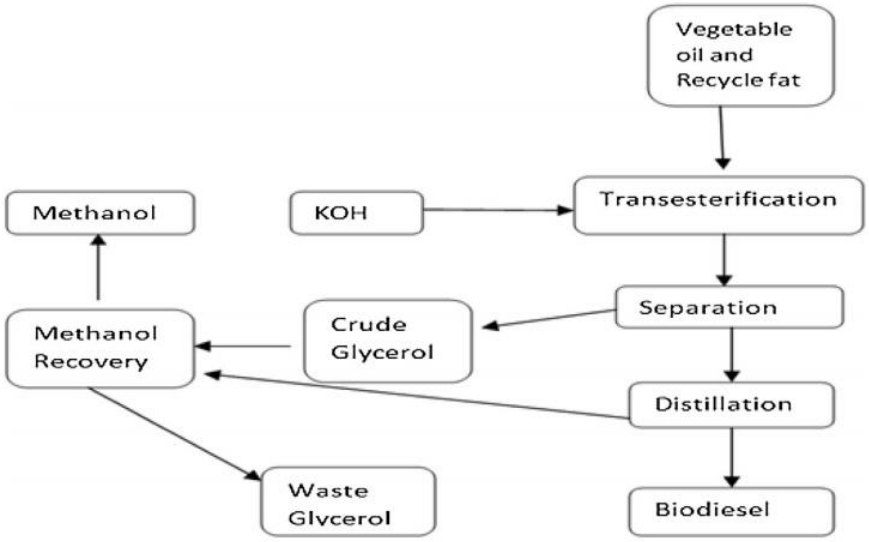

Figure 6 Biodiesel production through transesterification reaction
Source: Garlapati *et al.* (2016)

Table 6 Lipid production by oleaginous yeasts using crude glycerol as a carbon sources

Yeast strain	Carbon source	Nitrogen source	Lipid content (% W/W)
Cryptococcus curvatus ATCC 20509	Crude glycerol	Ammonium chloride	52.9
Cryptococcus curvatus ATCC 20508	Crude glycerol	Baker's yeast autolysate	43
Rhodosporidium toruloides AS2.1389	Crude glycerol from an enzyme-catalysed biodiesel production process	Yeast extract	74.1
Rhodosporidium toruloides AS2.1389	Crude glycerol from alkaline-catalysed biodiesel production process	Yeast extract	69.5
Trichosporonoides Spathulate	Crude glycerol	Ammonium sulphate	40.69

Source: Leiva-Candia *et al* (2014)

3.4 Lignocellulosic biomass

Another notable substrate analyzed for oleaginous yeasts is lignocellulosic biomass, typically a nonedible plant material consisting of dedicated crops, wood, grass and agro-forest residues. It is a principal building block of plant cell walls, made of carbohydrates, lignin, extractives and ashes. The composition these varies with plant type, species, age, growth conditions and plant parts, nonetheless carbohydrates and lignin make up the major fraction of lignocellulosic biomass (Kumar *et al*., 2017). Lignocellulosic materials are mainly composed of cellulose, hemicellulose, and lignin (Figure 7) (Verardi *et al*., 2012) with a concentration of 35–50%, 20–35% and 15–20%, respectively and others 15–20% (Mood *et al*., 2013). Cellulose and hemicellulose are polymers of glucose, xylose and other sugars which can be used as a renewable source of fermentable sugars, while lignin can be utilized to produce other valuable chemicals (Hassan *et al.,* 2018).

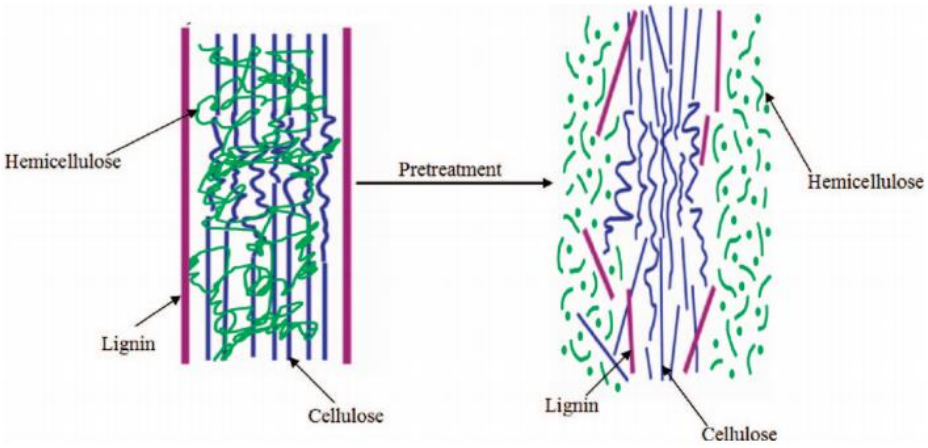

Figure 7 The composition of lignocellulosic materials and the resulting structure after pretreatment

Source: Kumar *et al*. (2009)

Cellulose is a chief homopolymer of lignocellulose, with an unbranched linear structure (Kumar *et al*., 2017). Cellobiose is a small repetitive unit formed by two glucose units comprising of β-D-pyranose units joined by β-1, 4-glycosidic bonds. The cellulose polymers are bundled and supported by hydrogen and van der Waals bonds with a degree of polymerization (number of repeating subunits) ranging from a few thousand to several thousand sub units as shown in the Figure 8 (Verardi *et al.*, 2012). The proportion of cellulose varies between different plant types. In the case of hardwood species cellulose represents approximately 39–54% of dry biomass; whereas in softwood species it is between 41–50%; and in agricultural residues, cellulose makes up 32–47% of the biomass (Kumar *et al*., 2017).

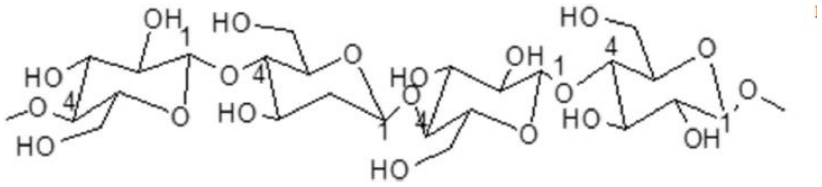

Figure 8 The structure of cellulose
Source: Pérez and Samain (2010)

Hemicellulose is a combination of several polysaccharides formed by pentoses, hexoses and uronic acids (Figure 9). It is a heteropolymer of several hexoses and pentoses such as, D-glucose, D-galactose, D-mannose, D-xylose and D-arabinose, & some organic acids like acetic and glucuronic acids. Hemicellulose has a linear and branched structure with backbone of repetitive units of the same sugars to form homopolymers, or heteropolymers by a mixture of different sugars (Kumari *et al*., 2018). In lignocellulose, hemicellulose is the most thermo-chemically sensitive structure. Xylan is an easily extractable component and is the major portion of hemicellulose. Glucomannan is the major composition of softwood hemicellulose rendering its difficulty to be extracted. High temperature pretreatment conditions or hydrolytic decomposition of hemicellulose sugars into respective products results in the production of other compounds such as furfural, acetic acid, etc. known to have inhibitory effect on downstream or microbial fermentation processes. (Larsson *et al*., 2000: Kumari *et al*., 2018).

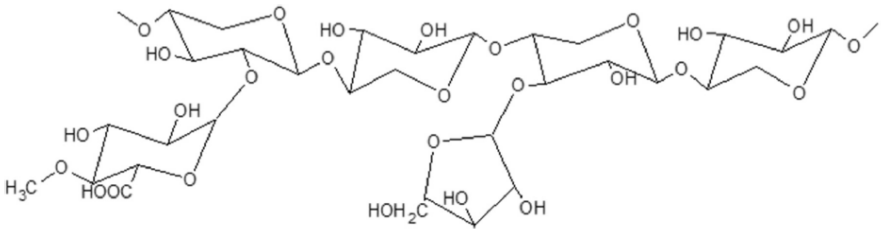

Figure 9 The structure of hemicellulose
Source: Chiaramonti *et al* (2007)

The third most abundant component of lignocellulose, lignin, is a natural amorphous heteropolymer made up of predominant building blocks of phenylpropane units that confer recalcitrance, impermeability, and structural support to lignocellulose (Agbor *et al*., 2011, Harmsen *et al*., 2010). Lignin structure is formed by three monomeric phenyl propane alcohols, p-coumaryl, coniferyl, and sinapyl, joined together in a large and very complex three-dimensional fashion (Figure 10) (Kumari *et al*., 2018). Table 7 shows the chemical composition of different lignocellulosic biomass.

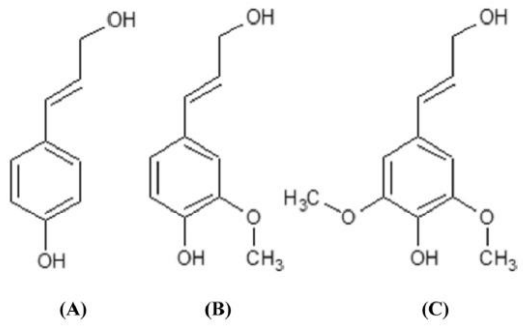

Figure 10 The structure of phenyl propane alcohols of lignin (A) ρ-coumaryl, (B) coniferyl and (C) sinapyl:

Source: Kumari (2018)

4. Hydrolysis of lignocellulosic biomass

There are different hydrolysis methods including dilute acid, concentrated acid or enzymatic. Different methods have different modes of actions and require various types of neutralizing steps to remove the inhibitors generated in the fermentation medium used by the oleaginous microorganisms. Figure 11 shows the overview of pretreatment of lignocellulosic biomass (Patel *et al*., 2016)

Table 7 The composition of different lignocellulosic feedstocks (% dry basis)

Source	Cellulose	Hemicellulose	Lignin
Eucalyptus	44.9	28.9	26.2
Oak	43.2	21.9	35.4
Rubber wood	39.56	28.42	27.58
Spruce	47.1	22.3	29.2
Pine	45.6	24.0	26.8
Bamboo	46.5	18.8	25.7
Natural hay	44.9	31.4	12.0
Hemp	53.86	10.60	8.76
Sunflower	34.06	5.18	7.72
Silage	39.27	25.96	9.02
Pine nut shell	31	25	38.0
Rice straw	38.14	31.12	26.35
Barley straw	35.4	28.7	13.1
Palm oil frond	37.32	31.89	26.05
Corn stover	43.97	28.94	21.82
Bamboo leaves	34.14	25.55	35.03
Hazel branches	30.8	15.9	19.9
Rice husk	40	16	26
Sugarcane peel	41.11	26.40	24.31

Source: Hassan *et al* (2018)

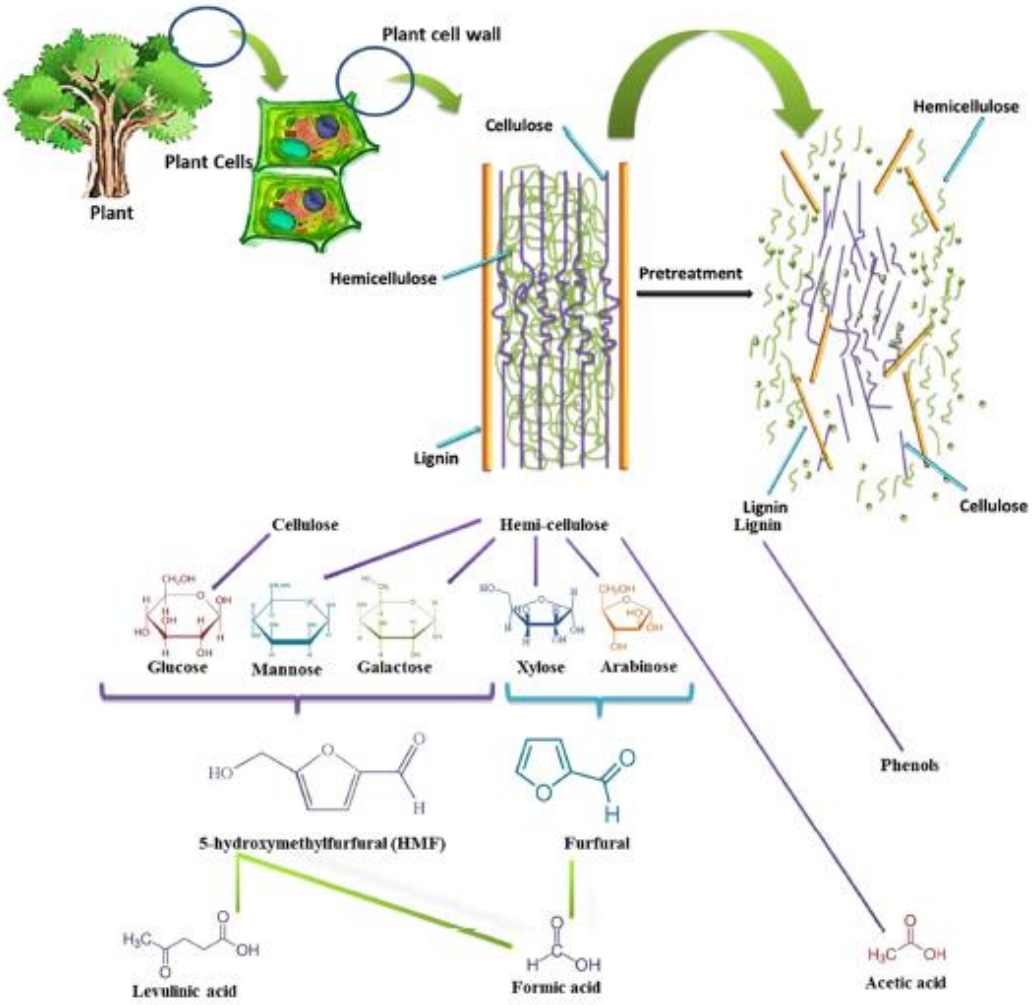

Figure 11 Overview of pretreatment of lignocellulosic biomasses
Source: Patel *et al*. (2016)

4.1 Acid hydrolysis

Acid hydrolysis is a well-established and most commonly used process for chemical pretreatment of the lignocellulosic biomass. The commonly used conditions are high acid concentration with low temperature and low acid concentration with high temperature (Sen *et al*., 2016). Depending on the acid concentration used, the acid hydrolysis process can be classified into: 1) Concentrated acid hydrolysis and 2) Dilute acid hydrolysis. Moreover, acid hydrolysis is the most applied process owing to its

advantageous capability of penetrating lignin without need of any primary pretreatment, resulting in the break down of cellulose and hemicellulose polymers into individual sugars (Verardi *et al.,* 2012).

1) Concentrated acids including H_2SO_4 and HCl are the most commonly used acids to treat lignocellulosic materials. (Lenihan *et al*., 2010). Pretreatment with acid hydrolysis can improve the further steps of enzymatic hydrolysis of lignocellulosic biomasses to release additional sugars. Although concentrated acids are powerful agents for cellulose hydrolysis, they are toxic, hazardous and corrosive, demanding specialized reactors that can resist corrosion and making the pretreatment process expensive. Additionally, economically feasible process requires the recovery of the concentrated acid after hydrolysis (Sun *et al.,* 2002: Kumar *et al*., 2009).

2) Another successfully developed method for pretreatment of lignocellulosic materials is diluted acid hydrolysis, the use of diluted sulfuric acid in a concentration range of 0.2–2.5 wt% is commonly used in pretreatment can obtain achieve high efficiency and significantly increase cellulose hydrolysis (Sun *et al.,* 2002). Hydrolysis of hemicelluloses requires mild operational temperature conditions (< 200 °C), but higher temperatures (> 220 °C) are needed to overcome its recalcitrance and for efficient hydrolysis.

The hydrolysis products of hemicellulose (mostly pentose and some hexose) are decomposed at higher temperatures and some of these products are known to have inhibitory effects on the subsequent processes. Continued breakdown of pentoses and hexoses generates several potential inhibitors like furfural, 5-hydroxymethylfurfural (HMF), formic acid, acetic acid, uronic acid, levulinic acid, , vanillic acid, 4-hydroxybenzoic acid, phenol, vanillin, cinnamaldehyde, and formaldehyde that interfere further processes (Larsson *et al*., 2000; Kumar *et al*., 2017). By-products generated during acidic pretreatment of lignocellulose can be categorized into groups based on their origin, chemistry and their effects on the microorganisms. Degradation products from carbohydrates, such as common aliphatic carboxylic acids, acetic acid, formic acid, levulinic acid and the furaldehydes such as furfural and HMF exert low inhibitory effects but their concentrations are high depending on the pretreatment conditions and the type of feedstocks (Jönsson *et al*., 2016) (Figure 10).

4.2 Enzymatic hydrolysis

Cellulases, a group of enzymes commonly used for enzymatic hydrolysis, which catalyze the depolymerization of cellulose to reducing sugars including glucose with great specificity (Kumar *et al*., 2017). The use of mild conditions (pH 4.8 and temperature 45–50 ºC) and absence corrosion problem makes the enzyme hydrolysis cost effective when compared to acid or alkaline hydrolysis (Sun *et al.*, 2002). Pretreatment methods employed greatly influences the efficiency of any typical method of hydrolysis. Several pretreatment methods combined with some of the operating conditions induce the production of inhibitory by-products that have negative effects on the downstream processes. Lignocellulosic biomass can be hydrolyzed by cellulase enzymes or by acids, however both methods have their own pros and cons associated with are listed in Table 8.

Table 8 Advantages and disadvantages with different hydrolysis methods

Hydrolysis Method	Advantage	Disadvantage
Dilute Sulfuric Acid Hydrolysis	-Requires low concentration of acid -Residence time is short -Mild hydrolysis conditions -Low cost of acidic catalyst	-Degradation of hemicelluloses sugar -Formation of inhibitory compounds -Requirement of high temperature -Corrosion of metallic reactors -Requirement of excess acid neutralization and detoxification of hydrolysates -Low sugar yield
Concentrated Sulfuric Acid Hydrolysis	-Operates at lower temperature -High yield of sugar	-Long residence time -Requirement of expensive reactors resistant to corrosion -High acid consumption -Excess acid must be neutralized
Enzymatic Hydrolysis	-Operates at low temperature (40–50 °C) -Optimal performance at only slightly acidic pH -No corrosion problem -High yield -Partial recycling and reuse of enzymes -Enzymes can be produced naturally	-High retention time -Product Inhibition -Reversion of glucose -Inhibition at high substrate to enzyme ratio -Requires addition of surfactants to prevent Irreversible binding of enzymes to lignin -Inhibitory effect of ionic surfactants -High cost of enzymes

Source: Kumar *et al.* (2018)

The lipid production and storage by oleaginous yeast employing lignocellulosic biomass-based sugar substrate is an attractive process considering the ability of some yeasts to ferment pentose sugars along with hexoses, producing high density lipids than ethanol. (Yu *et al*., 2011).

Table 9 Shows the effectiveness of lipid accumulation by several oleaginous yeasts to utilize lignocellulosic hydrolysates

Strain	Substrate	Pretreatment and hydrolysis	Inhibitory compounds	Lipid content (%)
Trichosporon fermentans	Rice straw	Acid Hydrolysis	Detoxified	40
Cryptococcus curvatus	Wheat straw	Dilute H_2SO_4	Detoxified	33.5
Cryptococcus curvatus	Wheat straw	Dilute H_2SO_4	Acetic acid, furfural, HMF	27.1
Lipomyces starkeyi	Wheat straw	Dilute H_2SO_4	Detoxified	29.1
Candida lipolytica	Corn cob	Steam explosion pretreatment and Enzymatic hydrolysis	Acetic acid, furfural, HMF	-
Lipomyces starkeyi	Wheat straw	Dilute H_2SO_4 pretreatment	Acetic acid, furfural, HMF	31.2
Yarrowia lipolytica	Defatted rice bran hydrolysate	Acid hydrolysis	Detoxified	48.02

Source: Kumar *et al.* (2017)

5. Factors affecting lipid accumulation

Several parameters such as carbon, nitrogen, C/N ratio, agitation rate, time of incubation, temperature, pH and concentration of mineral salts affects lipid accumulation in microorganisms. In addition, substrate cost, production rate and the ultimate lipid concentration are other crucial factors that determine the cost of microbial oil (Enshaeieh *et al*., 2012). Optimization of chemical parameters such as type of carbon and nitrogen sources, carbon and ammonium concentration as well as other physical factors, influence the lipid yield by oleaginous yeasts (Madani *et al.*, 2017).

5.1 Carbon source

Yeasts are chemoorganotrophic organisms acquiring carbon and energy sources from organic compounds and the most common of these compounds are sugars, whereas glucose is the most widely used by yeast. The evaluation of effect of different carbon sources on lipid production by oleaginous yeasts including *Rhodotorula glutinis, Rhodosporidium toruloides* and *Lipomyces starkey* has been reported. Glucose, among the various carbon sources tested, provided higher biomass yields and lipid content than the others. When compared to the evaluation of other yeasts, *Rhodotorula glutinis* grown on glucose had yielded highest biomass and lipid production of 10.21 g/L and 2.43 g/L corresponding to lipid content of 23.78% (Vijayakumar *et al.*, 2010). Table 10 shows a comparison of lipid production in various yeast strains.

Table 10 Lipid production rates in different types of carbon sources

Yeast strains	Carbon source	Lipid content (%)
Candida curvata	Xylose	48.6
Rhodosporidium toroloides	Glucose	22.54
Rhodotorula glutinis	Glucose	23.78
Yarrowia lipolytica	Sugarcane bagasse hydrolysates & peptone	58.5
Trichosporon dermatis	Corncob hydrolysate	40.1
Rhodotorula glutinis	Crude glycerol	60.7
Rhodotorula graminis	Corn stover hydrolysate	34
Trichosporon cutaneum	corn cob hydrolystae	32.0
Rhodotorola mucilaginosa	Glucose	60.09
Candida 14	Glycerol	22
Rhodotorula 110	Glucose	40
Yarrowia lypolitica	Glucose	47.5
Rhodotorola mucilaginosa	Corn stalk	36.91
Cryptococcus curvatus	Sorghum bagasse hydrolysate	43.3
Rhodotorula toruloides	Wheat straw hydrolysate	24.6
Rhodotorula glutinis	Wheat straw hydrolysate	25

Source: Madani *et al*. (2017)

5.2 Nitrogen source

Nitrogen makes up the 10% of yeast cell dry weight. Yeasts can utilize a wide variety of different organic and inorganic sources of nitrogen to make up the structural and functional nitrogenous components of the cells. Nitrogen enhances yeast cell growth and lipid biosynthesis irrespective of the nitrogen concentration in culture medium. Furthermore, nutrient variation in the culture medium was known to stimulate lipid accumulation and storage by oleaginous microorganisms. When the cell is under nutrient depletion state, especially lack of nitrogen, it will assimilate the excess substrate and converts into fat for storage, but it was observed that cell propagation is drastically depressed under nitrogen-limited conditions and in many cases restricting cell density. To overcome this problem various types of substrates and cultivation methods have been used to achieve a high-dense yeast cell culture for microbial lipids fermentation, (Li *et al*., 2007). Depending on the yeast species being examined, various results have been achieved when studied the effects of different nitrogen sources and levels on total lipids and on fatty acid profiles (Sitepu *et al*., 2014). It was found that the organic nitrogen sources had shown positive effect on lipid production and mineral nitrogen sources effect on biomass yield (Madani *et al*., 2017).

Enshaeieh *et al.* (2013) reported the effects of organic nitrogen sources such as peptone and yeast extract and inorganic nitrogen sources such as ammonium chloride and ammonium sulfate on the lipid production by *Rhodotorula* 110 (Yr2) strain and it was found that the yeast produced the highest lipids in the presence of yeast extract and ammonium sulfate (Table 11).

Table 11 Effect of nitrogen source for lipid production by oleaginous yeasts

Nitrogen source	Lipid production (g/l)	Biomass (g/L)	Lipid content (%)
Yeast extract and $(NH_4)_2SO_4$	6.29	17.57	35.78
Yeast extract and NH_4Cl	6.15	17.9	34.35
Peptone and $((NH_4)_2SO_4$	6.11	17.68	34.37
Peptone and NH_4Cl	6.18	17.88	34.55

Source: Enshaeieh *et al*. (2013)

5.3 Carbon to Nitrogen (C/N ratio)

Growth medium with varied C/N ratio known to have a great influence on lipid production in several yeast species (Figure 11). In many known oleaginous yeasts, more lipids are accumulated more lipids if the C/N ratio increased to a certain point, and lipid accumulation is influenced by nitrogen depletion in the medium (phase c in Figure 11). In the case of *Rhodotorula glutinis*, glucose-to-lipid conversion yield increased from 0.25 to 0.40 when the molar C/N ratio was raised from 150 to 350, but increase in the C/N ration further had inhibitory effects on yeast cell viability (Beopoulos *et al.*, 2009). Lipid productivity will be greatly influenced by the presence of more than a single carbon source in culture medium, for example if only glucose was used for *Y. lipolytica* with optimum C/N ratio, better lipid production was observed as 35, but 180 ratio is needed if a mixture of glucose plus glycerol was used (Sitepu *et al*., 2014).

Nevertheless, the effect residual C/N ratio triggers the production of citric acid resulting a shift in microbial metabolism into phase d as in Figure 12, this in turn results in the decrease in total conversion yield to produce lipids from carbon substrate. Thus, conversion yields and lipid accumulation in batch cultures are highly influenced by the ratio of biomass constitution. Controlling the consumption ratio of carbon to nitrogen is therefore necessary to prevent citric acid generation (Beopoulos *et al.,* 2009).

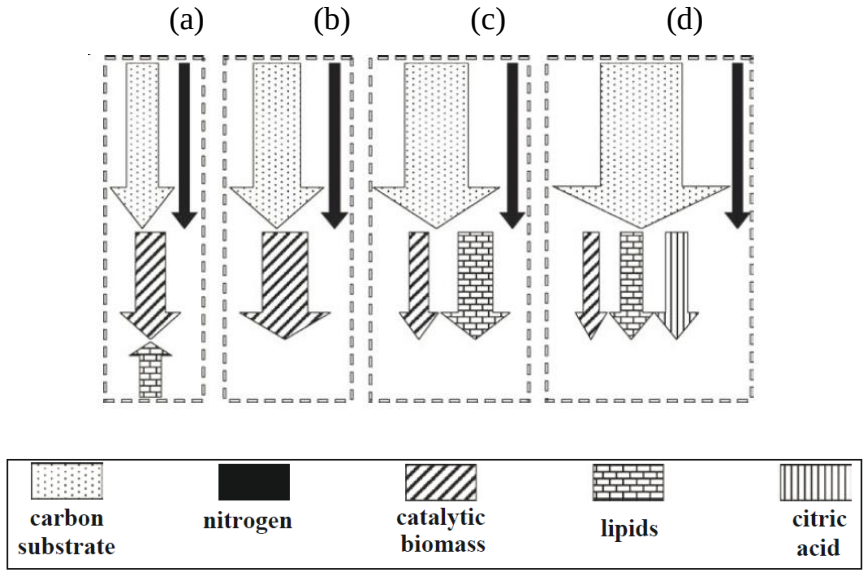

Figure 12 Yeast activity as a function of carbon flow rate for a fixed nitrogen flow rate. (a): pure growth and mobilization of stored lipids; (b): pure growth; state c: growth with lipid accumulation; (d): growth, lipid accumulation and citric acid production.

Source: Beopoulos *et al*. (2009)

Poli *et al.* (2014) studied the effect of C/N ratio on *Yarrowia lipolytica* QU21 – its cell growth and lipid accumulation using crude glycerol as a carbon source and the culture medium was added with 0.1% KH_2PO_4 and 0.05% $MgCl_2 \cdot 6H_2O$. The culture conditions were 150 rpm, 28 ºC for 4 days. It was found that the highest biomass values achieved when *Y. lipolytica* QU21 grew in C/N 276 and C/N 184, were 5.1±0.56 g/L and 4.9±0.68 g/L, respectively, with no significant difference between them. When cultivation on C/N 184 it was found that the lipid production increased up to 1.48 ± 0.27 g/L and gradually decreased when the C/N ratio increased. *Y. lipolytica* QU21 showed significant difference to all other treatments during its cultivation for lipid production in C/N 184.

5.4 pH

Most yeasts grow significantly in the acidic pH range of 4.5 to 6.5, excepting some yeasts that are able to grow in either more acidic (around pH 3) or alkaline (pH 8) media. Acidification of media with organic acids had shown to be highly inhibitory to yeast cell growth than the media acidified with mineral acids. This is due to the presence of undissociated organic acids that can translocate across the yeast plasma membrane and lower the intracellular pH (Walker, 1998). In industrial scale, the pH has to be controlled and this leads to the increased production costs. The use of acid-tolerant yeasts would be preferable. Acidified YM medium with pH of 3.9 is a simple selective medium that has been used since several decades to cultivate yeasts out of mixed groups comprising both yeasts and bacteria (Miller and Webb, 1954; Sitepu *et al.,* 2014). Ryu *et al.* (2013) reported the effect of initial pH on cell growth of oleaginous yeast *Cryptococcus curvatus* cultivated under different pH ranges from 4.8, 5.8, and 6.8 when using brewery industrial waste as a sole source of growth substrate. It was found that the highest lipid concentration and cell density of *C. curvatus* were observed at pH 5.8 (7.37 g/L and 16.3%).

5.5 Temperature

The oleaginous yeasts can be divided into two groups based on their optimal temperature requirements: mesophilic yeasts (25–30 ºC) and thermophilic group (35–45 ºC). Temperature also affects the variance of the fatty acid profiles of the lipid (Saxena *et al*., 2009). Besides, temperature also plays a crucial role in the regulation of fatty acid compositions of membranous lipids of a microorganisms. Oleaginous yeasts had shown a wide range of fatty acids (C7 to C18) in their fatty acid profiles when grown at different temperatures, probably due to the temperature-sensitive acyl-carrier protein, part of a main enzyme associated with the fatty acid chain elongation. Biosynthesis of long-chain fatty acids were predominant at optimal temperatures for growth (30-32 ºC) while short chain fatty acids were predominantly synthesized at a higher temperature of 38 ºC (Sexena *et al.,* 1998).

Fei *et al.* (2011) studied the effect of temperature on lipid accumulation by oleaginous yeast *C. albidus* using volatile fatty acids (VFAs) as a carbon source. The temperature was varied between 20, 25, 30, and 35 ºC. It was found that the yeast growth was well and a large amounts of lipids were accumulated in a temperature range of 20–30 ºC. Besides, growth was lasted up to 48 h and failed to accumulate lipids at 35 ºC. The optimal lipid concentrations of 0.27 g/L were obtained at 25 ºC, which also resulted in the optimum lipid content (25.9%).

5.6 Aeration rate

Complete absence of oxygen will disrupt the yeast cell growth as not only the provision of energy sources for respiratory enzymes during aerobic growth is needed, but oxygen is also required for certain growth-maintaining hydroxylation steps such as those involved in the synthesis of sterols and unsaturated fatty acids. Moreover, yeasts require molecular oxygen for the mixed-function oxidase mediated cyclization of 2,3-epoxysqualene to form lanosterol, and also for the synthesis of fatty acyl coenzyme-A esters. Therefore, oxygen should be considered as a crucial growth factor for yeast (Walker, 1998).

Papanikolaou *et al*. (2002) reported the effect of low and high aeration rates on the growth of *Yarrowia lipolytica* cultivated in an industrial saturated fatty acid (stearin). The yeast grew well and accumulated high lipid content at low levels of oxygen (5–15%), whereas at high aeration rate (oxygen saturation of 60–70%), the yeast grew better and tended to degrade its storage lipids, even when there were large amount of saturated stearic acid in the medium.

6. Modes of cultivation for high lipid accumulation

Three different types of culture modes of are employed: batch, fed-batch and continuous mode. Many of the processes described in literature relate to batch mode (Beopoulos *et al*., 2009).

6.1 Batch fermentation mode

In batch cultures, the bioreactors are added initially with a mixture of minerals and carbon substrates with a high initial C/N ratio to induce lipid accumulation. Active consumption of nitrogen right from the beginning of culture leads to the continual increase in the residual carbon to residual nitrogen ratio, trending to infinity. Batch fermentation maintains the exponential growth of the cells with unlimited nitrogen source (Beopoulos *et al*., 2009). Zhu *et al.* (2008) studied the lipid production by yeast *Trichosporon fermentans* in batch culture at a C/N molar ratio of 163 using peptone and glucose as nitrogen and carbon sources with maintaining a favorable initial pH of the medium and temperature at 6.5 and 25 °C. With these optimized conditions, the lipid content of 62.4% with maximum biomass of 28.1 g/L was achieved after 7 days cultivation. Kurosawa *et al.* (2010) studied the lipid production of oleaginous *Rhodococcus opacus* PD630 in batch cultivation and found that the increase in the concentrations of glucose and $(NH_4)_2SO_4$ resulted in a substantial increase in production of fatty acids. When pH was kept under control with an optimized production medium comprising 240 g/L glucose and 13.45 g/L $(NH_4)_2SO_4$ and with a C/N ratio of 17.8, yielded 77.6 g/L of dry cell weight comprising approximately 38% of triglycerides.

6.2 Fed-batch

To attain maximum metabolic state stability for lipid synthesis, the regulation of the environmental variables especially nutrient sources is required, which can be achieved through the definitive control of nutrient flow rate (Beopoulos *et al*., 2009). Chen *et al*. (2017) reported the cultivation of oleaginous yeast *Trichosporon oleaginosus* in fed-batch mode using sterilized crude glycerol solution equivalent to 10.28 g/L carbon with a C/N of 10. The results were found to be with a maximum concentration of biomass of 43.82 g/L and lipids of 21.87 g/L.

Chen *et al.* (2018) studied the oleaginous yeast *Trichosporon oleaginosus* in fed-batch cultivation and the effect of pH on the cell growth. It was observed that a neutral or weak acid pH was optimal for the cell growth of the yeast both in the wastewater sludge medium and yeast extract-peptone-dextrose medium. However, a marked growth inhibition was observed when using crude glycerol based medium due

to the high soap content, but the inhibition by soap could be prevented by converting it to free fatty acid (FFA) at pH 5. Fed-batch fermentation using crude glycerol and optimized pH at 5 was employed to produce lipids by this yeast. Fed-batch fermentation modes with a controlled pH showed to achieve remarkably high biomass (65.63 g/L) and lipid (35.79 g/L) concentration.

6.3 Continuous mode

Continuous culture mode operates at a constant C/N ratio and the residual carbon to residual nitrogen in the culture medium at a given dilution rate. Lower dilution rates with intermediate C/N ratios promoting lipid accumulation results in higher lipid and biomass concentrations than higher dilutions. Low specific growth rates, at similar residual carbon to residual nitrogen ratios, promote lipid accumulation. Therefore, optimization involves the determination of the optimal dilution rates with an optimal intermediate C/N ratio (Beopoulos *et al*., 2009).

Béligon *et al*. (2016) studied the oleaginous yeast *Cryptococcus curvatus* and acetic acid as its carbon source for cell biomass and lipid production. A model for oleaginous yeast grown on acetic acid in a fed-batch culture was first optimized which was later employed as a template to identify optimal dilution rates and C/N ratios for their use in continuous culture. By the application of this validated tool, cell biomass concentration was stabilized at 26.7 g/L with a lipid content of 48–53% and a maximum lipid productivity of 0.54 g/L.h at a steady state. This model could be credited as a mechanistic and predictive tool as it allow us to understand the underlying reactions for lipid production processes. The experimental data was on par with the model predictions confirming that the use of a model is a powerful and predictive tool that could aid the operator to analyze the parameters to improve the culture.

6.4 Repeated-batch

Repeated-batch cultivation is a continuous repetition of different cycles of batch cultivations with the fermented biomass being replaced with new substrate (Cheirsilp *et al*., 2015) to enhance the productivity of microbial process. Several advantages are associated with repeated-batch cultivations, such as complete medium exhaustion in the

reactor at the end of cultivation, the reusability of microbial cells for the next fermentation runs, high cell density in the culture, short operational times and process productivity. Also reported in further studies that the repeated-batch cultivation is influenced by the harvesting times of culture medium (Ganjali Dashti *et al*., 2014).

Dashti and Abdeshahian (2016) reported fungal lipid production by *Cunninghamella bainieri* 2A1, a locally isolated strain, comparing batch culture and repeated-batch culture applying a nitrogen-limited medium. The repeated-batch culture was performed at four harvesting culture volumes of 60%, 70%, 80% and 90% and at three harvesting times of 12, 24 and 48 h. In repeated-batch culture, at the first cycle of 48 h harvesting time using 70% harvesting volume, a lipid concentration of 3.30 g/L was produced, whereas the last cycle of 48 h harvesting time using 80% harvesting volume produced only 0.23 g/L gamma-linolenic acid (GLA).

Assawamongkholsiri *et al*. (2018) studied *Rhodobacter* sp. KKU-PS1 for its hydrogen and lipid production from sugar manufacturing plant wastewater (SMW). In the repeated-batch fermentation, the medium replacement was varied at ratios of 25, 50 and 75% to further study the production of hydrogen from SMW. At the medium replacement ratio of 75%., highest biomass and lipid concentration of 2.83 g/L and 685 mg lipid/L, respectively, were obtained. Long chain fatty acids of C18:0 (24.9%), C18:1 (51.2%), and C16:0 (9.1%) were found as significant fatty acids in the extracted lipids. It was observed that lactic acid followed by propionic, acetic and butyric acids present in SMW were the optimal carbon sources for biomass production of *Rhodobacter* sp. KKU-PS1.

7. Biodiesel characteristics based on fatty acid profile of oleaginous yeasts

Biodiesel is an important alternative fuel. It has excellent properties as diesel engine fuel. Moreover, the biodiesel can be used in compression-ignition diesel engines. Especially, its can be derived from various different source oils feedstock. (Lim et al., 2009). The advantageous of the application activities for the environmental friendliness and using biodiesel is that it is biodegradable and also used without modifying existing engines, produces less harmful gas emissions (sulfur oxide) (Van Garden, 2005).

Moreover, biodiesel reduces net carbon-dioxide emissions by 78% on a lifecycle basis when compared to conventional diesel fuel (Gunvachai et al., 2007). On the other hand, disadvantages of biodiesel production and usage indicated that is mainly produced from edible oil, which could cause shortages of food supply and increased prices, conflicts with food supply, less suitable for use in low temperatures and it can only be used in diesel powered engines (Viesturs et al., 2014).

Operating the diesel engines with the application of biodiesel is an environmental-friendly approach compared to fossil fuels. Biodiesel offers several advantages over conventional fossil fuels, such as its biodegradability, use in the existing engines without any modification and less harmful gas emissions such as sulfur oxide and reduction of carbon-dioxide emissions by 78% on a lifecycle basis when compared to conventional diesel fuel (Van Gerpen *et al.*, 2005, Gunvachai *et al*., 2007). Furthermore, biofuels present many advantages over fossil fuels, such as: (a) accessibility of renewable sources; (b) addressing CO_2 cycle in combustion; (c) eco-friendly; and (d) biodegradable and sustainable. In addition to these advantages biodiesel is well known for its: portability, abundance, lower sulfur and aromatic content, and high combustion characteristics (Puppan *et al.*, 2002; Atadashi *et al*., 2010).

The properties of biodiesel are highly influenced by the chemical constituent of used feedstocks (Sorate *et al*., 2015). Physicochemical characteristics of biodiesel are also determined by fatty acid profile including chain length and the presence of unsaturation are important factors (Verma *et al*., 2016). The purity of biodiesel must be higher and should meet the international biodiesel standard specifications set by American standard for testing materials (ASTM) and European Union (EU) standards for alternative fuels. According to the European Union (EU) standard specifications for biodiesel, fuel's water content, free fatty acids, and free and bound glycerine must be maintained at a minimal level and the purity of the fuel should be more than 96.5% (Atadashi *et al*., 2010). The methods and technologies employed for the purification of the feedstock and to convert them to biodiesel determine whether the produced biofuel will conform to the designed specification standards or not. Furthermore, the quality and purity of biodiesel fuel can be influenced greatly by a numerous of factors

including: feedstock quality of, fatty acid composition of the vegetable oils, animal fats and waste oils, production type and refining process and post-production parameters.

Table 12 shows the international standards requirements for biodiesel fuel (Atadashi *et al*., 2010). The characteristics of biodiesel can be categorized by several criteria - the most crucial are the ones that effect the processes occur in the engine like, quality of ignition, effortless starting, generation of fuel-air mixture, exhaust gas formation and emission quality and the heat value, etc, cold weather properties such as cloud point, pour point and cold filter plugging point, other properties including microbial contamination, oxidative and hydrolytic stability, induction period, flash point, limit temperature for filterability and wear and tear of engine parts (Barabás *et al*., 2011)

1) Cetane number (CN)

Cetane is an inverse functional indicator that defines ignition quality of biodiesel for compression ignition engines (CIE). Since the burning of the fuel-air mixture in CIE is initiated by compressed ignition of the fuel, the cetane number qualifies as a primary index of fuel quality as it describes the ease of its self-ignition. Theoretically, the cetane number is defined in the range of 15-100, which depends on its molecular structure of the substrate. The cetane number decreases when the number of double bonds in unsaturated fatty acid increases with the number of carbon atoms (Barabás and Todoruţ, 2011; Chuepeng *et al.*, 2010; Barabás and Todoruţ, 2010).

Table 12 International standard (EN 14214) requirements for biodiesel

Property	Units	Lower limit	Upper limit	Test-method
Ester content	% (m/m)	96.5	–	Pr EN 14103 d
Density at 15 ºC	kg/m^3	860	900	EN ISO 3675/EN ISO 12185
Viscosity at 40 ºC	mm^2/s	3.5	5.0	EN ISO 3104
Flash point	C	>101	–	ISO CD 3679e
Cetane number	–	51.0	–	EN ISO 5165
Water content	mg/kg	–	500	EN ISO 12937
Oxidation stability at 110 ºC	h	6	–	pr EN 14112 k
Iodine value	–	–	120	pr EN 14111
Polyunsaturated (P4 double bonds) methylester	% (m/m)	–	1	–
Monoglyceride content	% (m/m)	–	0.8	pr EN 14105m
Diglyceride content	% (m/m)	–	0.2	pr EN 14105m
Triglyceride content	% (m/m)	–	0.2	pr EN 14105m
Total glycerine	% (m/m)	–	0.25	pr EN 14105m
Methanol content	% (m/m)	–	0.2	pr EN 141101

Source: Demirbas *et al.* (2009); Atadashi *et al.* (2010)

. 2) Flash point

Biodiesel types have their flash point values higher than the minimum specifications prescribed by the U.S (93 ºC) and European (101 ºC) standards. Coconut-derived biodiesel is the one with lower flash point, but the value is still in the range. The main purpose to set the flash point specification standard is to ensure the FAME met with sufficient refinery standards and free of residual methanol, as methanol interfere with flash point (Hoekman *et al*., 2012).

3) Iodine value

Iodine value (IV) is a measurement of unsaturation in biodiesel. EN 14214 has set a maximum IV specification of 120 mg I_2/100 g FAME, whereas ASTM D6751(American Society for Testing and Materials) does not follow a specification standard. Biodiesels from soy and sunflower biomass are little over the standard limit, while biodiesel from rapeseed is slightly lower than the standard. Some biodiesels, such as those from camelina and safflower have even higher IV levels with respect to EN 14214 and are usually be qualified as "off-spec". Other biodiesel types showed lower than the 120 IV level, for example such as coconut-derived biodiesel is highly saturated and has an unusual low IV of about 19 (Hoekman *et al*., 2012).

4) Cloud point, pour point and cloud filter plugging point

Two principal parameters, at low-temperature define the application of biodiesel, are cloud point (CP) and pour point (PP). Cloud point is the temperature at which the wax in the diesel forms a cloudy appearance, inducing the thickening of oil and blocking the fuel injectors and fuel filters in engine. Pour point is the temperature at which the amount of wax is sufficient to solidify the fuel. Biodiesel has significantly high CP and PP values compared to the conventional diesels. Cold filter plugging point (CFPP) is the lowest temperature, expressed in 1°C, at which a certain volume of diesel fuel in a specified time and under cooled conditions still passes through a standardized filtration device. This property is very important as in cold temperature countries, a high CFPP valued diesels would clog up vehicle engines more easily (Su *et al*., 2011; Gopinath *et al*., 2009; Saxena *et al*., 2013)

5) Oxidative stability

Oxidative stability is one of the crucial fuel properties according in-use performance of biodiesel. The reason for this exception is that oxidative stability is determined by the compositional and functional properties of FAME and also by the age of the biodiesel and the conditions applied for its storage. Moreover, several biodiesel samples contain additives that confer stability without affecting the whole composition (Hoekman *et al.,* 2012). In general, oxidative stability is influenced by unsaturation leading to poorer stability, although the autoxidation of unsaturated fatty is highly dependent on the number of double bonds and their position (Refaat *et al*., 2009).

6) Other properties

Average chain lengths and average degree of unsaturation were also examined. Usually, biodiesels have an average chain lengths varying from 17 to 19. Camelina-derived fuel has the longest chain length of 19.1, whereas coconut biodiesel has a shorter chain length of only 13.4. The average degree of unsaturation varies significantly across the range – from as low as of 0.12 (for coconut) to a high of 1.81 (for camelina). These variabilities in unsaturation are the most important factors to explain many other differences in properties and performance capabilities among the wide range of biodiesel types (Hoekman *et al*., 2012).

Sankh *et al*. (2013) reported that the lipids produced by *Pichia kudriavzevii* MTCC 5493 cultivated on rotten fruits have high proportion of oleic acid followed by palmitic, linoleic acid, and stearic acids. When the extracted lipids were converted into biodiesel, its fuel properties were: acid value (EN14104) at 0.56, iodine value (EN14111) at 53, sulfated ash (%) (IS1448P:04) at 0.018, sediments (%) (D 2709) at 0.01 and conversion (%) (3/27 test) 100.

Munch *et al*. (2015) reported the mathematical estimation of the biodiesel derived from *Rhodosporidium babjevae and Rhodosporidium diobovatum* lipids. Their fuel properties were found to have good estimated cetane number (CE) values (57.3 for *R. babjevae* and 56.7 for *R. diobovatum*). However, the estimated cold-filter plugging point (CFP) (ºC) for both organisms were 6.5 and 11.4, respectively. The iodine value (IO) (gI_2/100g) at 78.0 for both organisms. The saponification (SA) were 191.1 and 195.4, respectively. The long-chain saturation factor (LCF) (wt.%) were 7.3 for *R. babjevae* and 8.9 for *R. diobovatum*.

8. Techno-economic analysis and environmental impact of biodiesel production

Triglycerides, for biodiesel production, are mainly derived from vegetable oils or animal fats. Recently, new oil sources have been investigated such as oil produced from oleaginous yeasts (Li *et al*., 2007) and microalgae (Scott *et al*., 2010). However, it should be noted that the new sources of oils in comparison to energy crops do not compete with the land for food plantation, thereby they can provide alternatives to the food vs. fuels land use (Rice, 2010). Biodiesel has been thoroughly tested for their energy production to be used as an alternative fuel in boilers and internal combustion engines either as in a pure form or blended with petroleum-based diesel. A number of techno-economic studies have increased with increase in the popularity of biodiesel and investigations on biodiesel industries were widely carried out in order to examine their economic influence and profitability.

The studied of techno-economic for biodiesel production technologies are important because it enables us to estimate the technical and economic efficiencies of alternative technologies and in order to choose the better performing options. The technical performances of biodiesel production are generally dependent on the energy usage and material input and output all of the production process (Gebremariam and Marchetti, 2018). In a different way, the technological evaluation can also be performed by assessing the technical benefits and limitations of the alternatives for specified quantity and quality of biodiesel production. The assessments include the process and equipment that required to achieve a given quantity and quality. This illustrates how simple and complex the whole production process of the alternative technology might be (Gebremariam and Marchetti, 2018).

Technological assessments offer the best scale for the economic performance assessment which should be performed based on the results obtained. To test if technically efficient production of alternative is cost-effective or not, various researchers used different economic indicators at a given specified market scenario. Zhang *et al.* (2003) used different fixed parameters, to evaluate the economic performances of four process alternatives, like total manufacturing cost, fixed capital cost, biodiesel break-even price, and after tax rate of return. However, the most widely used economic parameters are total investment cost and production cost to analyze which technology affordable.

As globally agreed, the emission of CO_2 from fossil fuel operated plants and the effect on the global environment is a major concern. Given the global concern over climate, new technologies needs to be introduced to manage and reduce the toxic emissions. Renewable sources, such as biomass, could replace the use of all or part of those fossil fuels and therefore plays an important factor in decreasing CO_2 emissions (Huang *et al*., 2013).

Singhabhandhu and Tezuka (2010) studied the cost and benefit of incorporation of a glycerin purification process to a biodiesel production. The cost and the benefit to evaluate the potential and have to compare the process with the original scenario. Moreover, they also examined different plant financial expenditure and governmental subsidies plans.

Zhang *et al*. (2003) studied the economic feasibilities of four continuous processes to produce biodiesel such as alkali and acid catalyzed processes when using the waste of cooking oil. It was found that the acid-catalyzed process when using waste cooking oil was less complex than the alkali-catalyzed process using the same oil character.

9. Assessment of environmental impact of biodiesel production

To assessment of environmental impact, the detailed of process and energy needs to make the biodiesel production in order to investigate the greenhouse gas emission at each stage of biodiesel production. The result determine the process that indeed emit less CO_2 than the use of fossil fuels (Gnansounou et al. 2009). Life cycle analysis consists of environmental impacts caused by a particular product or service throughout the process. The analyzes of the environmental impact including the flow materials chemical and energy. Analysis of the life cycle of a product consists of systematically examining the environmental impacts of products at every process of the cycle, and also include their final destination from extraction of raw materials to waste disposal (Varanda et al., 2011). There are four stages of LCA (Muralikrishna et al., 2017) (Figure 13)

(1) Goal and scope to define the life cycle in assessment and the end of the process. The criteria serving to system comparison and described in this step.

(2) Inventory analysis, this step could be description of material and energy flows within the product output system and especially the product interaction with environment.

(3) Details from inventory analysis serve for impact assessment. In this step show the result of impact categories. The importance of environmental impact is assessed by normalization and eventually also by weighting.

(4) Interpretation of a life cycle involves critical review and result presentation.

The inputs and outputs are then assessed for their adverse impacts on long-term sustainability resource, human health, and biodiversity. Once these are known, measures may be taken to mitigate the impact of the outputs that can impact on the environment. The utilization of LCA method are searching the most available life cycles with less negative environmental impact and choose important indicators of environmental behavior of organization (measurement and assessing techniques), with the assessment of the state of its environment. Moreover, marketing with the link on formulation of environmental declaration of eco-labeling (Muralikrishna *et al*., 2017).

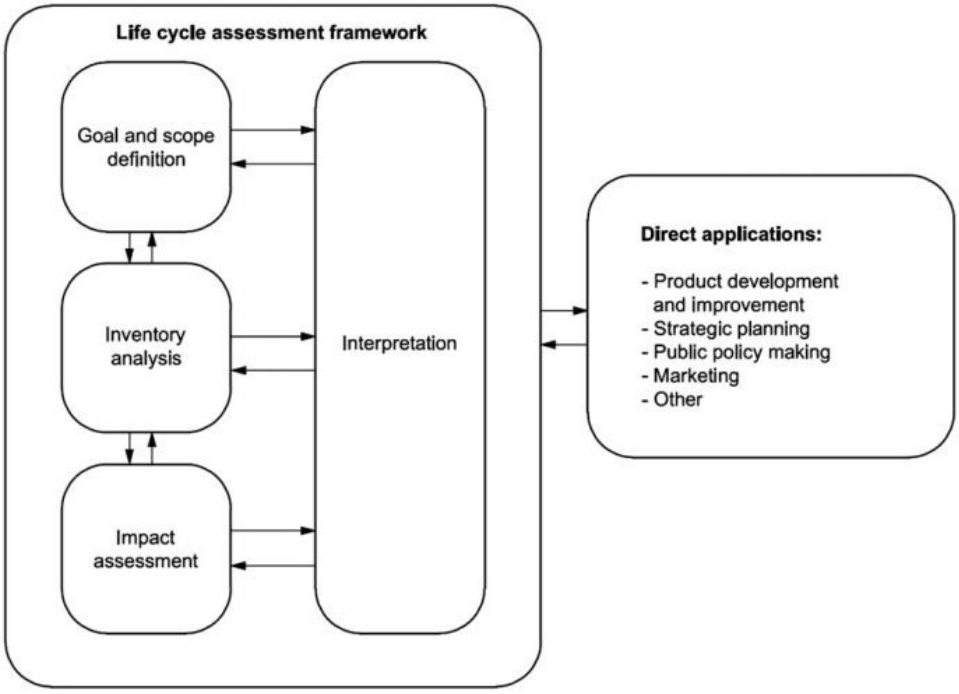

Figure 13 The LCA-framework according to ISO 14040.
Source: Heijungs *et al*. (2010)

CHAPTER 3

MATERIALS AND METHODS

Materials

1. Microorganisms and industrial wastes

Potent yeasts including *Trichosporonoides spathulata* JU4-57, *Kluyveromyces marxianus* X32 and *Rhodotorula mucilaginosa* G43 were obtained from the Bioprocess Engineering Laboratory (Faculty of Agro-Industry, Prince of Songkla University). *Yarrowia lipolytica* TISTR 5054 and *Yarrowia lipolytica* TISTR 5151 were purchased from Thailand Institute of Scientific and Technological Research (TISTR).

Industrial wastes were used as low-cost nutrient sources for cultivation of the yeasts. These included the wastes from carbonated soft drinks industry, brewery industry and biodiesel plant. Expired soft drinks were obtained from Haadthip company (Songkhla, Thailand). They were sonicated for 30 min to remove residual carbon dioxide before use. Crude glycerol was collected from the Biodiesel Plant (Faculty of Engineering, Prince of Songkla University, Thailand). For brewers' spent grain (BSG) and spent yeast cell (SYC), they were acid hydrolyzed before use.

2. Medium preparation and cultivation

2.1 Inoculum medium

The stock cultures were transferred to 250 mL Erlenmeyer flasks containing 50 mL of YPD culture medium (g/L, glucose 10, peptone 5, yeast extract 3, pH 6.0) followed by the incubation of the flasks at room temperature (30±2 ºC) with shaking at 200 rpm for 24 h and used as seed culture.

2.2 Expired soft drinks based medium

The effect of nitrogen-rich and nitrogen limitation conditions was studied by using expired soft drinks (total sugar at 40 g/L) added with and without ammonium sulfate at 0.5 g/L. The initial pH was 6.0. The seed culture was transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h.

2.3 Brewers' spent grain and spent cell yeast based medium

Brewers' spent grain (BSG) and spent yeast cell (SYC) were acid hydrolyzed and the total sugar concentration was adjusted to 40 g/L. The medium was added with $MgS0_4 \cdot 7H_2O$ 0.2 g/L, KH_2PO_4 0.5 g/L and $CaCl_2 \cdot 2H_2O$ 0.1 g/L, pH 6.0. The seed culture was transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h.

2.4 Crude glycerol based medium

For the crude glycerol based medium, the glycerol concentration was prepared at 40 g/L added with $MgS0_4 \cdot 7H_2O$ 0.2 g/L, KH_2PO_4 0.5 g/L and $CaCl_2 \cdot 2H_2O$ 0.1 g/L, pH 6.0. The seed culture was transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h.

3. Instruments

All the instruments, used in this work are listed and shown in Table 13, were in Faculty of Agro-Industry, Prince of Songkla University, Thailand.

Table 13 List of instruments

Instrument	Model	Source
Autoclave	SS-325	Tomy Seiko Co., Ltd, Japan
Balance	HF-1200	A&D, USA
Centrifuge	CF-10	Wise Spine, Korea
Haemacytometer		Diamond, Taiwan
Hot air oven	ED 115	Binder, USA
Laminar flow	-	Clean, USA
pH Meter	EF-201	Mettler Toledo, China
pH controller	4801	DIN, Taiwan
Shaker	VRN-480	Gwmmmy Industrial Corporation, Germany
Sonicator	E30 H	Elma, Germany
Spectophotometer	U-2000	Technical Cooperation, USA
Stirrer tank bioreactor, 5 L	-	S.T.S, Taiwan
Vortex	VM-19	Wise Mix, Korea

Analytical methods

1. Biomass concentration

The cell mass in culture broth was harvested by centrifugation at 8,000 rpm for 15 min. The cell pellets were washed with distilled water and once with acetone when cultivated on crude glycerol. After washing, the cell pellets were dried at 60°C in hot air oven until weighed to constant weight after cooling in a desiccator (Kavadia *et al.*, 2001).

2. Lipid extraction

The cell dry mass was blended with 2 mL chloroform/methanol (2:1) and mixing for 20 min in an orbital shaker at room temperature and the cell mass was broken by sonicator for 30 min. The same process was repeated three times. The whole solvent was evaporated and the extracted lipids were dried under vacuum (Bligh and Dyer, 1959).

$$\text{Lipid content (\%)} = \frac{\text{Oil weight (g)}}{\text{Cell dry weight (g)}} \times 100 \qquad (1)$$

3. Carotenoids determination

The carotenoids in the yeast wet cells were extracted using acetone. The suspension of cells in acetone was centrifuged and the supernatant was collected. The absorbance at 452 nm of acetone phase was measured using a spectrophotometer and the carotenoids content was calculated using the following formula (Sartory, 1982)

$$\text{Carotenoids (mg/g-cell)} = \frac{E_{452} \times \text{volume of extracted sample (mL)}}{A^{1\%}_{1cm} \times \text{volume of sample (mL)}} \qquad (2)$$

where $A^{1\%}_{1cm} = 2592$ (carotenoid absorption efficiency)

4. Characterization of crude glycerol

The glycerol concentration was determined using the method described by Kosugi *et al.* (1994). One mL of sample was mixed with 0.2 mL of potassium periodate (KIO_4) concentration at 0.0025 M for 5 min and then added with 0.5 M sodium arsenate of 0.05 mL. After 10 min, chromotropic acid reagent at 2 mL (110 mg of chromotropic acid disodium salt in 10 mL water with 120 mL of 50% H_2SO_4) was added and the mixture was boiled for 30 min. After cooling, the total volume was adjusted to 5 mL using water and the glycerol concentration was determined by measuring the absorbance at 570 nm. The glycerol concentration was calculated using pure glycerol standard concentration.

For methanol content, the crude glycerol solution was heated at 60ºC for 15 min. The weight loss of the solution was considered due to the methanol evaporation. For soap content, the pH of 50 mL crude glycerol solution was lowered to 1 by adding 85% H_3PO_4 to convert the soap to free fatty acid (FFA). The solution obtained was centrifuged at 5000 rpm for 20 min, and the top layer (FFA) was collected and weighed. The amount and content of soap was then calculated according to Eqs. (3) and (4) (Chen *et al*., 2017).

$$\text{Soap amount (g)} = 304 \times \text{FFA amount}/282 \quad (3)$$

$$\text{Soap content (\%w/v)} = \text{g of soap}/50\ \text{mL of crude glycerol solution} \times 100\% \quad (4)$$

Where 304 represents average soap molar mass and 282 is average FFA molar mass.

5. Determination of fatty acid composition by GC analysis

To convert the extracted lipids into fatty acid methyl esters (FAME), hydrolysis of the lipids followed by transesterification reaction were performed. The sample of lipids at 50 µL was added with 1 mL of KOH/MeOH (0.5 M) and heat up to 100 ºC for 5 min. After that the sample mixture was added with 400 µL of aq. HCl/MeOH (4:1, v/v) and heat up to 100 ◦C for 15 min in an oil bath. Afterwards, the sample was cooled before added with 2 mL water and then extracted with petroleum ether (2 mL).

The organic layer was dried over anhydrous Na_2SO_4 evaporated and redissolved with $CHCl_3$ in 500 µL. The sample at 0.5 µL was used for measurement by gas chromatography (GC). The fatty acid profiles was 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. The operating conditions were as follows: inlet temperature 290 °C, oven temperature initial 210 °C hold 12 min ramp to 250 °C at 20 °C/min hold 8 min and detector temperature 300 ºC. The fatty acids were identified by comparison of their retention times with those of standard and the quantification being based on their respective peak areas and normalized (Jham *et al*.,1982).

6. Estimation of biodiesel fuel properties using fatty acid compositions of yeast lipids

The biodiesel fuel properties were calculated from empirical equations based on fatty acid compositions of yeast lipids (Ryu *et al*., 2013; Munch *et al.,* 2015). These include cetane number (CE), iodine value (IO), saponification value (SA) and cold filter plugging point (CFP). Following GC analysis, the molecular structures of the fatty acid methyl esters (FAME) of the yeast lipids were used to estimate the important fuel properties of biodiesel. The IO and SA were estimated using Eqs. (5) and (6), where P is the percentage of each individual FAME component by weight, M is the molecular mass of the FAME, and D is the number of double bonds present in the FAME:

$$SA = \sum (560 \times P)/M \quad (5)$$

$$IO = \sum (254 \times DP)/M \quad (6)$$

One these two values were obtained, Eq. (7) was used to estimate the CE:

$$CE = 46.3 + (5458/SA) – (0.255 \times IO) \quad (7)$$

The CFP calculate by the long-chain saturation factor (LCS) is required. It is estimated using Eq. (8):

$$LCS = (0.1 \times C16:0 + (0.5 \times C18:0) + (1\times C20:0) + (1.5\times C22:0) + (2 \times C24:0) \quad (8)$$

Using this value, the CFP can be estimated according to Eq.(9):

$$CFP = (3.1417 \times LCS) - 16.477 \quad (9)$$

7. Chemical Oxygen Demand (COD) and total nitrogen (TN) determination

Chemical oxygen demand (COD) was determined according to the procedures used in standard methods (APHA, 2005). Total nitrogen was measured by combustion method (AOAC Official Method).

8. Total sugar

The total sugar was analyzed by phenol-sulfuric method (Dubois, 1956). One mL of 5% phenol solution was added to 1 mL of sample and stay chill for 3 min and then added with 5 mL of H_2SO_4 98%. The mixture was vortexed for 5 s. Lastly, the optical density (OD) at 490 nm was measured by spectrophotometer with the blank of 0.5 mL DI water.

9. Statistical analysis

The data from triplicate experiments were used to calculate mean values and standard deviations (mean ± SD). Statistical significance of the data was evaluated by one way ANOVA (analytical of variance) and Duncan's multiple range tests ($P < 0.05$) using SPSS version software 20.

Experimental methods

1. Cultivation of yeasts in the wastes from soft drinks industry

1.1 Screening of oleaginous yeasts under nitrogen-rich condition

The effect of nitrogen-rich condition was studied by using expired soft drinks (total sugar at 40 g/L) added with ammonium sulfate at 0.5 g/L, $MgSO_4 \cdot 7H_2O$, 0.2 g/L; H_2PO_4 0.5 g/L and $CaCl_2 \cdot 2H_2O$ 0.1 g/L. The initial pH was 6.0. The seed cultures

(1×10^7 cells/mL) were transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h. The samples were taken every 12 h and determined for dry cell mass weight and lipid production. The supernatant was analyzed for pH and total sugar.

1.2 Screening of oleaginous yeasts under nitrogen-limitation condition

The effect of nitrogen-limitation condition was studied by using expired soft drinks (total sugar at 40 g/L) added with $MgSO_4 \cdot 7H_2O$, 0.2 g/L; H_2PO_4 0.5g/L and $CaCl_2 \cdot 2H_2O$ 0.1 g/L. The initial pH was 6.0. The seed cultures (1×10^7 cells/mL) were transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h. The samples were taken every 12 h and determined for dry cell mass weight and lipid production. The supernatant was analyzed for pH and total sugar.

2. Cultivation of oleaginous yeasts in the wastes from brewery industry

2.1 Acid hydrolysis of brewers' spent grain and spent cell yeast

BSC and SYC were suspended in water at solid loadings of 5%, 10%, 15% and 24% and pH adjusted to 2 using 3 M sulfuric acid solution prior to thermal treatment at 121 ºC for 20 min (Ryu *et al.*, 2013). The pH was adjusted back to neutral using 10 N of NaOH. The samples were taken and determined for total nitrogen and total sugar. The solid loadings that gave the highest total sugar were chosen as nutrient sources for the yeasts. After hydrolysis process of these wastes, the inhibitory compounds such as furfural, 5-hydroxymethyl furfural (5-HMF) and acetic acid in hydrolysate were determined (Galafassi *et al.*, 2012).

2.2 Biomass and lipid production of yeast using BSC and SYC hydrolysate

The BSC and SYC hydrolysate with a total sugar concentration of 40 g/L were added with $MgSO_4 \cdot 7H_2O$, 0.2 g/L; H_2PO_4 0.5g/L and $CaCl_2 \cdot 2H_2O$ 0.1 g/L. The initial pH was 6.0. The seed cultures (1×10^7 cells/mL) were transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h. The samples were taken every 12 h and determined for dry

cell mass weight and lipid production. The supernatant was analyzed for pH, total sugar, HMF and furfural.

3. Cultivation of oleaginous yeasts in crude glycerol from biodiesel industry

For crude glycerol based medium, the glycerol concentration was prepared at 40 g/L added with $MgSO_4 \cdot 7H_2O$ 0.2 g/L, KH_2PO_4 0.5 g/L and $CaCl_2 \cdot 2H_2O$ 0.1 g/L. The initial pH was 6.0. The seed cultures (1×10^7 cells/mL) were transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h. The samples were taken every 12 h and determined for dry cell mass weight and lipid production. The supernatant was analyzed for pH and glycerol.

4. Fed-batch cultivation

The fed-batch fermentation was attempted to supply more nutrient sources for the selected yeasts to increase the lipid productivity. Start of the process was the same with the batch operation. The feeding started in the middle of the exponential phase and at every 12 h to maintain the concentration of nutrients at optimal level obtained from batch fermentation**.** The initial pH was 6.0. The seed cultures (1×10^7 cells/mL) were transferred into 50 mL sterilized medium in a 250 mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 72 h. Samples (2 mL) were collected every 12 h and centrifuged, and the supernatants were analyzed for pH, biomass and lipid production**.**

5. Repeated-batch fermentation

Repeated-batch fermentation was carried out in a 2 L bioreactor. The bioreactor contained 1 L sterilized medium and incubated at room temperature (Figure 14). The culture conditions were 10% (v/v) seed culture. The agitation rate was 200 rpm. The aeration rate was 0.5 ppm. The pH was maintained at 6.0. The culture broth was replaced with new medium every 48 h with the percent replacement of 80% (Cheirsilp *et al.*, 2015). Samples (2 mL) were collected every 48 h and centrifuged. The supernatants were analyzed for pH, biomass and lipid production.

Figure 14 The stirred tank bioreactor for oleaginous yeast cultivation under repeated-batch cultivation

6. Fatty acids composition analysis and evaluation of biodiesel fuel properties

The method for determination fatty acid methyl ester (FAME) was carried out on a HP6850 Gas Chromatograph. To convert the lipid extracted from the yeast to fatty acid methyl esters (FAME), hydrolysis of the lipids followed by transesterification reaction were performed. The sample of lipids at 50 µL was added with 1 mL of KOH/MeOH (0.5 M) and heat up to 100 ºC for 5 min. After that, the sample mixture was added with 400 µL of aq. HCl/MeOH (4:1, v/v) and heat up to 100 ºC for 15 min in an oil bath. The sample was cooled before added with 2 mL water and then extracted with petroleum ether (2 mL).

The organic layer was dried over anhydrous Na_2SO_4 evaporated and redissolved with $CHCl_3$ in 500 µL. The sample at 0.5 µL was used for measurement by Gas Chromatography (GC). The fatty acid profiles was 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. The operating conditions were as follows: inlet temperature 290 °C, oven temperature initial 210°C hold 12 min ramp to 250 °C at 20°C/min hold 8 min and detector temperature 300 ºC. The fatty acids were identified by comparison of their retention times with those of standard and the quantification being based on their respective peak areas and normalized (Jham *et al*.,1982).

7. Techno-economic Feasibility and Environmental Impact Analysis

For analysis the techno economic and environmental in this study, laboratory data were used to validate the engineering process flow and system model. Figure 13 shows the system model that was modularly constructed. The model could help to facilitate the analysis of alternative processing methods and processing technologies. The methods below present details on the baseline process for alternative processing of lipid production by oleaginous yeast until the biodiesel production process. The system model serves as the foundation for techno-economic analysis (TEA) and life cycle assessment (LCA) calculations which provide the basis for determining sustainability.

7.1 Techno analysis

This section begins by summarizing the options for lipid production from various industrial wastes by the suitable oleaginous yeast followed by description of the steps used, including acid hydrolysis of biomass into fermentable sugars, yeast cultivation, harvesting of yeast cells, and direct transesterification of yeast lipids into biodiesel. The mass balance employed for the economic analysis. The following lipid production models were studied;

- Model I: the use of lignocellulosic wastes (brewers' spent grain from brewery industry)

- Model II: the use of carbohydrate-rich wastes (spent cell yeast from brewery industry)

- Model III: the use of sugars-based liquid wastes (expired soft drinks from beverage industry)

- Model IV: the use of glycerol wastes (crude glycerol from biodiesel industry)

7.2 Economic analysis

A scale factor of 1,000 L or 1 m^3 was used in order to adjust the investment for the electricity. The capacity was selected based on an economic case study where the specific investment cost reaches a constant value, the price of inputs, capacity and operation costs as well as key financial assumptions along with sources. All the values are presented in US$. The capital costs for the process units are based on estimates and

the corresponding units from the literature. The operating costs are estimated based on typical industrial-scale operation.

7.3 Assessment of greenhouse gas emissions of biodiesel production

A life cycle inventory analysis is completed for each biodiesel production model. The environmental interventions studied are energy requirements and GHG emissions. The GHG taken into account includes three types of gases, namely, CO_2, CH_4 and N_2O. In the assessment of GHG emission reduction, the GHG emission gases was calculated in units of carbon dioxide equivalent (CO_2-eq) using the Global Warming Potential (GWP) values according to the 2006 IPCC Guidelines for National Greenhouse Gas Inventories (IPCC, 2016) and aggregated using Intergovernmental Panel on Thailand Greenhouse gas Management Organization (Public organization) (PGO).

$$\text{GHG emission} = \text{activity data} \times \text{emission conversion factor}$$

There are four step for the LCA methodology according to the ISO 14000 series (ISO 14041-43) including (1) goal and scope definition; (2) inventory analysis; (3) impact assessment; and (4) interpretation. The case study was based on a lab-scale lipid production from different types of industrial wastes by oleaginous yeasts.

(1) Goal and scope definition

The aim and scope of this study is to determine the environmental impact of biodiesel production using oleaginous yeasts grown on agro-industrial wastes and to determine which model has a greater environmental impact. This study does not cover the entire life cycle (cradle-to-grave) evaluation of the product, but rather it studied a gate-to-gate system boundary beginning with the acid hydrolysis of biomass into fermentable sugars, cultivation, harvesting (integrated lab-scale) and lipid extraction (lab-scale with estimated energy requirements) and did not get to the end of life process (disposal). Overall goal of the preliminary study was to compare life cycle energy and life cycle CO_2. The functional unit for this system boundary is one kilo gram biodiesel. A flow chart of the process of biodiesel production is shown in Figure 13. The use phase, disposal phase and transportation phase of the products are omitted in this study.

The whole CO_2 emission values were investigated from Models I, II, III and IV.

(2) Inventory analysis

The results of the LCA environmental impact have been quantified in relation to the functional unit, the functional unit must be quantifiable. The same functional unit can allow the comparison of results with similar LCA studies. The functional unit provides a reference that relates inputs and outputs (Okoro *et al*., 2012). The functional unit performed by a product and shows how much of this function is considered necessary to ensure that the LCA results are comparable. The inventory data for the biodiesel production were collected and calculation were made to quantify the input from the environment and the output to the environment by 1 kg biodiesel. This study focused on resources usage, energy usage, chemical, wastes and by-products from biodiesel production. The LCA covers the fundamental processes of cultivation, harvesting, drying and lipid extraction. The overall LCA system boundary is shown in Figure 15. Almost all LCA carried out on microorganisms to date have looked at the direct process related impacts from microorganisms with none fully considering indirect effects (Collet *et al*., 2015).

The system boundary and LCA study according to the following parameter:

-From gate-to-gate, start from yeast cultivation and ends with biodiesel production as the main product.

-The biodiesel production from lipid is carried out via direct transesterification.

-Main energy inputs will be included throughout the life cycle.

-Emissions of wastewater and other types of air pollutants are not covered in the LCA. (Khoo *et al.*, 2011)

-Wastes and among the wastes used (solids or wastewater) treatment is not covered in the LCA

(3) Impact assessment

This study focuses on emissions of greenhouse gases (GHG) from renewable energy sources from biodiesel production. The greenhouse gas emissions were calculated in carbon dioxide equivalent (CO_2-eq) which is a standard unit. The impact of GHG emissions in terms of the CO_2 would create the same amount of warming.

The amount CO_2- equivalent can be expressed from a carbon footprint of all the process (Peng *et al*., 2013). This is necessary to undertake detailed of the life cycle calculations of the processing energy needed to make the biodiesel production, in order to quantify the GHG emissions at each stage of the process of environmental impact.

(4) Interpretation

The interpretation of the environmental impact, a normalization step relates the indicator values to reference values (Brentrup *et al*., 2004). The results and recommendations should be analyzed, concluded and explained during the interpretation phase in accordance with the results of the previous LCA phases and the results of the LCA should be reported.

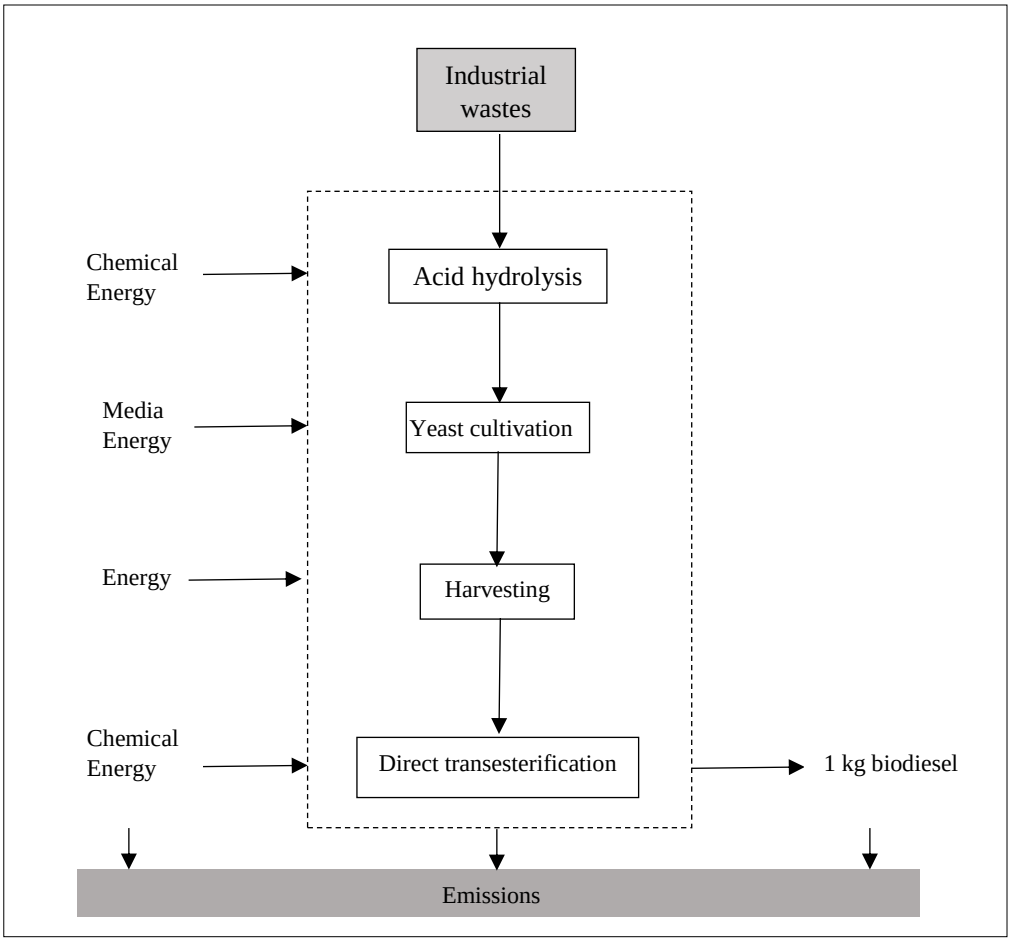

Figure 15 Life cycle system boundary of the lipid production by oleaginous yeasts

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Screening of oleaginous yeasts on agro-industrial wastes

4.1.1 Cultivation of oleaginous yeasts on expired soft drinks (ES)

As the expired soft drinks (ES) contained high amount of sugars (48.04±0.97 g/L), it was considered as a low-cost nutrient source for cultivation of oleaginous yeasts. The sugar compositions in the ES were: sucrose, glucose and fructose at 5.41±0.42 g/L (12.73 %), 11.67±0.02 g/L (27.46 %) and 25.41 g/L (59.80 %), respectively. The nitrogen concentration was only 0.135±0.01 g/L similar sugar compositions have been reported by Dwidar *et al.* (2012). In addition to the availability of sugars, carbon-to-nitrogen (C/N) ratio is one of important factors affecting both cell growth and lipid accumulation. Although high C/N ratio can facilitate the accumulation of lipids as carbon reserve material (Ratledge *et al.*, 2002) too high C/N ratio or very low nitrogen will depress cell propagation, which in many cases restricts biomass production (Cescut *et al*., 2014). The low biomass concentration then led to the low overall lipid production. To evaluate the suitable condition for attaining high biomass with lipid content, the cell growth and lipid content of the yeasts were evaluated under nitrogen-rich (ES added with ammonium sulfate) (C/N 74) and nitrogen limitation (using ES as a sole nutrient source) (C/N 126).

The effect of nitrogen-rich condition on the yeast cell growth and lipid content are shown in Figure 16a. All yeasts could grow well on ES added with ammonium sulfate (nitrogen-rich condition) but in different levels. The highest value of biomass was obtained with *Y. lipolytica* TISTR 5151 (12.4±0.7 g/L) at 72 h, followed by *T. spathulata* JU4-57 and *R. mucilaginosa* G43 (7-9 g/L). While *Y. lipolytica* TISTR 5054 and *K. marxianus* X32 gave relatively lower biomass of 4-5 g/L. The lipid contents of five oleaginous yeasts were evaluated in two phases: mid-log phase (12-36 h) and late-log phase (48-72 h) (Figure 17a). It was found that the lipid contents during mid-log phase were higher than that during late-log phase. The decrease in lipid content during late-log phase could be due to the unsuitable C/N ratio change which led to the conversion of accumulated lipid into other metabolites such as citric acid (Beopoulos *et al*., 2009). Braunwald *et al.* (2013) reported that the lipid production by red

oleaginous yeast *Rhodotorula glutinis* increased with increasing C/N ratio. While Wu *et al.* (2010) reported that the lipid accumulation by *Rhodosporidium toruloides* Y4 was effective regardless of the presence of high or low concentration of nitrogen sources. When the nitrogen concentration was used at 20 g/L, its lipid content was 62.2% and lipid production was high as 0.205 g/g glucose.

The effect of nitrogen limitation on the yeast cell growth and lipid content are shown in Figures 16b and 17b, respectively. Among the oleaginous yeasts tested, *R. mucilaginosa* G43 and *Y. lipolytica* TISTR 5151 could grow under nitrogen limitation better than other strains (Figure 15a). The highest value of biomass was obtained with *R. mucilaginosa* G43 at 72 h (5.2±0.77 g/L) followed by *Y. lipolytica* TISTR 5151 (2.7±0.05 g/L). While other oleaginous yeasts could not grow and gave very poor biomass of 0.75-1.45 g/L. It should be noted that although the yeasts could not grow well under nitrogen limitation but they could accumulate lipids at higher levels (21-46%, especially *Y. lipolytica* TISTR 5151 and *T. spathulata* JU4-57) than those under nitrogen-rich condition (16-28%).

It was obvious that the yeasts could grow well under nitrogen-rich condition and gave high biomass but with low lipid content indicating that the high nitrogen content stimulated the cell growth rather than the lipid synthesis. While under nitrogen limitation the yeasts grew less but accumulated higher lipid content. The sugar consumptions by the yeasts cultivated under nitrogen-rich condition were higher than those cultivated under nitrogen limitation. These results are consistent with those of Munch *et al.* (2015) found that two oleaginous yeasts, *R. babjevae* and *R. diobovatum*, accumulated high lipids under nitrogen limitation. In addition to nitrogen, many factors such as the medium composition, C/N ratio and culture conditions including temperature and pH may also have significant influences on cell growth and lipid accumulation of oleaginous yeasts. It has been reported that when oleaginous yeasts were cultivated at the pH near neutral they could give higher lipid production (Papanikolaou *et al*., 2004). In this study, the final pH in the culture medium of five oleaginous yeasts strains under nitrogen-rich condition, were in the range of 5.6-7.5 which were not significantly different to those under nitrogen-limitation condition. The use of high sugar concentration and high C/N ratio, principally channel sugar catabolism towards synthesis of intra-cellular lipids (Saenge *et al.*, 2011; Saenge *et al.*,

2011; Fei *et al*., 2011). Based on the above results, it was clear that all yeasts responded to nitrogen limitation in the typical manner of oleaginous yeasts.

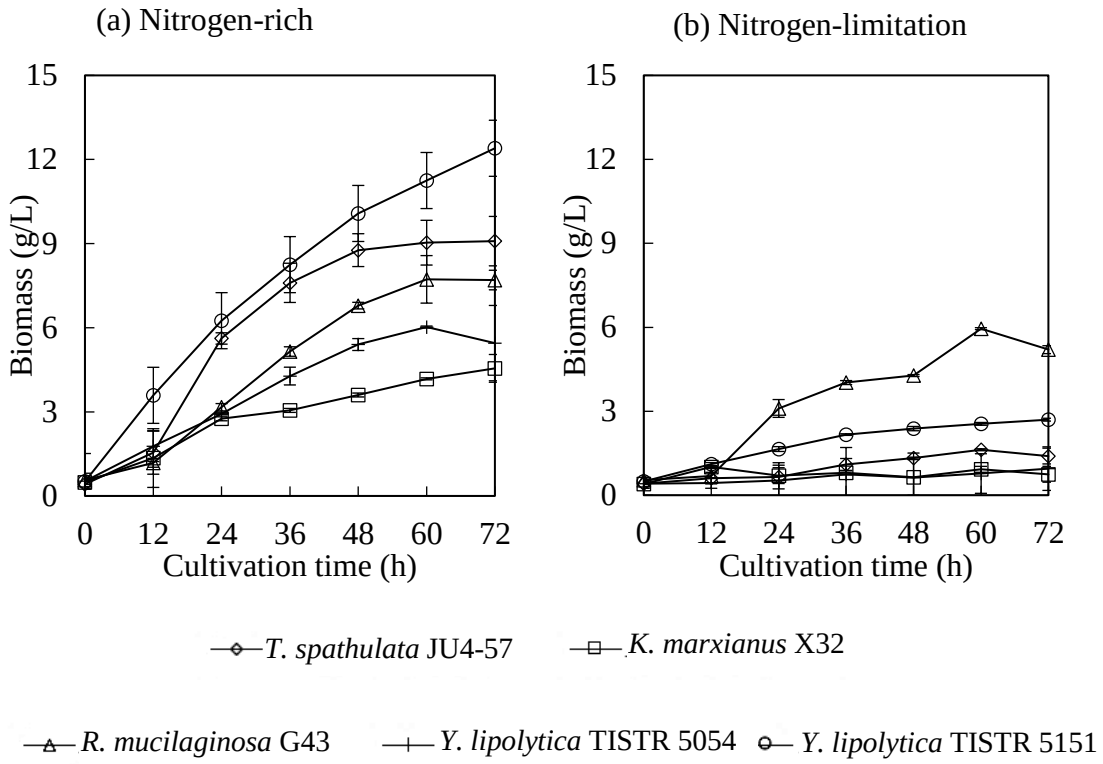

Figure 16 Effect of nitrogen-rich (a) and nitrogen-limitation (b) conditions on cell growth of five oleaginous yeasts. The yeasts were cultivated in expired soft drinks added with ammonium sulfate as a nitrogen source under nitrogen-rich condition. Under nitrogen-limitation, the yeasts were cultivated in expired soft drinks as a sole nutrient source.

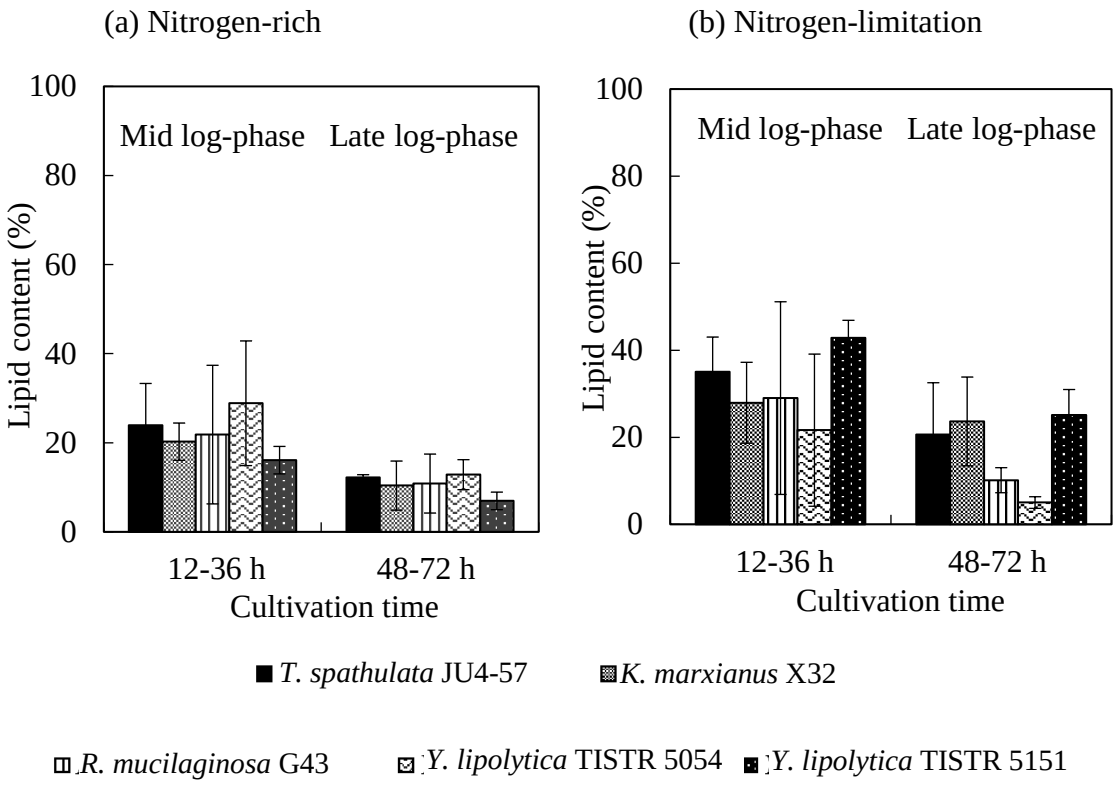

Figure 17 Effect of nitrogen-rich (a) and nitrogen-limitation (b) conditions on lipid content of five oleaginous yeasts.

These results suggest that all oleaginous yeasts had the ability to grow well on ES supplied with nitrogen source but only some of them could grow and accumulate lipids when using only ES as a sole nutrient source. It should be noted that the use of only ES for cultivation of the yeasts is more cost- effective. At this point, *R. mucilaginosa* G43, *Y. lipolytica* TISTR 5151 and *T. spathulata* JU4-57, were selected as potential yeast strains for SCO production using ES due to their ability to grow and produce considerable amount of lipids without the addition of external nitrogen source.

4.1.2 Cultivation of oleaginous yeasts on solid wastes from brewing industry

4.1.2.1 Acid hydrolysis of brewers' spent grain (BSG) and spent yeast cell (SYC)

Figure 18 illustrates the preparation of brewers' spent grain (BSG) and spent yeast cell (SYC) as the nutrient sources for cultivation of oleaginous yeasts. Table 14 shows the BSG composition that contained mainly 24.49±0.91 % w/w hemicellulose, 20.86±0.60% w/w cellulose, 23.05% w/w protein and 9.62 % lignin. The composition of BSG used in this study was similar with those previously reported (Sitepu *et al.*, 2014; Senthilkumar *et al*., 2010; José *et al*., 2013; Plaza *et al*., 2017). These compositions may be varied relate to the kind of barley, collecting time, malting and mashing condition and the quality and type of engine added in the brewing production process (Mussatto *et al.*, 2006; Vieira *et al.*, 2017; Pejin *et al*., 2015). The spent yeast cell (SYC) from brewing process is one of interesting nutrient sources due to its high content of carbohydrate and protein. The SYC used in this study comprised of 46.97±0.07 %w/w protein, 21.32±0.08 %w/w carbohydrate and 5.73±0.57 %w/w lipid (Table 14). The high protein content in SYC indicates its prospect use as inexpensive protein source (PODPORA *et al.*, 2016). It has been reported that SYC also contained high amounts of vitamins and minerals (Chen *et al.*, 2011). Both BSG and SYC were hydrolyzed by acid to produce fermentable sugars and release nutrients.

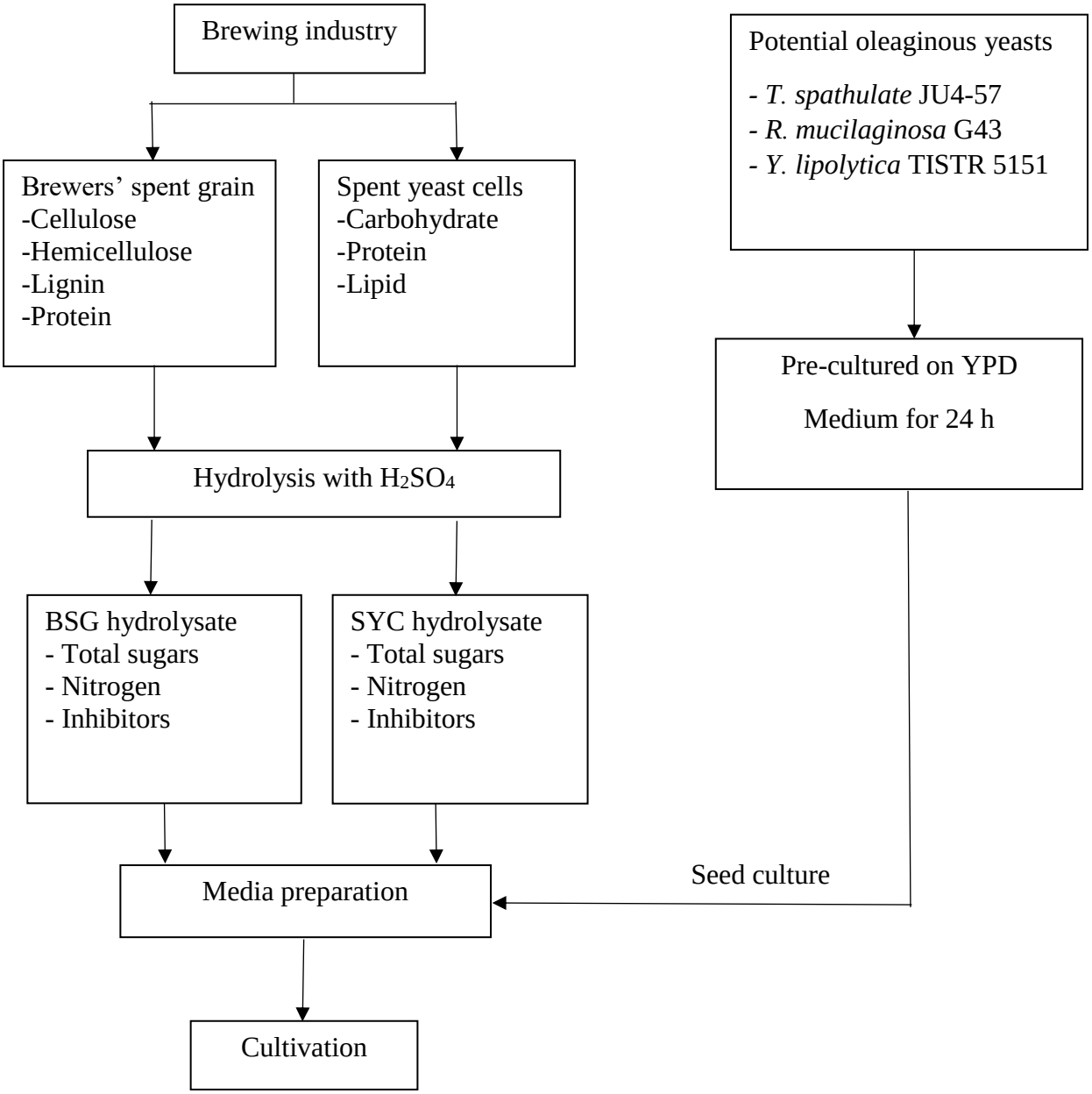

Figure 18 Flow chart for the preparation of Brewers' spent grain (BSG) hydrolysate and spent yeast cells (SYC) hydrolysate for cultivating oleaginous yeasts.

Table 14 Chemical composition of Brewers' spent grain (BSG) and Spent yeast cell (SYC)

Composition (% w/w)	BSG	SYC
Hemicellulose	24.49±0.91	-
Cellulose	20.86±0.60	-
Lignin	9.62±0.40	-
Carbohydrate	59.48±0.63	21.32±0.08
Protein	23.05±0.56	46.97±0.07
Lipid	5.05±0.02	5.73±0.57

Figure 19 shows the effect of BSG loadings on total sugar, total nitrogen and C/N ratio of the acid hydrolysate. With increasing the BSG loading, the concentrations of total sugar and total nitrogen in the hydrolysate increased. The highest total sugar obtained was 33.76 ± 0.67 g/L at 15% BSG loadings. The higher BSG loading at 24% gave a lower total sugar of 28.10 ± 0.45 g/L but a higher total nitrogen of 1.28 ± 0.103 g/L than those at 15% BSG loading. It has been reported that the concentration of the inhibitory compounds in the hydrolysate went up along with the increase in solid loading (Qureshi *et al.,* 2012). The use of high BSG concentration is attractive because it enables a high concentration of hydrolyzed products obtained (Plaza *et al*., 2017). BSG hydrolysate with the highest total sugar concentration 33.76 ± 0.67 g/L was used as nutrient source for cultivation of the selected yeasts *T. spathulate, R. mucilaginosa* G43 and *Y. lipolytica* TISTR 5151. The initial C/N ratio of BSG hydrolysate used for nutrient source was 47. The BSG hydrolysate was composed of glucose (22.02±0.8%) from cellulose, xylose (45.83±1.53%) and arabinose (32.13±2.3%) from arabinoxylan in hemicellulose. The BSG hydrolysate also contained furfural, 5-HMF and acetic acid at 1.58±0.07 g/L, 0.73±0.01 g/L and 1.07±0.04, respectively.

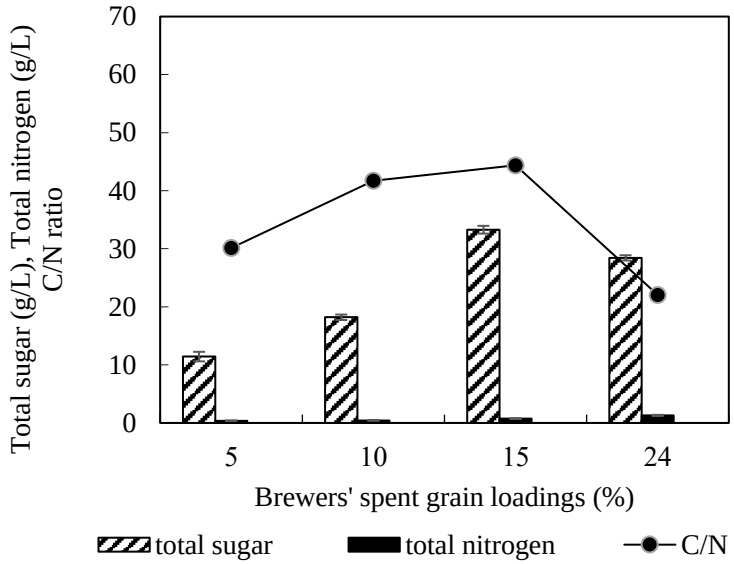

Figure 19 The effect of BSG loadings on the concentrations of total sugar, total nitrogen and C/N ratio in the hydrolysate

Figure 20 shows the effect of SYC loadings on the concentrations of total sugar, total nitrogen and C/N ratio in the hydrolysate. It was found that the SYC loading at 24% gave the highest total sugar at 62.12±0.15 g/L followed by 15% (44.97±0.79 g/L). Similarly, the SYC loading at 24% also gave the highest total nitrogen of 7.02±0.01 g/L followed by that at 15% (5.84±0.73 g/L) corresponding to the low C/N ratio of 8.84. The main sugars found in the SYC hydrolysate were glucose (31.43±0.38%) and mannose (69.57±1.04%) which released from β-glucan and mannan, respectively (Table 14). The SYC hydrolysate also contained furfural, 5-HMF and acetic acid at 1.88±0.01 g/L, 1.22±0.06 g/L and 1.03±0.03, respectively. Yang *et al*. (2014) also reported that the sugar components in the yeast cells are mainly glucose and mannose. In this study, the SYC hydrolysate with the highest total sugar and total nitrogen concentration was used as a nutrient source for cultivation of three selected yeasts.

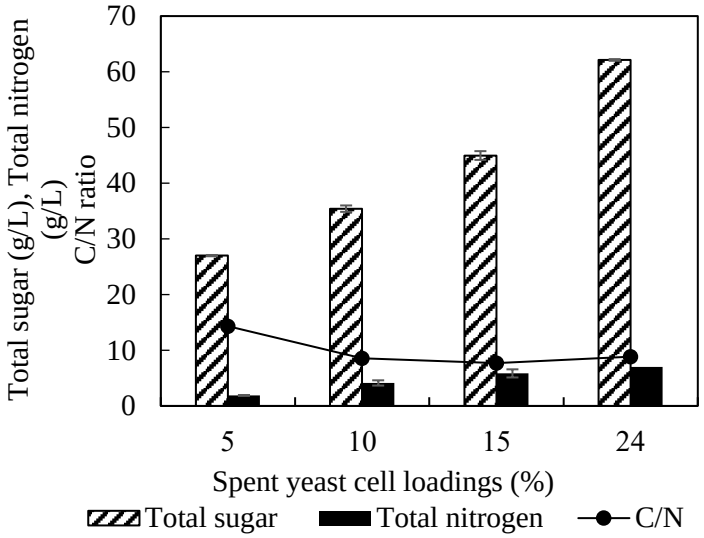

Figure 20 The effect of SYC loadings on the concentrations of total sugar, total nitrogen and C/N ratio in the hydrolysate

4.1.2.2 Cultivation of oleaginous yeasts on brewers' spent grain (BSG) and spent yeast cell (SYC)

Solid wastes from brewery industry used in this study were Brewers' spent grain (BSG) and spent yeast cell (SYC). The utilization of these wastes from brewery industry as a valuable product stream could help to increase the environmental and economic sustainability of the brewery business. The BSG is considered as a potential source of hemicellulose and cellulose that could be further converted into sugar. The hemicellulose, cellulose and lignin comprise of BSG used in this study were 24.49±0.91% (w/w), 20.86±0.60% (w/w) and 9.62±0.40% (w/w), respectively. BSG also contained protein at a high level of 23.05%. After acid hydrolysis, the BSG hydrolysate contained sugars at a concentration of 33.76±0.67 g/L and nitrogen at 1.28±0.103 g/L. The initial C/N ratio of 10.32. The sugar compositions were xylose (45.83±1.53%) and arabinose (32.13±2.3%) from hemicellulose and glucose (22.02±0.8%) from cellulose. Another solid waste from brewery industry is SYC containing protein, carbohydrate and lipids at 46.97±0.07%, 21.32±0.08% and 5.73±0.57%, respectively. The SYC hydrolysate was composed of sugars at 62.12±0.15 g/L and total nitrogen of 7.02±0.01 g/L. The initial C/N ratio of 3.5. The main sugars

found in the SYC hydrolysate were glucose (31. 43±0. 38%) and mannose (69.57±1.04%) which released from β-glucan and mannan, respectively.

Figure 21 shows the biomass and lipid content of three oleaginous yeasts using BSG hydrolysate. Three yeasts could grow on BSG hydrolysate and gave similar biomass concentration of 6-7 g/L. The lipid content of three oleaginous yeasts ranged from 13% to 24% during mid-log phase and slightly decreased when entering late-log phase. The concentration of furfural and HMF concentration detected in BSG hydrolysate were 1.11±0.03 g/L and 0.54±0.07, respectively. It has been reported that these furfurals and HMF (sugar derivatives) could hinder the mass cell growth and lipid accumulation of oleaginous yeasts. They could damage cell walls, cell membranes and inhibit RNA synthesis process in microorganisms (Hu *et al*., 2009; Heer *et al*., 2009; Liu *et al*., 2010). Compared to furfural, HMF was less toxic and several species of oleaginous yeasts can grow even at a high HMF concentration (2.5 g/L) (Galafassi *et al*., 2012: Liu *et al*., 2010). Depending on the sources of biomass and types of hydrolysis employed, the among of these sugar derivatives concentration in the lignocellulose hydrolysate can range between 0.5 and 11 g/L (Almeida *et al*., 2009; Tampitak *et al*., 2015). *T. spathulata* JU4-57 consumed more sugars of 24.18±0.49 g/L than the other two yeasts (17.45-19.02 g/L). The final pH in the medium of three oleaginous yeasts strains were slightly increased from 6.0 to 6.15-6.87.

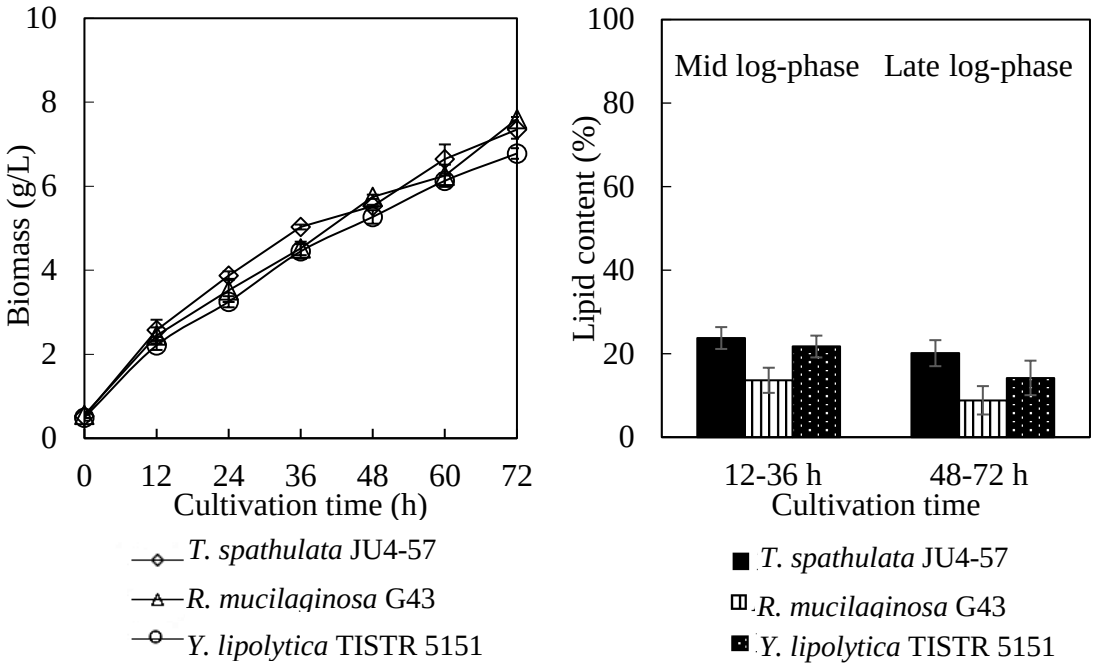

Figure 21 Cell growth and lipid content of three selected oleaginous yeasts cultivated on BSG hydrolysate.

The biomass and lipid content of oleaginous yeasts cultivated on SYC hydrolysate are shown in Figure 22. All yeasts could grow on SYC hydrolysate and gave the biomass in the same range of 4.0-4.8 g/L. Among three oleaginous yeasts, *T. spathulata* JU4-57 and *Y. lipolytica* TISTR 5151 produced lipids higher than *R. mucilaginosa* G43. The lipid content of three oleaginous yeasts ranged at 29-39% in the mid-log phase before decreased to 14-26% in the late-log phase. The concentrations of furfural and HMF in the SYC hydrolysate were 1.13±0.002 g/L and 0.76±0.01 g/L, respectively. The sugar consumptions by the yeasts were in the range of 21.41-28.77 g/L. The final pH in the media of three oleaginous yeasts strains increased up to 7.59-8.43. Ryu *et al*. (2013) reported that oleaginous yeast *Cryptococcus curvatus* grew well and accumulated lipids up to 37% on SYC hydrolysate. This study showed that it was possible to use the solid wastes from brewing industry as low-cost nutrient sources for cultivation of oleaginous yeasts. It should be noted that the yeasts grew better on BSG hydrolysate than SYC hydrolysate possibly due to the difference in their sugar compositions. Most of microorganisms metabolize sugars sequentially and normally

glucose is metabolized first followed by other sugars. The sequential utilization of the sugars by the microorganisms depends on strains and culture conditions (Hu *et al.*, 2011; Yu *et al*., 2014).

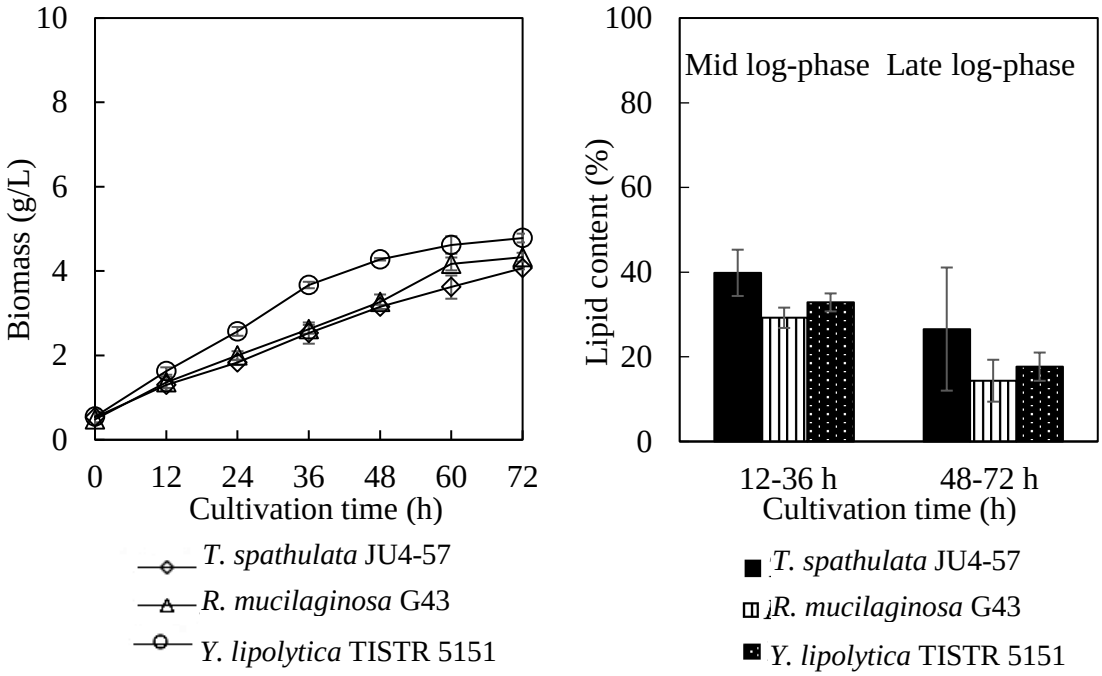

Figure 22 Cell growth and lipid content of three selected oleaginous yeasts cultivated on SYC hydrolysate.

4.1.3 Cultivation of oleaginous yeasts on by-product from biodiesel industry

The main by product from biodiesel production of oil is crude glycerol, its amount of 10% by mass of oil production. (Poli *et al*., 2014). As the biodiesel plants are increasing their production, the future supplies of crude glycerol are expected to increase. The compositions of crude glycerol vary due to the variation in feedstocks, types of catalyst, efficiency of reaction and downstream processes used during the biodiesel production (Uprety *et al*., 2017). The COD of the crude glycerol used in this study was as high as $3{,}062 \pm 0.87$ g/L but its nitrogen content was only 1.86 ± 0.17 g/L. The initial C/N ratio in this study at 67, which is suitable for lipid accumulation by the yeasts. The glycerol content in crude glycerol was 32.35 ± 0.19 % w/v. The pH was dramatically high approximately 9.19 ± 0.04 that is because the alkali which is catalyst was used during biodiesel production.

The biomass and lipid content of three oleaginous yeasts cultivated on crude glycerol based medium are shown in Figure 23. Only *R. mucilaginosa* G43 could grow well on crude glycerol and gave the highest biomass of 4.12 ± 0.10 g/L. While *T. spathulata* JU4-57 and *Y. lipolytica* TISTR 5151 could not grow well and gave relatively low biomass of 1.4-2.2 g/L. It has been reported that the presence of the impurities in crude glycerol might negatively affect the morphology and biochemical processes of the cells (Xu *et al*., 2012; Samul *et al*., 2014). The main impurities in crude glycerol included methanol, free fatty acid, soap and catalyst (Chen *et al*., 2017; Hájek *et al*., 2010; Liang *et al*., 2010). The methanol in crude glycerol could negatively influence cell membrane and its permeability (Samul *et al*., 2014; Venkataramanan *et al*., 2012). The impurities such as salts, which is decrease the van der waals effort in lipid membrane and cause swelling of the cell membrane. That was a negative effect of nutrition transportation through the cell membrane (Samul *et al*., 2014; Petrache *et al*., 2006). Moreover, soap (or detergent) is also one of inhibitors to microorganisms by decreasing their motility parameters, impairing their orientation and transforming their morphology (Kosmela *et al*., 2017; Rahman *et al*., 2017). Valerio *et al.* (2015) reported the compositional analysis of crude glycerol samples and found that these samples contained free glycerol (15.4%), free fatty acids (FFA) (2.1%), soaps (22.4%), fatty acid methyl esters (FAMEs) (30.9%) and others like methanol, water and ash. This

variability in glycerol samples can be attributed to the heterogeneous nature of the feedstock and process employed in each particular biodiesel factory

Among three yeasts, *R. mucilaginosa* G43 gave the highest lipid of 2.63±0.10 g/L at 48 h. While the lipid productions of *Y. lipolytica* TISTR 5151 and *T. spathulata* JU4-57, were lower at about 1.6 g/L. It should be noted that the lipid contents during mid-log phase (52-61%) were close to those during late-log phase (50-63%). *R. mucilaginosa* G43 also consumed glycerol (23.17±0.54 g/L) higher than *Y. lipolytica* TISTR 5151 (19.57±0.20 g/L) and *T. spathulata* JU4-57 (15.82±0.10 g/L). The final pH in the cultures of three oleaginous yeasts slightly increased up to 6.96-7.08. It has been reported that some fatty acids present in the crude glycerol could improve the level of lipids produced by the yeasts. It was also believed that the fatty acids might act as surfactants and stimulated the uptake rate of the fatty acids together with the nutrients (Munch *et al.*, 2015; Poli *et al*., 2014; Xu *et al*., 2012).

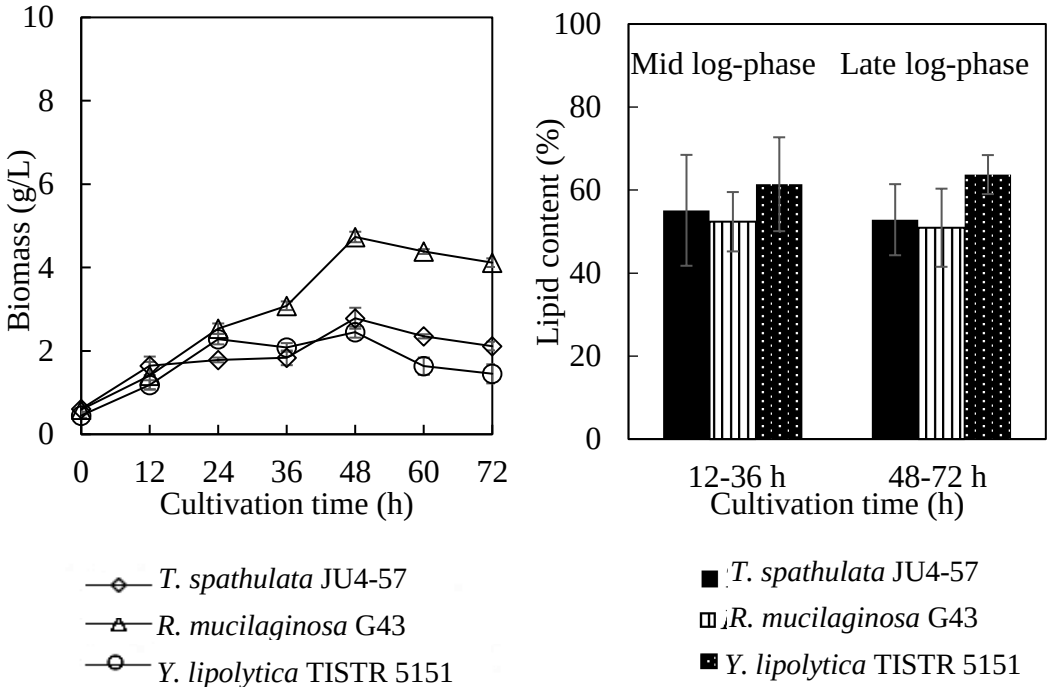

Figure 23 Cell growth and lipid content of three selected oleaginous yeasts cultivated on crude glycerol based medium.

4.1.4 Comparison of agro-industrial wastes as nutrient sources for oleaginous yeasts

Table 15 summarizes the performance of the most potent three yeasts cultivated on each agro-industrial waste for 72 h. This study has revealed that the expired soft drinks (ES) were good nutrient sources for cell growth and biomass production by oleaginous yeast *R. mucilaginosa* G43. *R. mucilaginosa* G43 grew well and gave higher biomass (5.2±0.77 g/L) than other two yeasts. It was possible that *R. mucilaginosa* G43 could assimilate fructose, the main sugar found in ES, better than other two yeasts. However, *Y. lipolytica* TISTR 5151 was more suitable for lipid production from ES due to its higher lipid content and lipid production than *R. mucilaginosa* G43. Among two solid wastes from brewery industry, brewers' spent grains (BSG) were better nutrient source for all oleaginous yeasts than spent yeast cells (SYC) possibly due to the suitable sugar compositions (glucose, xylose and arabinose) of BSG hydrolysate for the yeast cell growth. However, the yeasts accumulated lipids less than 15%. Although the yeasts could grow on SYC hydrolysate, they reached lower amount of biomass than those grown on BSG hydrolysate. As SYC hydrolysate mainly contained glucose and mannose, it was possible that mannose was not favorable sugar for the yeasts. Among three agro-industrial wastes, it was obvious that crude glycerol (CG) was the most suitable nutrient source for lipid accumulation by oleaginous yeasts. The oleaginous yeasts could accumulate lipids as high as 50-52% of their biomass when grown on CG. These levels were much higher than those cultivated on other three wastes. It could be concluded that among the yeasts and the wastes tested, *R. mucilaginosa* G43 cultivated on CG and *Y. lipolytica* TISTR 5151 cultivated on ES were the most suitable combinations for lipid production. In addition to lipids, *R. mucilaginosa* G43 also contained carotenoids at 3.78±0.29 mg/g-cell and protein at 16.46±0.17 %w/w. It then has high potential not only as single cell oil but also as a source of pigment and protein.

Table 15 Potential use of low-cost agro industrial wastes for cultivation of oleaginous yeasts

Potent yeasts	Agro-industrial wastes	Biomass (g/L)	Lipid content (%)	Lipid production (g/L)
T. spathulata JU4-57	ES	1.40±0.28	20.64±11.9	0.289±0.034
	BSG	7.35±0.22	20.12±3.12	0.650±0.020
	SYC	4.07±0.03	26.55±14.5	0.485±0.071
	CG	2.12±0.10	52.85±8.57	0.770±0.066
R. mucilaginosa G43	ES	5.20±0.77	10.14±2.88	0.527±0.015
	BSG	7.60±0.05	8.83±3.40	0.671±0.023
	SYC	4.33±0.10	14.36±4.95	0.622±0.031
	CG	4.12±0.10	50.92±9.41	2.098±0.197
Y. lipolytica TISTR 5151	ES	2.70±0.05	25.13±5.84	0.836±0.049
	BSG	6.78±0.13	14.20±4.13	0.670±0.028
	SYC	4.78±0.10	17.67±3.33	0.671±0.022
	CG	1.45±0.23	63.73±4.70	0.720±0.034

ES: Expired soft drinks (C/N 126); BSG: Brewers' spent grain hydrolysate (C/N 10.32); SYC: Spent yeast cell hydrolysate (C/N 3.5); CG: Glycerol (C/N 67)

The growth rate is an important factor affecting overall lipid production. Since lipids are accumulated in the cells, it would be more desirable to have yeast with a shorter lag phase, higher specific growth rate and a high cell density. Furthermore, rapid growth would speed up the condition of nitrogen limitation and the metabolic shift (Galafassi *et al*., 2012). Table 16 summarizes the kinetics of yeast cultivation on agro-industral wastes. The specific growth rates (h^{-1}) for *Y. lipolytica* TISTR 5151 cultivated on ES was comparable to that of *R. mucilaginosa* G43 cultivated on CG at 0.04 h^{-1}. While, *R. mucilaginosa* G43 cultivated on CG reached the highest lipid productivity of 0.040 g/L.h. The rapid growth and high lipid productivity of *R. mucilaginosa* G43 would be advantageous during larger-scale production of yeast-based biodiesel, as it would allow for shorter batch times and increased turnover of production.

In order to achieve high cell densities and high lipid accumulation, it is necessary to use high initial carbon source concentration in the batch culture. The lipid yield by *Y. lipolytica* TISTR 5151 cultivated on ES and *R. mucilaginosa* G43 cultivated on CG were evaluated. It was found that the lipid yield by *R. mucilaginosa* G43 was as high as 64.74 (mg-lipid/g-substrate). Moreover, the fatty acid metabolic pathway and overexpression of the key genes that can promote in lipid uptake and degradation are expected to give efficient cell growth and bioconversion of oil wastes into the oil products (Xie *et al*., 2017).

Table 16 Specific growth rate, lipid productivity, growth yield and lipid yield and cultivated on low-cost agro industrial wastes of oleaginous yeasts

Potent yeasts	Agro-industrial wastes	Specific growth rate μ (h^{-1})	P_L (g/L.h)	$Y_{x/s}$ (g-cell/g-substrate)	$Y_{P/s}$ (mg-lipid/g-substrate)
T. spathulata JU4-57	ES	0.017±0.005	0.0069±0.007	0.03±0.01	2.22±0.10
	BSG	0.06±0.002	0.027±0.002	0.28±0.006	28.67±0.48
	SYC	0.04±0.009	0.024±0.002	0.12±0.0004	7.45±0.62
	CG	0.03±0.006	0.03±0.004	0.10±0.003	50.53±2.94
R. mucilaginosa G43	ES	0.05±0.004	0.011±0.002	0.19±0.01	4.01±2.65
	BSG	0.05±0.006	0.011±0.0007	0.39±0.002	14.16±4.006
	SYC	0.04±0.003	0.015±0.002	0.16±0.01	10.20±2.89
	CG	0.04±0.005	0.040±0.009	0.14±0.006	64.74±1.51
Y. lipolytica TISTR 5151	ES	0.04±0.003	0.02±0.001	0.08±0.005	15.32±3.59
	BSG	0.06±0.005	0.021±0.003	0.32±0.004	33.38±2.83
	SYC	0.05±0.008	0.026±0.0002	0.20±0.003	26.99±1.57
	CG	0.04±0.004	0.037±0.001	0.04±0.003	37.93±2.67

*μ= specific growth rate; P_L = lipid productivity; $Y_{x/s}$ = growth yield (gram biomass/gram substrate); $Y_{P/s}$ = lipid yield (gram lipid/gram substrate).

* The data used for calculation of specific growth rate and lipid productivity were the data during 0-36h. The data used for calculation of biomass yield were the data at 72h.

4.1.5 Fatty acid composition of oleaginous yeast lipid produced from industrial waste

Conversion of the yeast lipids to FAMEs followed by GC analyses revealed the fatty acid compositions of the yeasts cultivated under different carbon sources. Table 16 shows the fatty acid compositions and their contents. The FAMEs profile of lipids from *T. spathulate* JU4-57 cultivated on BSG showed that the yeast accumulated mainly palmitic (C16:0) 13.53%, stearic acid (C18:0) 5.74%, oleic acid (C18:1) 53.26% and linoleic acid (C18:2) 22.27%. While *Y. lipolytica* TISTR 5151 cultivated on SYC accumulated mainly palmitic acid (C16:0) 10.33%, stearic acid (C18:0) 3.34%, oleic acid (C18:1) 47.02% and linoleic acid (C18:2) 30.57%. Moreover, the lipids from *Y. lipolytica* TISTR 5151 cultivated on ES were mainly composed of oleic acid (C18:1) as the predominant fatty acid (52.77%) followed by linoleic acid (C18:2) (15.92%) and palmitic acid (C16:0) (15.43%). While the lipids from *R. mucilaginosa* G43 cultivated on CG were mainly palmitic acid (C16:0) (40.47%) followed by oleic acid (C18:1) (26.86%), linoleic acid (C18:2) (20.62%). The comparative analysis showed that used *R. mucilaginosa* G43 grown on CG could produce FAMEs with the highest level of palmitic acid (C16:0) at 40.47% compared to 10.33%, 13.53% and 15.43% observed on SYC, BSG and ES, respectively. Whereas, *T. spathulate* JU4-57 cultivated on BSG gave the FAMEs with the highest level of oleic acid (C18:1) at 53.26% followed by ES and BSG that used the same yeast of *Y. lipolytica* TISTR 5151 (52.77% and 47.02%, respectively). While *R. mucilaginosa* G43 grown on CG contained oleic acid only 26.86%. These results indicate that the fatty acids mainly depend on the yeast strains and nutrient sources rather than the fermentation modes. Moreover, the fatty acid composition of oleaginous yeast is completely dependent on provided of medium composition and cultivation conditions

The similar fatty acid composition profiles of lipids from all models to those of plant oils indicate the potential use of yeast lipids as biodiesel feedstocks. The long-chain fatty acids with 16 and 18 carbon atoms also found in other oleaginous yeasts lipids profile. Li *et al.* (2010) has reported that the lipids from *Rhodotorula mucilaginisa* TJY15a which was grown on hydrolysate of cassava starch were mainly composed of palmitic acid and oleic acid higher than 85%, especially oleic acid was as high as 63.5%. *Rhodosporidium toruloides* ATCC 10788 also contained mainly 47.16%

of oleic acid when using crude glycerol as a substrate (Uprety *et al.*, 2017). Patel *et al.* (2014) reported that the oleaginous yeast *Rhodosporidium* spp. cultivated on glycerol showed high content of oleic acid (C18:1) 37.30% followed by stearic acid (C18:0) 25.10%, arachidic acid (C20:0) 22%, behenic acid (C22:0) 6.5%, palmitic acid (C16:0) 5%, and heptacosylic acid (C27:0) 3%.

A large number of saturated fatty acids present in FAMEs mitigate the biodiesel to auto-oxidation and also increase its shelf-life. While, the amount of unsaturated fatty acids determines its cold flow plugging properties (CFPP) (Patel *et al*., 2017) and a higher instability due to the easier oxidation of unsaturated fatty acid. The fatty acids produced from yeast that used the yeasts grown on BSG, SYC and ES contained more unsaturated fatty acid (77-80%) indicating a lower cold filter plugging point (CFP). Whereas, that from the yeast grown on CG had the highest ratio of saturated to unsaturated fatty acids indicating longer shelf-life.

Patel *et al.* (2017) reported that the biodiesel quality of the FAME profile of transesterification reaction relate to the fatty acids chain length and the degree of unsaturation which directly influences the quality of biodiesel. Biodiesel properties that are directly influenced by FAMEs profile including cetane number (CE), iodine value (IO), the cold-filter plugging point (CFP), the saponification value and the long-chain saturation factor (LCS). The main factor of biodiesel quality is the CE which is show the ignition quality of a diesel fuel. The most diesel engines are required a value above of 40 (Knothe *et al.*, 2005; Munch *et al*., 2015). A large number of CE imparts is better for ignition of biodiesel for example cold start behavior, smooth engine run and the complete combustion (Patel *et al*., 2016). Table 17 shows the biodiesel properties from the yeasts grown on BSG, SYC, ES and CG as nutrient sources, respectively. The observed CE values were 49.51, 47.84, 51.43 and 55.04, respectively. The numbers of double bonds in the fatty acid represent of the tendency of the biodiesel to react with oxygen and also relate to the number and position of double bonds and measure by the IO. The IO on BSG, SYC, ES and CG lipids 93.39, 99.88, 85.10 and 68.54 g I_2/100g, respectively which meet the European standard (EN-14214) that defines a maximum IO value of 120 g I_2/100g oil.

The important parameter for low temperature applications of a fuel is cold-filter plugging point (CFP). The long-chain saturation factor (LCS) estimates the impact of each long-saturated FAMEs on the CFP (Munch *et al*., 2015; Knothe *et al.,* 2008; Ramos *et al.,* 2009). The CFP is used to predict the behavior of the biodiesel at the low temperature. The cloud point is the temperature at that the wax first becomes visible when the fuel is cooled. The oil then can move at the lowest temperature. (Kaya *et al*., 2009). The FAMEs from BSG, SYC, ES and CG lipids would have CFP of -2.67, -7.97, -0.56 and 3.92 ºC, respectively. The saponification (SA) was also required. The SA value represents the milligrams of KOH needed to saponify 1 g of oil. A low SA indicates the high content of long chain fatty acids in the lipids. The SA of BSG, SYC, ES and CG lipids were from 201 to 208 mg KOH/g oil which were similar to those of *R. toruloides* (204.7 mg KOH/g oil) (Xu *et al*., 2012). These properties depend on the fatty acid profiles of lipids from oleaginous yeasts. Most oleaginous yeasts derived biodiesel could be alternative petroleum diesel fuel.

Table 17 Fatty acid compositions (%) of the yeast lipids grown on different carbon sources

Fatty acid compositions	Brewers' industry		Beverage industry	Biodiesel industry
	BSG	SYC	ES	CG
	T. spathulate JU4-57	*Y. lipolytica* TISTR 5151	*Y. lipolytica* TISTR 5151	*R. mucilaginosa* G43
Myristic acid (C14:0)	0.32	0.21	-	1.91
Palmitic acid (C16:0)	13.53	10.33	15.43	40.47
Palmitoleic acid (C16:1)	1.61	2.22	8.82	2.70
Stearic acid (C18:0)	5.74	3.34	7.04	4.69
Oleic acid (C18:1)	53.26	47.02	52.77	26.86
Linoleic acid (C18:2)	22.27	30.57	15.92	20.62
Linolenic acid (C18:3)	1.29	-	-	1.16
Lignoceric acid (C24:0)	0.26	-	-	0.86
Saturated fatty acids	19.85	13.88	22.47	47.93
Unsaturated fatty acids	78.43	79.81	77.51	51.34
SA	201.96	202.052	203.40	208.30
IO (g I2/100 g)	93.39	99.88	85.10	68.45
CE	49.51	47.84	51.43	55.04
LCF (wt.%)	4.39	2.7	5.06	6.49
CFP (ºC)	-2.67	-7.97	-0.56	3.92

SA, saponification value; IO, iodine value; CE, cetane number; LCF, long-chain saturation factor; CFP, cold-filter plugging properties: <0 (summer) <-10 (winter).

4.2 Fed-batch cultivation

4.2.1 Fed-batch cultivation of *Y. lipolytica* TISTR 5151 and *R. mucilaginosa* G43

From batch cultivation, it could be concluded that only *Y.lipolytica* TISTR 5151 could use ES as low-cost nutrient source for production of lipids (lipid content >20%). While BSG and SYC were more suitable nutrient sources for single cell protein rather than for single cell oil. Among four agro-industrial wastes, CG was the most suitable nutrient source for lipid production by all yeasts but only *R. mucilaginosa* G43 could grow and gave considerable amount of biomass with high lipid content. Therefore to produce lipids from agro-industrial wastes, *Y. lipolytica* TISTR 5151 and *R. mucilaginosa* G43 were cultivated on ES and CG, respectively and the fed-batch cultivations were performed to increase the lipid productivity of the yeasts. An exponential feed model was chosen and the feeding times were set in function of the yeasts' growth rate leading to different feeding times (24 h for *Y. lipolytica* TISTR 5151 and 36 h for *R. mucilaginosa* G43). Figure 24 shows the time-course of biomass, lipid production, lipid content and sugar concentration in the fed-batch fermentation of ES by *Y. lipolytica* TISTR 5151. The yeast continuously grew until 96 h. The lipid content increased to the maximum level of 40-46% during 24-48 h before decreased to 19.85% at 96 h. This indicated that ES could be a nutrient source for lipid production only during mid-log phase (until 48 h). The decrease of lipid content during late-log phase was possibly due to the lipid metabolism during unsuitable condition (Zhang *et al*., 2012). The feeding of the substrate increased the biomass of the yeast up to 3.78±0.16 g/L at 96 h. The productivity of lipids was also increased from 0.871±0.036 g/L.h in the batch to 0.921±0.036 g/L.h in the fed-batch. This could be because the supplementation of the nutrients could increase the growth rate and prolong the growth period (Li *et al*., 2007). Another explanation would be the increase of C/N ratios at each feeding in the fed-batch could enhance lipid accumulation process and resulted in higher lipid content than that of the batch (Louhasakul and Cheirsilp, 2013).

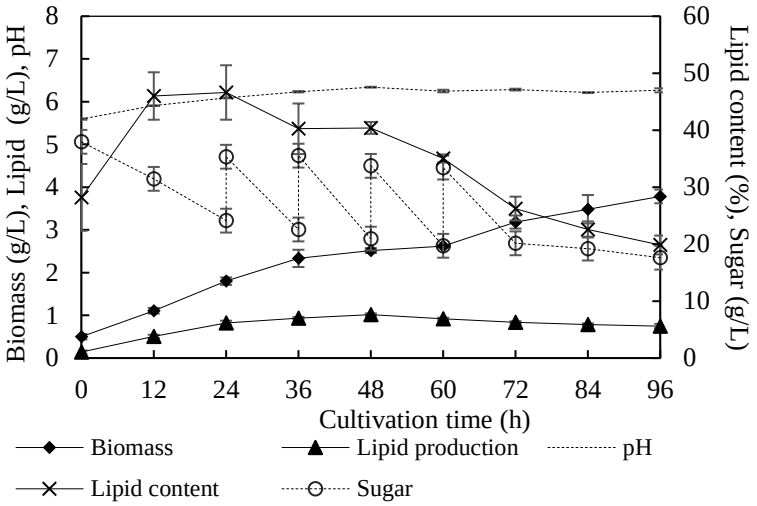

Figure 24 Fed-batch cultivation of *Y. lipolytica* TISTR 5151 using expired soft drinks (ES) The wastes were added to maintain concentration of carbon source.

Figure 25 shows time course of biomass, lipid production, lipid content and glycerol concentration in the fed-batch fermentation of CG by *R. mucilaginosa* G43. The yeast grew continuously until 96 h and gave the maximum biomass concentration of 6.13±0.22 g/L. The maximum lipid production of 1.85±0.10 g/L was obtained at 60 h before slightly decreased to 1.53±0.13 g/L at 96 h. The lipid content increased up to 61.84% at 24 h then slightly decreased to 26.89% at 96 h. When the yeast became acclimated to the CG, the biomass increased rapidly. The CG consumption rate became faster along with the increased biomass. Although the biomass was enhanced with the fed-batch, the lipid production from the fed-batch was lower than that from the batch. These results indicated that the fed-batch mode was not suitable for bioconversion of CG into lipids by *R. mucilaginosa* G43.

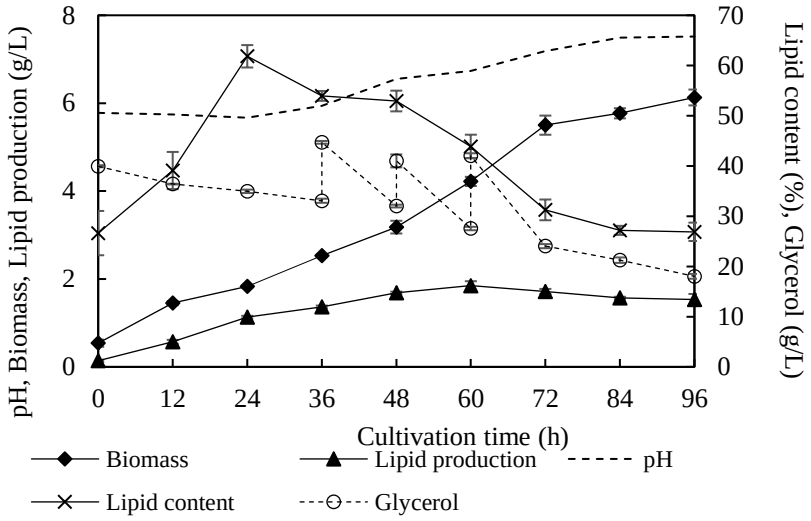

Figure 25 Fed-batch cultivation of *R. mucilaginosa* G43 using crude glycerol (CG) . The wastes were added to maintain concentration of carbon source.

However, the lipid production by the fed-batch was lower than that of the batch fermentation. The lipid content and the productivity of lipids in the fed-batch culture decreased to 41.80±9.66 % and 1.37±0.03 g/L.h, respectively (Table 18). This might be due to the inhibitory effect from the impurities in crude glycerol that accumulated at every feeding (Gao *et al*., 2016). It has been reported that the presence of the impurities in crude glycerol had a very negative effect on the morphology and biochemical processes of the cells (Samul *et al*., 2014). The main impurities in crude glycerol depend on the kind of raw glycerol used to produce biodiesel and condition of transesterification process (Hájek and Skopal, 2010). These include methanol, free fatty acid, soap and catalyst (Chen *et al.*, 2017; Liang *et al.*, 2010). The methanol in crude glycerol could negatively influence cell membrane and its permeability (Venkataramanan *et al*., 2012; Samul *et al*., 2014). Another group of impurities is salts, which could decrease van der waals force in lipid membrane and cause swelling of the cell membrane. This swelling exerts has a negative effect transportation of nutrition factors through the cell membrane (Petrache *et al.*, 2006; Samul *et al.*, 2014). These results indicated that the fed-batch mode was not suitable for bioconversion of crude glycerol into lipids by *R. mucilaginosa* G43. Therefore, another fermentation mode should be designed.

Table 18 Comparison of batch and fed-batch fermentation for production of biomass and lipids by *Y. lipolytica* TISTR 5151 and *R. mucilaginosa* G43

Yeast strain and industrial waste	Parameter	Batch	Fed-batch
Y. lipolytica TISTR 5151 cultivated on ES	Biomass (g/L)	2.70±0.005	3.18±0.15
	Lipid production (g/L)	0.87±0.03	0.93±0.03
	Lipid content (%)	25.13±5.84	33.89±7.14
	Specific growth rate (h^{-1})	0.044±0.003	0.046±0.0007
	Productivity of lipids (g/L.h)	0.871±0.036	0.921±0.036
R. mucilaginosa G43 cultivated on CG	Biomass (g/L)	4.12±0.10	5.50±0.22
	Lipid production (g/L)	1.86±0.08	1.36±0.03
	Lipid content (%)	50.92±9.41	41.80±9.66
	Specific growth rate (h^{-1})	0.043±0.005	0.042±0.0004
	Productivity of lipids (g/L.h)	1.821±0.034	1.37±0.03

* The data used for calculation of specific growth rate and lipid productivity were the data during 0-36h. The data used for calculation of biomass yield were the data at 72h.

4.2.2 Fatty acid composition and estimation of biodiesel properties of yeast lipids

Conversion of the yeast lipids to fatty acid methyl esters (FAMEs) followed by GC analyses revealed the fatty acid composition of the yeasts cultivated under different cultivation modes (Table 19). The lipids from *Y. lipolytica* TISTR 5151 cultivated on expired soft drinks in both batch and fed-batch fermentation were mainly composed of long-chain fatty acids with 16 and 18 carbon atoms including oleic acid as the predominant fatty acid (52-56%) followed by linoleic acid (15-21%) and palmitic acid (approximately 15%). It should be noted that the fatty acids of *Y. lipolytica* TISTR 5151 lipids were mainly unsaturated fatty acids (63-77%). While the lipids from *R. mucilaginosa* G43 cultivated on crude glycerol in both batch and fed batch mode were mainly palmitic acid (approximately 40%) followed by oleic acid (26-30%), linoleic acid (16-20%). These results indicate that the fatty acid compositions of the yeast lipids mainly depend on the yeast strains and nutrient sources. The similar fatty acid composition profiles of *R. mucilaginosa* G43 to those of plant oils indicate the potential use of its lipids as biodiesel feedstocks. The lipid compositions of the oleaginous yeasts in this study were also similar to those previously reported. The lipids from *Rhodotorula mucilaginisa* TJY15a which was grown on hydrolysate of cassava starch were mainly composed of palmitic acid and oleic acid higher than 85%, especially oleic acid was as high as 63.5% (Li *et al*., 2010). *Rhodosporidium toruloides* ATCC 10788 also contained mainly 47.16% of oleic acid when using crude glycerol as a substrate (Uprety *et al*., 2017).

Biodiesel properties are directly influenced by the fatty acid compositions of the oil sources. Cetane number (CE), iodine value (IO), cold filter plug point (CFP), saponification value and long-chain saturation factor (LCS) are biodiesel properties that can be estimated from the compositions of fatty acids. CE is considered as the main factor determining the quality of biodiesel. It represents a diesel fuel's ignition quality. For most diesel engines, the CE value should be higher than 40 (Munch *et al*., 2015; Knothe *et al*., 2005). Table 19 shows the biodiesel properties from the yeasts grown on expired soft drinks and crude glycerol as nutrient sources in different fermentation modes. The calculated CE value for lipids from *R. mucilaginosa* G43 in both fermentation modes were 55-56 while those from *Y. lipolytica* TISTR 5151 were 51-57. The IO is a measure of the biodiesel 's tendency to react with oxygen and depends

on the number and position of two bonds in the FAMEs. The IO is the iodine mass which reacts with a substance of 100 g. The IO of *R. mucilaginosa* G43 lipids were 63-68 I_2/100g oil and those of *Y. lipolytica* TISTR 5151 lipids were 63-85 g I_2/100g oil which meet the European standard (EN-14214) that defines a maximum IO value of 120 g I_2/100g oil. The important parameter for low temperature applications of a fuel is cold-filter plugging point (CFP). The long-chain saturation factor (LCS) estimates the impact of each long-saturated FAMEs on the CFP (Munch *et al*., 2015; Knothe *et al*., 2005; Ramos *et al*., 2009). The CFP is used to predict the behavior of the biodiesel at the low temperature. The cloud point is the temperature at which wax first becomes visible when the fuel is cooled. The pour point is the lowest temperature at which the oil specimen can still be moved (Kaya *et al*., 2009). The FAMEs from *R. mucilaginosa* G43 lipids would have CFP of 3.9-4.9, while those from *Y. lipolytica* TISTR 5151 lipids would have more varied CFP. The saponification (SA) value represents the milligrams of KOH needed to saponify 1 g of oil. A low SV indicate that the percent of long chain fatty acids in the lipids. The SV of *R. mucilaginosa* G43 lipids were approximately 208 mg KOH/g oil and that of *Y. lipolytica* TISTR 5151 lipids were 201-203 mg KOH/g oil which were similar to those of *R. toruloides* (204.7 mg KOH/g oil) (Xu *et al*., 2012). Based on the fatty acid profiles of *R. mucilaginosa* G43 lipids, it could conclude that the biodiesel produced oleaginous yeasts could also be used as a substitute for petroleum-based diesel fuel.

Table 19 Fatty acid compositions and estimated biodiesel properties of the yeast lipids

Fatty acid compositions	*Y. lipolytica* TISTR 5151		*R. mucilaginosa* G43	
	Batch	Fed-batch	Batch	Fed-batch
Myristic acid (C14:0)	-	-	1.91	2.53
Palmitic acid (C16:0)	15.43	15.73	40.47	39.24
Palmitoleic acid (C16:1)	8.82	-	2.70	2.09
Stearic acid (C18:0)	7.04	21.08	4.69	4.93
Oleic acid (C18:1)	52.77	56.90	26.86	29.03
Linoleic acid (C18:2)	15.92	6.28	20.62	16.48
Linolenic acid (C18:3)	-	-	1.16	1.22
Lignoceric acid (C24:0)	-	-	0.86	-
Saturated fatty acids	22.47	36.81	47.93	46.7
Unsaturated fatty acids	77.51	63.18	51.34	48.82
SA	203.40	201.20	208.30	208.67
IO (g I_2/100 g)	85.10	62.54	68.45	63.49
CE	51.43	57.47	55.04	56.26
LCF (wt%)	5.06	12.11	6.49	6.8
CFP (ºC)	-0.56	21.58	3.92	4.89

SA, saponification value; IO, iodine value; CE, cetane number; LCF, long-chain saturation factor; CFP, cold-filter plugging properties: <0 (summer) <-10 (winter).

The ratio of saturated fatty acids and unsaturated fatty acids of yeast lipids are different depended on the yeast strains and nutrient sources. More unsaturated fatty acid would lead to lower cold filter plugging point (CFP) and higher instability of biodiesel due to the easier oxidation of unsaturated fatty acids (Yen *et al*., 2015). 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 (Talebi *et al*., 2013). Figure 26 (a) shows the effect of different yeasts cultivated on different wastes on the content of saturated fatty acids and unsaturated fatty acids, namely *T. spathulate* JU4-57 cultivated on BSG hydrolysate under batch cultivation (BSG/T), *Y. lipolytica* TISTR 5151 cultivated on SYC hydrolysate under batch cultivation (SYC/Y), *Y. lipolytica* TISTR 5151 cultivated on ES under batch cultivation (ES/Y), *R. mucilaginosa* G43 cultivated on CG under batch cultivation (CG/R), *Y. lipolytica* TISTR 5151 cultivated on ES under fed-batch cultivation (ES/Y/F) and *R. mucilaginosa* G43 cultivated on CG under fed-batch cultivation (CG/R/F). After cultivation, at least 48% of the total FAMEs present in each culture were unsaturated fatty acids. The total unsaturated fatty acid (UFA) contents were recorded at 48.82% (CG/R/F), 51.34% (CG/R), 63.18% (ES/Y/F), 77.51% (ES/Y), 78.43% (BSG/T) and 79.81% (SYC/Y. Patel *et al.* (2015) reported the biodiesel production from a low cost non-edible lignocellulosic biomass from aqueous extract of *Cassia fistula* L. fruit pulp by yeast *Rhodosporidium kratochvilovae* HIMPA1. It was indicated that the fatty acid profile was changed when the oleaginous yeast was grown in non-edible lignocellulosic biomass of *Cassia fistula* L. fruit pulp. Interestingly, when this oleaginous yeast was grown on pulp and paper industry effluent as a culture medium it synthesized high quantity of long chain monounsaturated fatty acid (45.43%) and polyunsaturated fatty acid (15.91%) (Patel *et al*., 2017). When oleaginous yeast cultivated on crude glycerol, the percentage of saturated fatty acid increased while that of unsaturated fatty acid decreased possibly due to the composition of feedstocks that contained high content of palmitic (44%) (Mancini *et al.*, 2015). Fatty acid metabolism is also dependent on the carbon chain length, with two fatty acyl-CoA synthetase (ACS I and II) involved. In the peroxisome, ACS II converts the short-chain fatty acids into fatty acyl-CoAs. Inthe cytosol, ACS I converts the long-chain fatty acids into fatty acyl-CoAs, which can be used as substrates for lipid synthesis or further transported into the peroxisome for β-oxidation (Liu *et al*.,

2015; Xie *et al*., 2017). The results of this study also indicate that fatty acid composition can be affected from the type of nutrient source and mode of cultivation. The biodiesel quality under low temperature condition in terms of CFP (Figure 26b) is directly related with contents of saturated fatty acid and can be obtained by correlation between CFP and long chain saturated factor. The biodiesel based on yeast oil found to have poor CFP properties, as it contained the limited amount of long chain saturated fatty (Ryu *et al.*, 2013). Although, the CFP is a bit suboptimal but all these results implied that the yeast oil is quality suitable for the production of biodiesel production along with good oxidative stability and cetane number as per ASTM D6751-02 and EN 14214 guidelines.

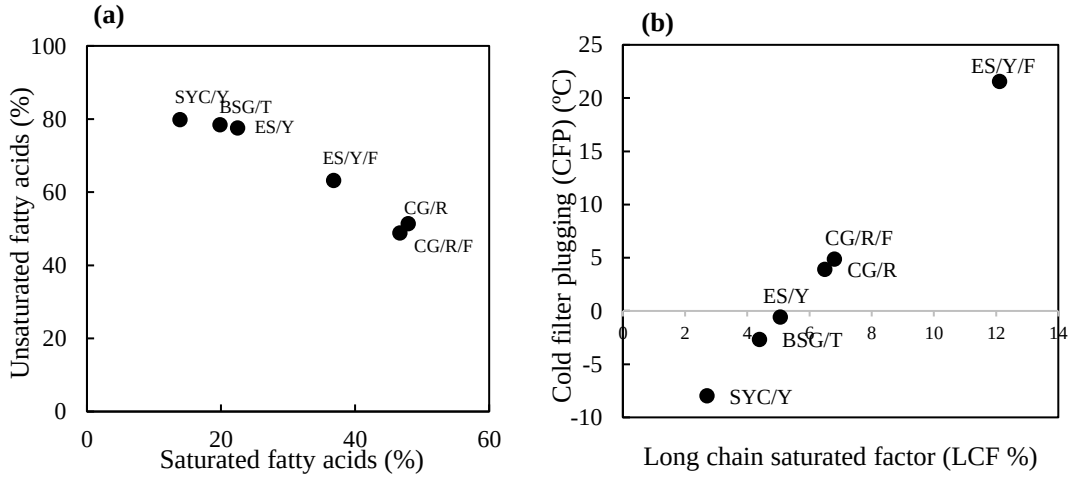

Figure 26 Saturated fatty acids and unsaturated fatty acids of the yeast lipids grown on different industrial wastes. BSG/T: *T. spathulate* JU4-57 cultivated on BSG hydrolysate under batch cultivation, (SYC/Y): *Y. lipolytica* TISTR 5151 cultivated on SYC hydrolysate under batch cultivation, (ES/Y): *Y. lipolytica* TISTR 5151 cultivated on ES under batch cultivation, (CG/R): *R. mucilaginosa* G43 cultivated on CG under batch cultivation, (ES/Y/F) *Y. lipolytica* TISTR 5151 cultivated on ES under fed-batch cultivation and (CG/R/F): *R. mucilaginosa* G43 cultivated on CG under fed-batch cultivation

4.3 Scale-up of *R. mucilaginosa* G43 cultivation in 2L bioreactor

4.3.1 Repeated-batch fermentation in bioreactor

As the oleaginous yeast *R. mucilaginosa* G43 cultivated on crude glycerol gave the highest lipid production, especially in batch mode, its fermentation was scaled up in 2 L-bioreactor. Moreover, the batch fermentation was repeated to increase lipid productivity by reusing the cells as inoculums for subsequent fermentation cycles. This could then reduce both time and cost required for inoculum preparation (Dashti *et al*., 2016). After 48 h of the first batch, the culture broth was replaced with the fresh medium at 80% replacement rate. Five cycles of batch cultivation were repeated and the fermentation proceeded for 228 h. The profiles of biomass, lipid production, lipid content and carotenoid production are shown in Figure 27. The biomass and lipid production continued during 4 cycles of repeated-batch but gradually decreased in each repeated batch. The biomass obtained at the end of each cycle was 4.62 ± 0.97 g/L, 3.91 ± 0.05 g/L, 3.18 ± 0.01g/L and 1.78 ± 0.08 g/L. This indicated that increased batch cycle had an adverse effect on biomass concentration. The lipid content varied from 20-60% of dried biomass. The highest lipid production of 2.52±0.03 g/L was obtained at the first cycle and decreased to 1.11 ± 0.01 g/L at the second cycle and those in the subsequent batch were only 0.6-0.7 g/L. With the repeat-batch fermentation, *R. mucilaginosa* G43 could produce total biomass and lipids of 10.8 g/L and 4.01 g/L, respectively. It was of interest that the carotenoid content in the cells from the third cycle was highest at 21.85±0.58 mg/g-cell which was comparable to those in the literature (Manowattana *et al*., 2018).

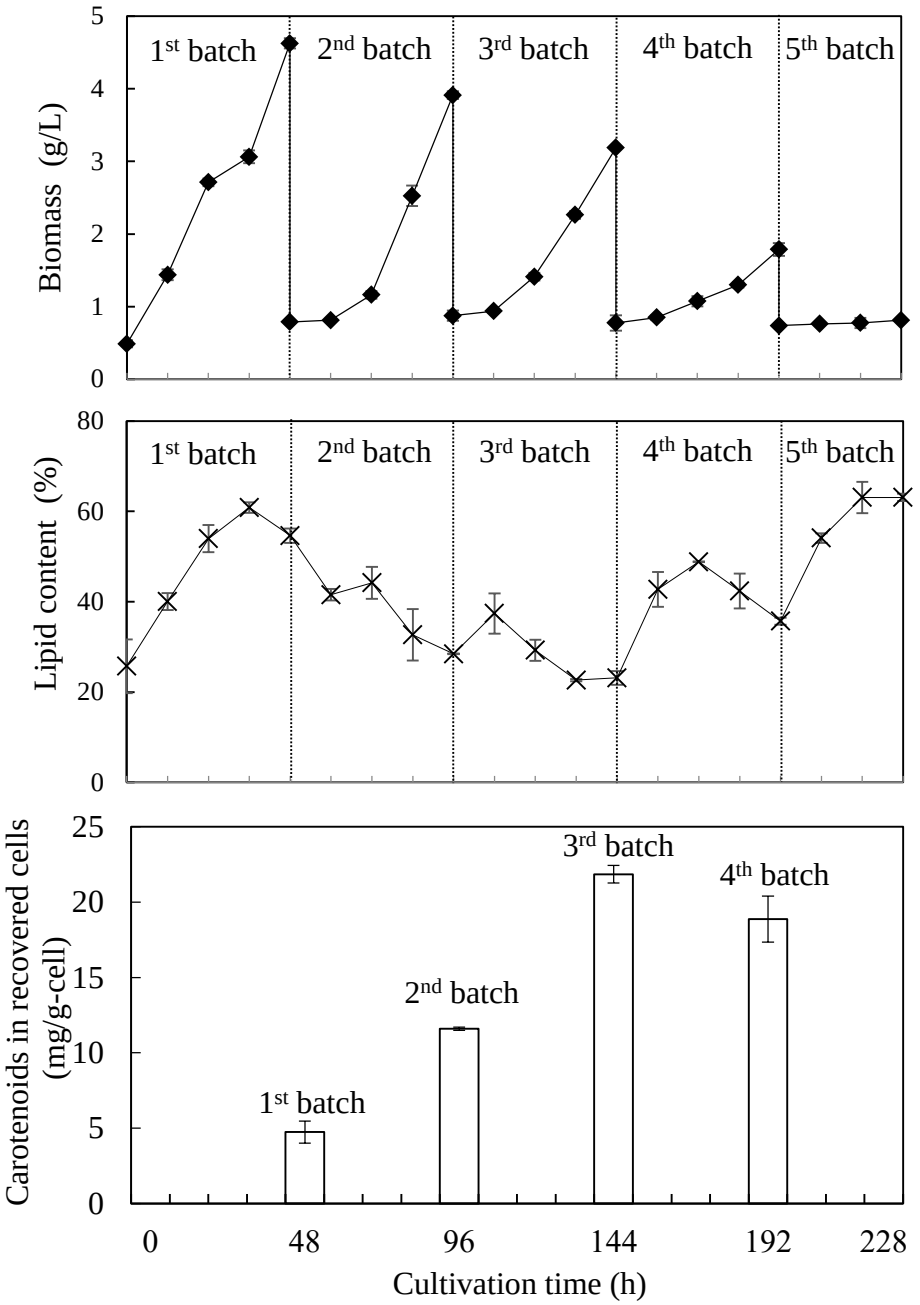

Figure 27 Repeated-batch of *R. mucilaginosa* G43 using crude glycerol (CG) in 2-L bioreactor. The initial pH was 6.0 and the aeration rate was 0.5 vvm.

4.3.2 Fatty acid composition and estimation of biodiesel properties of *R. mucilaginosa* G43 cultivated in repeated-batch mode

The lipids from *R. mucilaginosa* G43 cultivated on crude glycerol in repeated-batch mode were mainly composed of long-chain fatty acids with 16 and 18 carbon atoms including palmitic acid as the predominant fatty acid (40.58%) followed by oleic acid (30.02%) and linoleic acid (approximately 17 %) (Table 20). The number of SFA and USFA play a key role of biodiesel properties, especially cetane number, flash point, viscosity, oxidation stability, and fluidity at low temperature (Piligaev *et al*., 2015). Specifically, the high amount of saturated and monounsaturated fatty acids (SFA and MUFA) can give high value of cetane number (CE) which denotes better combustion quality and easy start of the engine. The similar fatty acid composition profiles of *R. mucilaginosa* G43 to those of plant oils indicate the potential use of its lipids as biodiesel feedstocks. More than 96% of fatty acids were C16-C18, which are suitable as biodiesel feedstocks (He *et al*., 2013).

The calculated CE value for lipids from *R. mucilaginosa* G43 was 56.26. The IO of *R. mucilaginosa* G43 lipids was 63.49 I_2/100g. The important parameter for low temperature applications of a fuel is cold-filter plugging point (CFP). The FAMEs from *R. mucilaginosa* G43 lipids would have CFP of 4.89The SA of *R. mucilaginosa* G43 lipids was approximately 208 mg KOH/g oil.

Table 20 Fatty acid compositions and estimated biodiesel properties of the yeast lipids

Fatty acid compositions	*R. mucilaginosa* G43
Myristic acid (C14:0)	2.62
Palmitic acid (C16:0)	40.58
Palmitoleic acid (C16:1)	2.16
Stearic acid (C18:0)	5.10
Oleic acid (C18:1)	30.02
Linoleic acid (C18:2)	17.04
Linolenic acid (C18:3)	1.26
Lignoceric acid (C24:0)	-
Saturated fatty acids	48.3
Unsaturated fatty acids	50.48
SA	208.67
IO (g I_2/100 g)	63.49
CE	56.26
LCF (wt%)	6.8
CFP (ºC)	4.89

SA, saponification value; IO, iodine value; CE, cetane number; LCF, long-chain saturation factor; CFP, cold-filter plugging properties: <0 (summer) <-10 (winter).

4.4 Techno-economic analysis and environmental impact

4.4.1 Overview of biodiesel process

There are several different substrates to choose for lipid production by oleaginous yeasts. Some of those substrates have seriously restricted the development of industrialization. Nowadays, lipid productions by the oleaginous yeasts are still in lab scale. More research and development are required to establish an industrial route. It is necessary to study the crucial steps of the whole process (Chen *et al*., 2017). In this study, there are four crucial steps for lipid production from industrial wastes by oleaginous yeasts including acid hydrolysis of biomass into fermentable sugars, oleaginous yeast cultivation, harvesting of yeast cells, direct transesterification of lipids into biodiesel as illustrated in Figure 28.

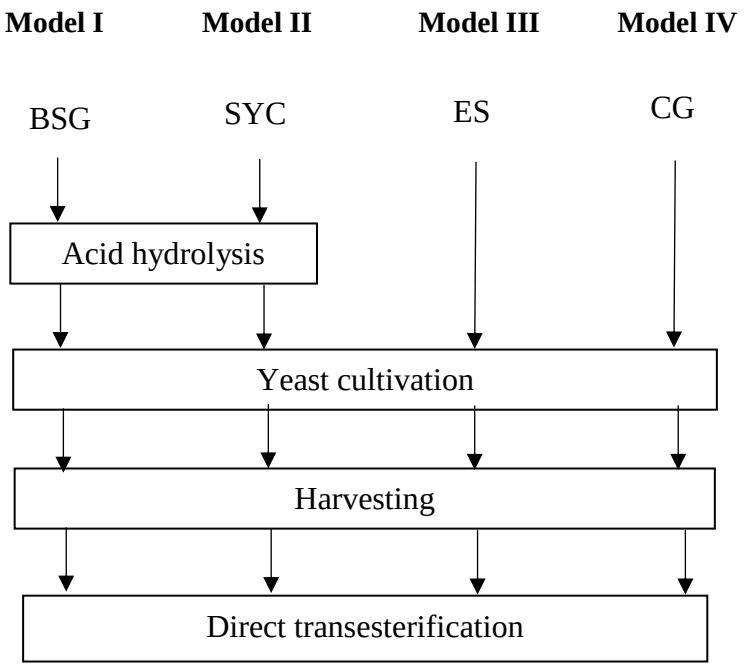

Figure 28 Production process of biodiesel using industrial wastes by oleaginous yeast

(1) Acid hydrolysis of biomass

Among four wastes, brewers' spent grain (BSG) and spent yeast cell (SYC) needed to be acid hydrolyzed before use. BSC and SYC were added into water and pH adjusted to 2 using 3 M sulfuric acid solution prior to thermal treatment at 121 ºC for 20 min (Ryu *et al*., 2013). It has been proved that acid hydrolysis is very well-known and effective process for hydrolysis of biomass. The effectiveness of acid hydrolysis depends on type of acid, acid concentration solid to liquid ratio and temperature (Behera et al., 2014). In addition, the most advantage of the acid hydrolysis is that acids can penetrate lignin without any preliminary pretreatment of biomass and the acid hydrolysis is faster reaction than enzyme hydrolysis (Lenihan *et al*., 2010). Zaldivar *et al*. (2001) and Lenihan *et al*. (2010) reported that both dilute and concentrated acids could break down the lignocellulosic especially, the polymer of cellulose and hemicellulose biomass to form individual sugar molecules that can be the carbon source for yeast cultivation. Several types of acids, concentrated or diluted, can be used, including sulfurous, sulfuric, hydrochloric, hydrofluoric, phosphoric, nitric and formic acids (Galbe and Zacchi, 2002). Among them, sulfuric acid is the most commonly used catalyst for hydrolysis of lignocellulosic biomass (Lenihan *et al*., 2010). Sulfuric acid has been extensively used due to its high catabolic activity (Chiaramonti *et al*., 2012). The optimal condition for acid concentration used for acid hydrolysis process is in the range of 1-10% and at the temperature between 100-150ºC (Wingren *et al*., 2003; Lenihan *et al*., 2010).

(2) Yeast cultivation

The use of industrial wastes as cheap source of nutrients for the cultivation of yeasts with high lipid content has been considered as a cost-effective way to produce biodiesel feedstocks (Louhasakul *et al*., 2013). As soft drinks contain high amounts of sugars such as glucose, fructose and sucrose, when left unsold and expired they cannot be disposed directly into general wastewaters and cannot be treated immediately. Brewers' spent grain (BSG) and spent yeast cell (SYC) are the wastes from brewery industry. It is the major by-product from brewing and it is mainly composed of lignocellulosic materials (Vieira *et al*., 2017; Celus *et al*., 2006). Another organic waste that has attracted special attention as it is an unavoidable by-product of biodiesel production is crude glycerol. The biodiesel industry generates approximately 10%

(w/w) of glycerol with every batch of biodiesel produced (Uprety *et al* 2017). It has been reported that crude glycerol could be used by microorganisms without a requirement for any purification (Qin *et al*., 2017).

As expired soft drinks (ES) and crude glycerol (CG) can be used directly without acid hydrolysis, the process started with yeast cultivation. Only solid wastes like brewers' spent grain (BSG) and spent yeast cell (SYC) have to be acid hydrolyzed before use.

(3) Harvesting

Commonly used harvesting technologies are flocculation, centrifugation, and filtration. Membrane filtration often uses modified cellulose as the filter, which is easy to be polluted, though the counter current operation may improve the efficiency to some extent. Centrifugation is a commonly used method for cell separation, which uses centrifugal separation without introducing other chemical reagents. The advantage of centrifugation is fast harvesting (Chen *et al*., 2017). Flocculation is an industrial separation technology. This method requires the addition of $AlCl_3$, $FeCl_3$ or chitosan as flocculant to fix cells into flakes. However, the flocculant is difficult to be removed during the downstream separation processes (Chen *et al*., 2017). Although, there are a number of available techniques for harvesting of the microorganisms, the most commonly used harvest methods for the oleaginous cells are still the centrifugation (Xia *et al*., 2011).

(4) Direct transesterification

Microbial lipids can be converted to fatty acid methyl esters, biodiesel by direct transesterification by using alkaline or acid catalysts (Kiran *et al*., 2014). Recently, Louhasakul et al. (2018) reported the direct transesterification of yeast lipids into biodiesel in vigorously stirred tank reactor (VSTR). As this study did not perform the direct transesterification of the yeast lipids, the experimental data from Louhasakul *et al.* (2018) were used for calculation. The reaction conditions were as follows:

i) The direct transesterification process was performed in 2-L VSTR with a liquid volume of 0.5 L.

ii) After oleaginous yeast cultivation on industrial wastes in bioreactor, the yeast cells mass were concentrated by self-flocculation for 1 h and then harvested by centrifugation at 1585×g for 15 min.

iii) The wet yeast cells were washed and transferred into VSTR and then added with glass beads as cell disrupting agents at a bead: biomass ratio of 1:1 (w/w) and methanol as a lipid extracting solvent and feedstock at a methanol: biomass ratio of 4:1 (v/w). The total volume of the mixture was 0.6–0.7 L. The agitation speed was set at 1000 rpm and the reaction temperature was set at 50 ºC. Sulfuric acid was used as catalyst and its concentration in methanol of 2% (v/v).

iv) To determine the biodiesel yield, the direct transesterification lipids were extracted by adding hexane to the reactants at the volume ratio of 2:1 and then vigorously mixed at 1000 rpm in an Eppendorf vortex (Daihan Scientific, Korea) for 30 min. Solvent was recovered by evaporation.

4.4.2 Technical performance and mass balance

Four industrial wastes for lipid production and subsequently biodiesel production were investigated in this study. These include Model I: brewers' spent gain waste from brewers' industry, Model II: spent yeast cell waste from brewers' industry, Model III: expired soft drinks waste from beverage industry and Model IV: crude glycerol from biodiesel industry. All these technological processes were evaluated to fulfill their requirement for industrial scale application. The investigations were made to see the efficient and affordable technological options for biodiesel from industrial wastes.

Model I: the process flow diagram describing the preparation of brewers' spent grain hydrolysate as nutrient sources for oleaginous yeast cultivation is shown in Figure 29. The optimum condition at 15% BSG loadings was taken and pH was adjusted to 2 using 3 M sulfuric acid solution prior to thermal treatment at 121 ºC for 20 min (Ryu *et al*., 2013). The BSG is comprised of hemicellulose (24.49%), cellulose (20.86%) and lignin (9.62%). After acid hydrolysis, the BSG hydrolysate composed of glucose (22.02%), xylose (45.83%) and arabinose (32.13%). For yeast cultivation, *T. spathulate*

JU4-57 was the most suitable oleaginous yeast for bioconversion of BSG hydrolysate into lipids. The cultivation conditions were set to be: initial cell concentration of 0.5 kg/m^3 in stirred tank bioreactor at room temperature (30±2ºC) for 72 h. Under these conditions, the highest biomass and lipid production achieved were 7.35 kg/m^3 and 1.5 kg/m^3, respectively. The centrifugation was used for harvesting the yeast cells at 8,000 rpm for 20 min. The harvested yeast cells were assumed to be directly used in direct transesterification without drying (Louhasakul *et al*., 2018)

In order to evaluate the energy consumption of the designed scenarios, energy demand to produce 1 kg of biodiesel was accessed. The conversion efficiency of lipids into biodiesel at 97 % recovery was referred from Louhasakul *et al*. (2018). To obtain 1 kg of biodiesel, 1.46 kg of *T. spathulate* JU4-57 lipids are required. The total energy input required for Model I was 37.04 kW·h. It seemed that heat required for heating operation appeared to be the most energy intensive in the acid hydrolysis stage which accounted of 23.97 kW·h. In the harvesting stage, the centrifugation step was 1.281 kW·h. The energy required for direct transesterification was 2.68 kW·h. In this study it was found that the harvesting process required large amount of energy. It should be noted that amount of removed water during harvesting process was 99.69% of the initial water (Ventura *et al*., 2013). Moreover, the glycerol is assumed to be sold at the market and could then offset the production cost of the biodiesel.

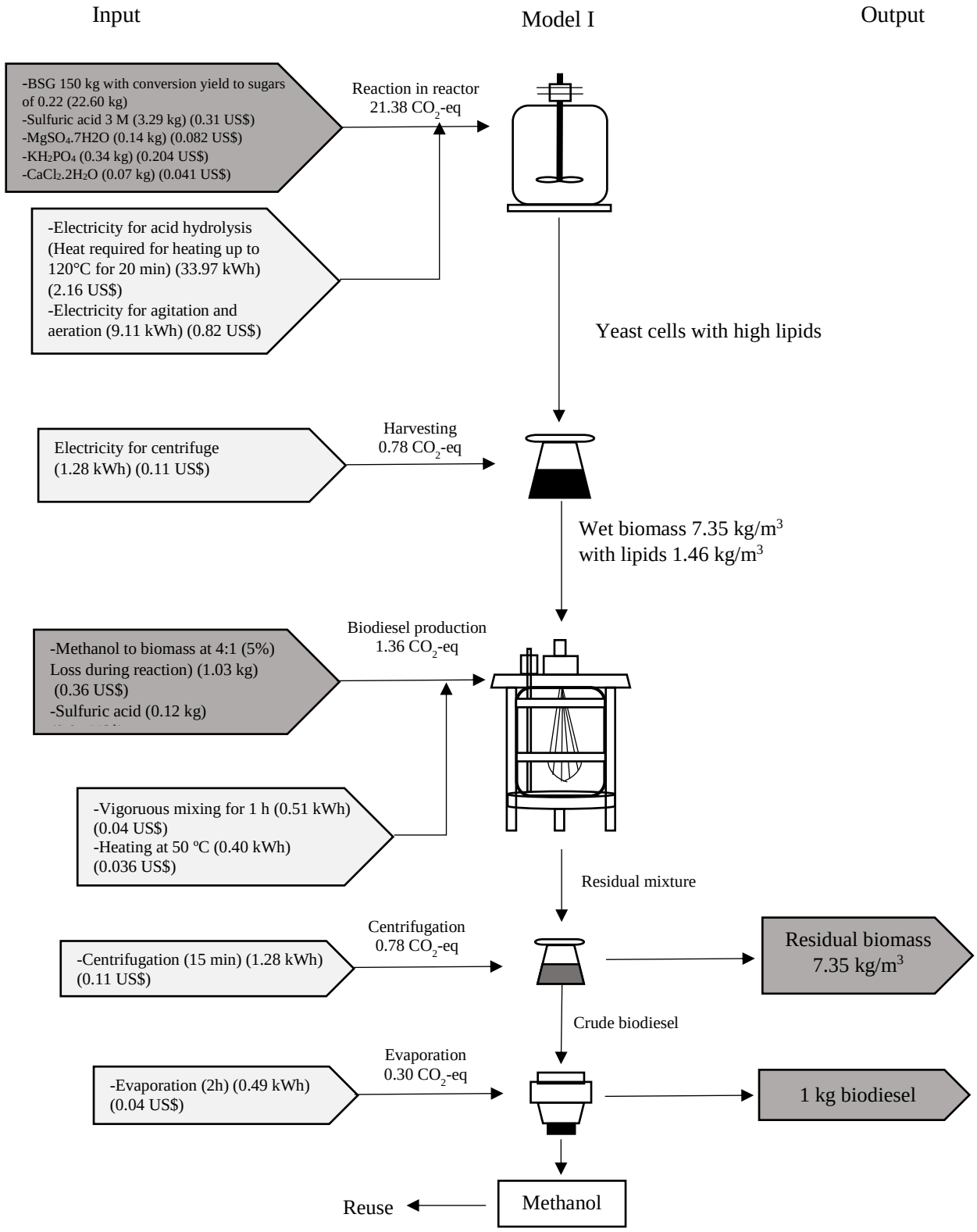

Figure 29 Overall mass balance of brewer's spent grain hydrolysate as feedstock for lipid production by *T. spathulate* JU4-57

Model II: the process flow diagram describing the preparation of spent yeast cell hydrolysate as nutrient sources for oleaginous yeast cultivation is shown in Figure 30. The optimum conditions were 24% SYC loadings and pH adjusted to 2 using 3 M sulfuric acid solution prior to thermal treatment at 121 ºC for 20 min (Ryu et al., 2013). The SYC is comprised of protein (46.97%), carbohydrate (21.32%) and lipids (5.73%). After acid hydrolysis, the SYC hydrolysate composed of the main sugar of glucose and mannose at 31.43% and 69.57%, respectively. For yeast cultivation, *Y. lipolytica* TISTR 5151 was the most suitable oleaginous yeast for bioconversion of SYC hydrolysate into lipids. The cultivation conditions were set to be: initial cell concentration of 0.55 kg/m^3 in stirred tank bioreactor at room temperature (30±2ºC) for 72 h. Under these conditions, the highest biomass and lipid production achieved were 4.07 kg/m^3 and 0.71 kg/m^3, respectively. The centrifugation was used for harvesting the yeast cells at 8,000 rpm for 20 min. The harvested yeast cells were assumed to be directly used in direct transesterification without drying (Louhasakul *et al*., 2018). With these conditions, 0.68 kg biodiesel would be produced. The total energy input required for Model II was 79.74 kW·h. Heat required for heating operation appeared to be the most energy intensive in the acid hydrolysis stage which accounted of 51.47 kW·h. In the harvesting stage, the centrifugation step was the least energy required at 1.281 kW·h. The energy required for direct transesterification was 2.68 kW·h.

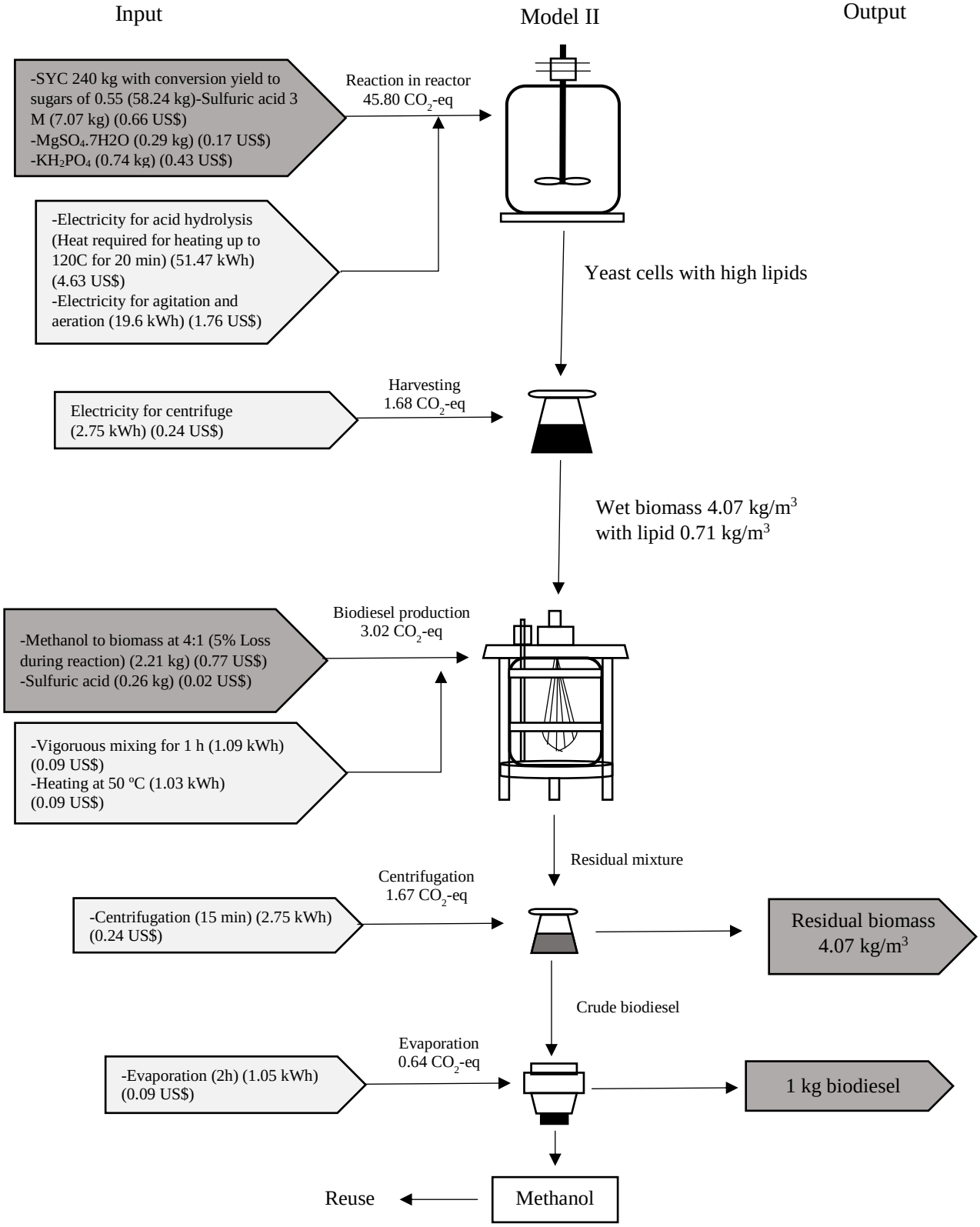

Figure 30 Overall mass balance of spent yeast cell hydrolysate as feedstock for lipid production by *Y. lipolytica* TISTR 5151

Model III: the process flow diagram describing the expired soft drink as nutrient sources for oleaginous yeast cultivation is shown in Figure 31. The ES is comprised of 27.46% glucose, 12.73% sucrose and 59.80% fructose, respectively. For yeast cultivation, *Y. lipolytica* TISTR 5151 was the most suitable oleaginous yeast for ES conversion into lipids. The cultivation conditions were: initial cell concentration of 0.49 kg/m^3 in stirred tank bioreactor at room temperature (30±2ºC) for 72 h. Under these conditions, the highest biomass and lipid production achieved were 2.70 kg/m^3 and 0.67 kg/m^3, respectively. The centrifugation was used for harvesting the yeast cells at 8,000 rpm for 20 min. The harvested yeast cells were assumed to be directly used in direct transesterification without drying (Louhasakul *et al*., 2018). Under these conditions, the biodiesel achieved from *Y. lipolytica* TISTR 5151 was 0.64 kg. The total energy input required for Model III was 29.9 kWh.

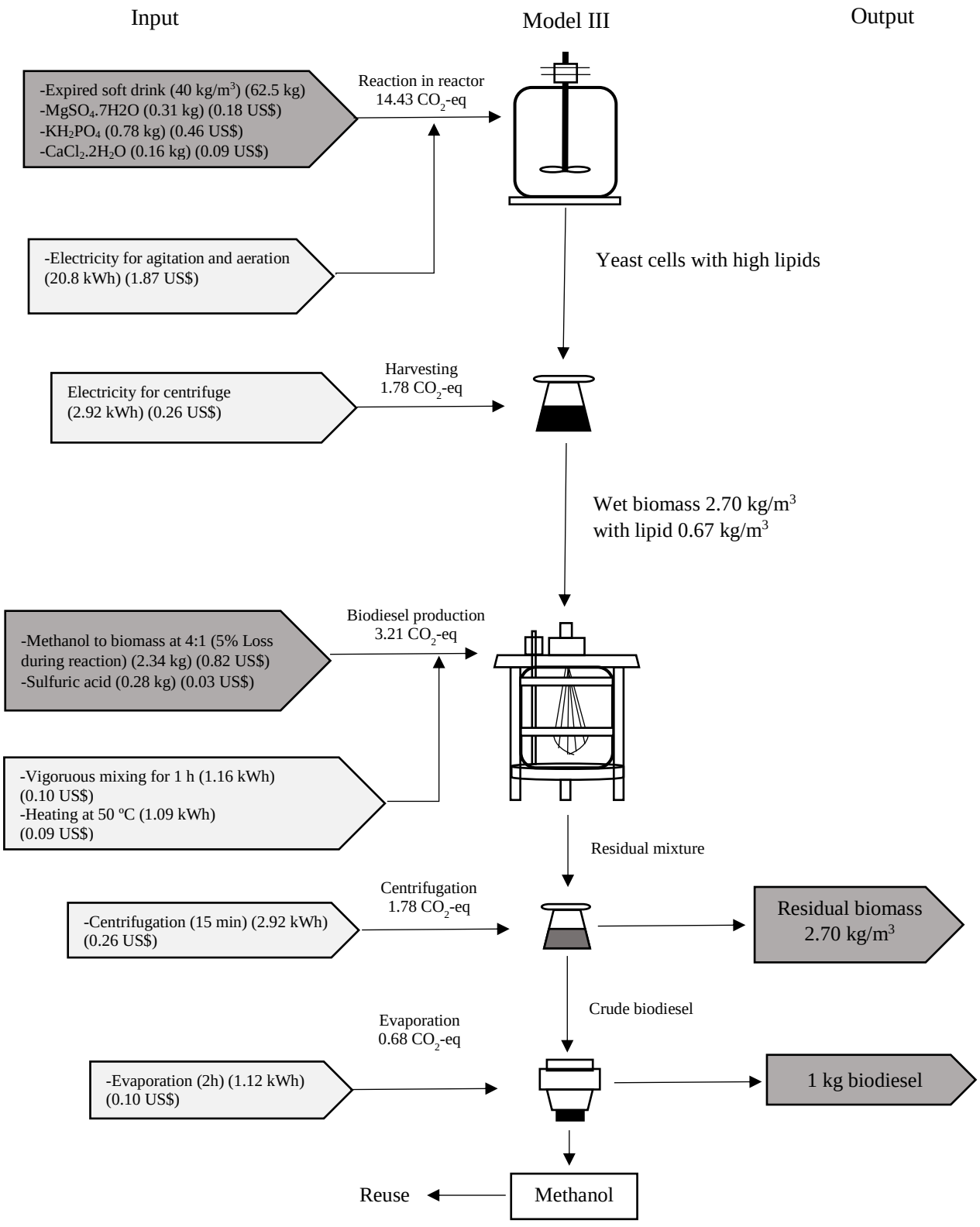

Figure 31 Overall mass balance of expired soft drinks as feedstock for lipid production by *Y. lipolytica* TISTR 5151

Model IV: the process flow diagram describing the crude glycerol as nutrient sources for oleaginous yeast cultivation is shown in Figure 32. For yeast cultivation, *R. mucilaginosa* G43 was the most suitable oleaginous yeast for CG conversion into lipids. The chemical composition of crude glycerol used in this study showed that it comprised of high amount COD at 3,062±0.87 g/L but low nitrogen content (1.86±0.17 g/L). The content of glycerol in crude glycerol was 32.35±0.19 %w/v. The cultivation conditions were: initial cell concentration of 0.59 kg/m^3 in stirred tank bioreactor at room temperature (30±2ºC) for 72 h. Under these conditions, the highest biomass and lipid production achieved were 4.12 kg/m^3 and 2.09 kg/m^3, respectively. The biodiesel achieved was 2.02 kg. The total energy input required for Model IV was 9.52 kWh.

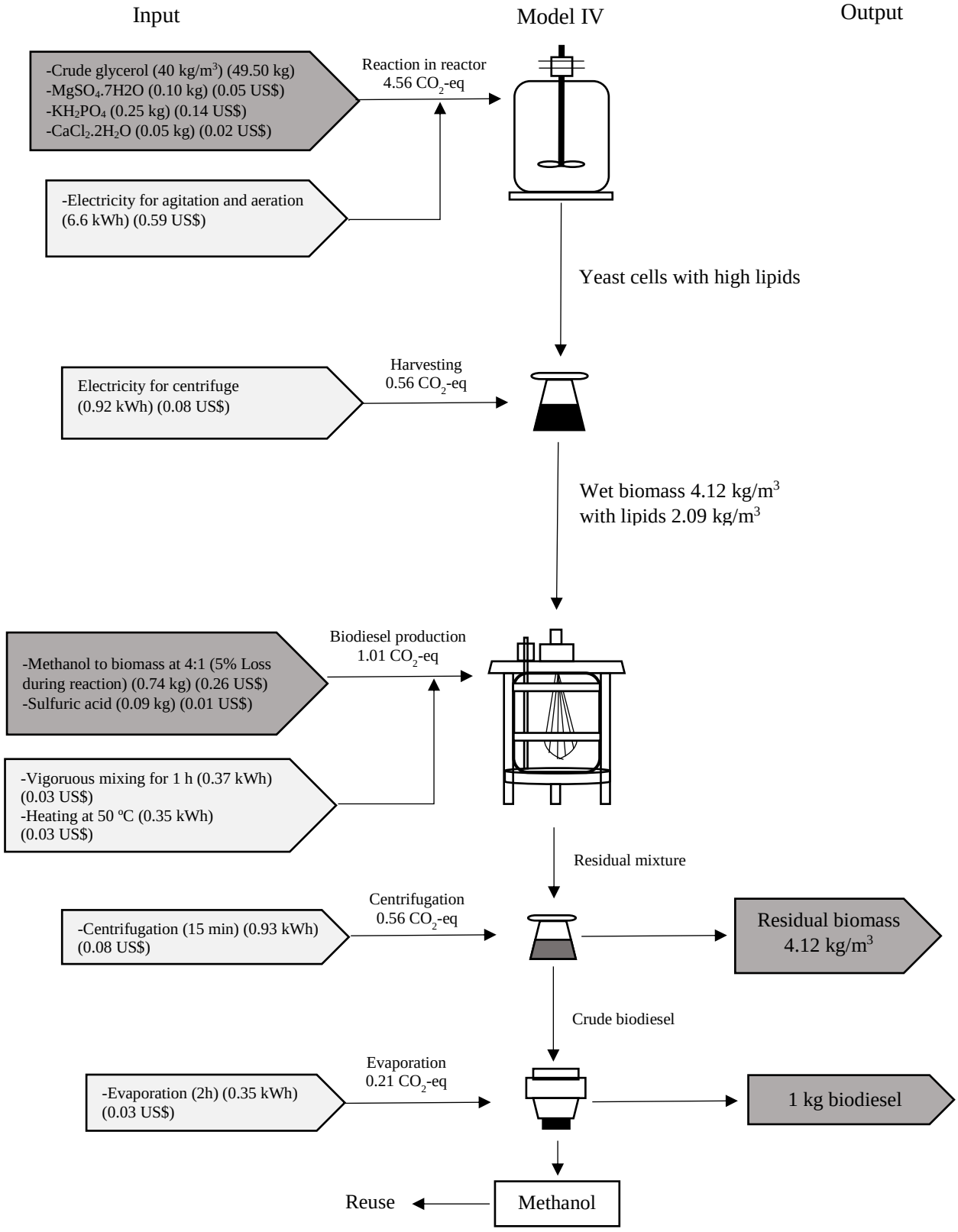

Figure 32 Overall mass balance of crude glycerol as feedstock for lipid production by *R. mucilaginosa* G43

Among the four models, Model IV could give biodiesel as high as 2.02 kg followed by Model I (1.46 kg), Model II (0.68 kg) and Model III produced the lowest biodiesel (0.64 kg). Therefore, Model IV showed the lowest energy demand of 9.52 kWh/kg-biodiesel (Figure 33a). It could be because Model IV required lower energy. The highest energy consumption was by acid hydrolysis and cultivation approximately 69-89%. It could be due to large volume fermentation and fermentation time requited. As the solid wastes, namely brewers' spent grain and spent yeast cell had to be hydrolyzed into fermentable sugars before use, the energy consumption by Model I and II were then higher than those by Model III and Model IV (Figure 33a).

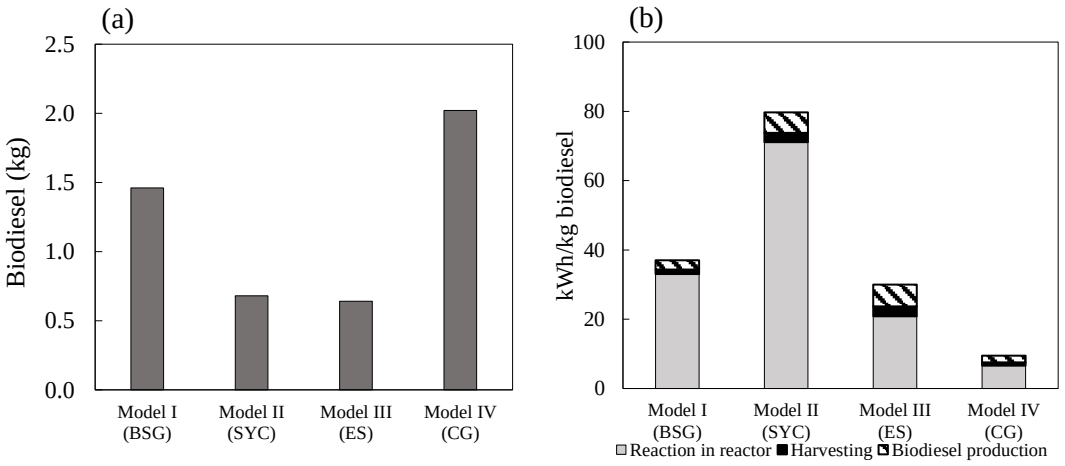

Figure 33 Lists and energy equipment involved in four models for lipid production by oleaginous yeasts.

4.4.3 Economic assessment

Economically viable biofuel should be cost competitive with petroleum fuels. There are many factors that involve in the high production cost of microbial based biodiesel (Subramaniam *et al*., 2010). Specifically, the cost of substrate or carbon source required approximately 60-75% of the total costs of the biodiesel production. Therefore, the economics of single cell oil production can be improved by using low-cost carbon in agro-industrial wastes (Fakas *et al*., 2009). Another important aspects in lipid production technology are the efficiency and economic feasibility to achieve an economical and sustainable biofuel production (Japar *et al*., 2017). This study had conducted economic analysis of biodiesel production from agro-industrial wastes including Model I (BSG), Model II (SYC), Model III (ES) and Model IV (CG). To determine the economic feasibility of the four models, cost analyses were carried out at the basis of 1 kg biodiesel. The capital, operation, equipment, raw material costs base on the market price (US$). The installation and instrumentation costs were not included and the calculations of these cost categories are indicated under Model I, Model II, Model III and Model IV. Figure 32a indicates capital cost categories including acid hydrolysis cost, cultivation cost, harvesting cost, direct transesterification and total capital investment cost for each model as well as the heat and electricity costs were accounted for comparison. From the overall net cost analysis in each model (Figure 34b), the total cost of Model II (9.34 $/kg-biodiesel) was the highest among four models followed by Model I (4.34 $/kg-biodiesel) and Model III (4.29 $/kg-biodiesel). The lowest cost production was 1.36 $/kg-biodiesel of Model IV. The high cost for biodiesel production in Model II was due to the electricity cost for heating in acid hydrolysis process which was approximately 83% of the total capital cost. The electricity costs was almost identical in all models. However, no heat was needed in Model III and Model IV. The cost of heating in Model I and Model II (2.19 US$/kg-biodiesel and 4.72 US$/kg-biodiesel) were relatively higher than the electricity cost (1.12 US$/kg-biodiesel and 2.42 US$/kg-biodiesel). For oleaginous cultivation, minor nutrient costs were 0.32, 0.70, 0.74 and 0.23 US$/kg-biodiesel in Model I, II, III and IV, respectively. While the chemicals for acid hydrolysis and direct transesterification (methanol) costed 0.67, 1.43, 0.82 and 0.26 US$/kg-biodiesel in Model I, II, III and IV, respectively. In addition, the expenses for reaction in a reactor were the highest operating cost (60–

83%). It is interesting that, carotenoid in Model IV from *R. mucilaginosa* G43 (15.5 g/kg-cell) (Figure 34b), could be sold back to the market for commercial use (Ventura *et al*., 2013). Microbial residual mass and crude glycerol that are generated as side streams in the microbial lipids and biodiesel production processes could be utilized as useful feedstocks for other products and can then contribute significant revenues (Koutinas *et al*., 2014).

The economics of biodiesel production from single cell oils relate to the cost-competitive technologies used in the process. Alabi *et al*. (2009) reported the production cost for lipid production from algae was between $7.64/L and $14.44/L. While Ratledge and Cohen (2008) reported a very high cost of $21/kg. However, other studies have reported lower production costs of lipids from algae in the range of $2.25/L to $3.37/L (Davis *et al*., 2011; Richardson *et al*., 2012). The production of lipids from oleaginous microorganisms including yeast and algae should be more developed in order to evaluate the potential industrial implementation of microbial based biodiesel production.

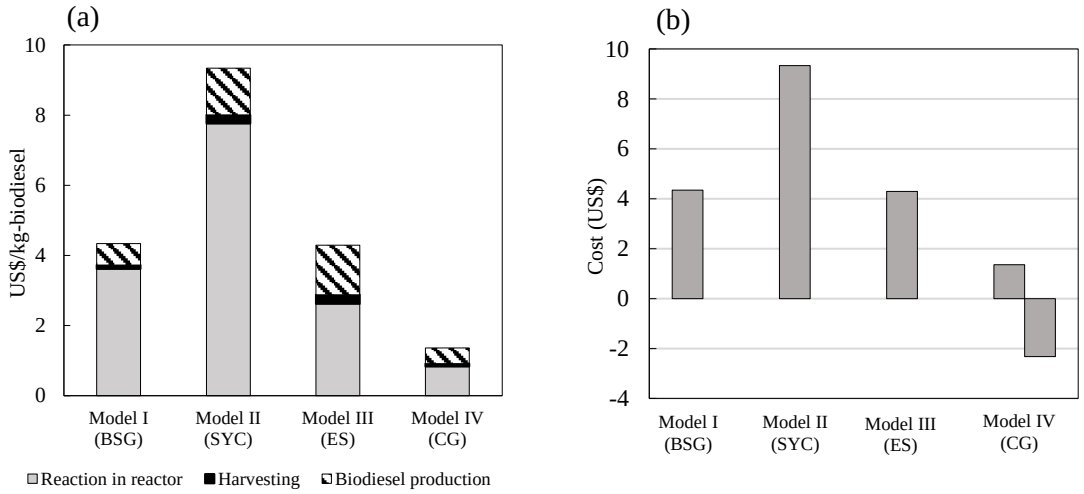

Figure 34 Lists and cost equipment involved in four models for lipid production by oleaginous yeasts.

4.4.4 Assessment of GHG emissions of biodiesel production

The rising concern of global warming has caused an increased interest in commercial production emissions. Carbon dioxide is produced during the fermentation process and other forms in the life cycle of biodiesel generation have certain environmental results. In this case, the life-cycle assessment of biodiesel production must be evaluated in order to find the areas of the environmental hotspot. and should produce biofuel that in traduce substantially less GHG emissions into the atmosphere than comparative fossil fuels (Okoro *et al*., 2012). The primary greenhouse gas emissions considered in this paper are carbon dioxide, methane and nitrous oxide. The emissions and environmental impact of the system were quantified through the metric of global warming potential. The greenhouse gas potential (GWP) factors measure how much a given mass of greenhouse gas contributes to global warming, in comparison to carbon dioxide. This allows to sum the CO_2-eq (carbon dioxide equivalent) units of all the emissions together to allow direct comparisons between different model. (Campbell *et al*., 2011).

The net greenhouse gas was determined leveraging the inputs and outputs of the engineering process model with life cycle inventory data. In the lipids production unit, the CO_2 emission of the biodiesel was determined and calculated. Figure 35 shows the pioneer CO_2 emissions from each model of biodiesel production. The majority of the emissions are directly related to the Model II (52.84 kg CO_2-eq) and Model I (24.60 kg CO_2-eq). followed by Model III (21.90kg CO_2-eq) and the lowest CO_2 emissions was Model IV (6.93kg CO_2-eq) and The CO_2 equivalents of the input chemicals (nutrients and methanol), heat and electricity inputs were calculated. For all models, it was found that GHG emission from acid hydrolysis and cultivation process approximately 65-86 % of total GHG emissions. It could be due to large volume fermentation and electricity, chemical raw material requited.

Alternative scenarios were considered which focused on reducing the environmental impact of the system. Potential reductions in emissions are possible through co-product utilization and optimization. It is theorized that the wastes from fermentation would be able to be used as a basic fertilizer. The left-over bio-waste could represent a credit as it would replace the need for farmers to use traditional fertilizers (Edlund *et al*., 2018). In addition, the assuming the dry biomass emissions could be

removed as a sun dry. Moreover, the use of liquid substrate such as expired soft drinks and crude glycerol as the media carbon source represents a free carbon source as it is expected to be a waste product from the agro-industry.

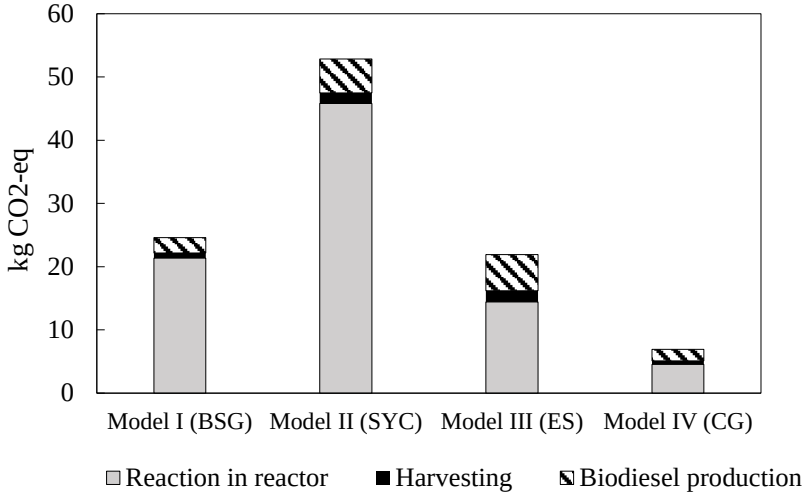

Figure 35 Pioneer CO_2 emissions of four models for lipid production by oleaginous yeasts.

4.4.5 Technical cost and environmental analysis

The lipid production process simulator has been successfully used to perform technical, environmental and economic analyses for all of case studies. As each model, the biodiesel process is assumed to operate following the electricity load. This process is scaled 97% biodiesel recovery. Figure 36 shows the technical cost and environmental analysis of industrial waste including brewers' spent grain (Model I), spent cell yeast (Model II), expired soft drinks (Model III) and crude glycerol (Model IV) based medium for biodiesel production. In Model I, the total available energy was 36.84 kWh with the total CO_2 emissions of 24.54 kg CO_2-eq and the total cost was 4.34 $/kg-biodiesel. In Model II, the waste used is SYC with the same electricity generated with Model I, the overall efficiency of energy use in Model II was 79.74 kWh with the CO_2 emissions of 52.84 kg CO_2-eq and the total cost was 9.34 $/kg-biodiesel. In Model III and Model IV, the feed stock used is ES and CG with the same electrical output, the fuel consumption from process were 29.99 kWh and 9.54 kWh, respectively with the CO_2 emission of 21.90 kg CO_2-eq and 9.52 kg CO_2-eq, respectively. The total cost were 4.29 $/kg-biodiesel and 1.36 $ $/kg-biodiesel in Model III and Model IV, respectively. Moreover, to improve economic feasibility, the higher biomass productivity in the cultivation system must be achieved. Oleaginous yeast lipid composition also needs to be enhanced to increase the biodiesel production.

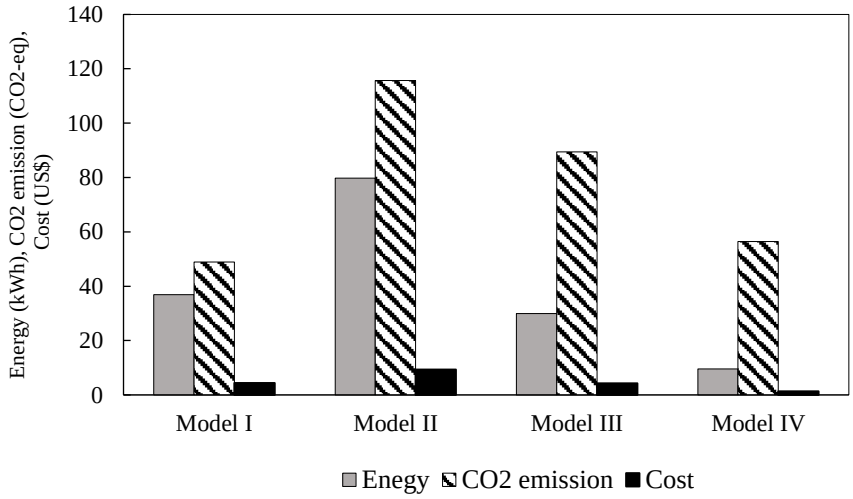

Figure 36 Calculation basis of energy requirement of unit processes and its comparison

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

CONCLUSIONS

This study has shown that the agro-industrial wastes could be used as low-cost nutrient sources for cultivation of oleaginous yeasts. Among the wastes tested, the expired soft drinks could be a good nutrient source for lipid production by *Y. lipolytica* TISTR 5151 without the addition of nitrogen source. While brewers' spent grains and spent yeast cells were more suitable for biomass production rather than lipid production by the yeasts. Crude glycerol was not suitable for the yeast cell growth but it could be used as nutrient source for lipid production by the specific yeast, i.e. *R. mucilaginosa* G43. The repeated-batch fermentation with 80% replacement with fresh medium could continuously produce biomass and lipids. Furthermore, the prospect fuel properties of yeast lipids suggested their potential use as biodiesel feedstocks. This study may contribute greatly to the low-cost production of biodiesel feedstocks and environment friendly waste utilization.

This work has directly evaluated the techniques economic and greenhouse emissions viability of the production of lipids through oleaginous yeasts. Experimental data was used to validate process that are integrated into a mass balance. The model of lipid production serves as the foundation for sustainability assessment through mass balance and techno-economic analysis. TEA indicates that CG could give the highest biodiesel 2.02 kg-biodiesel. The economic assessment shows a lower profitability in the case of SYC. The majority of the emissions are directly related to the SYC was 52.84 kg CO_2-eq.

SUGGESTIONS

1. The use of the agro-industrial wastes which contain high nitrogen source to replace ammonium sulfate should be attempted because the high cost of nitrogen source was considered to have a negative impact on its economic when use in industrial scale.

2. For the future work, the lipid production should be integrated with wastewater treatment system to reduce the energy cost for yeast cultivation.

3. The effective method for simultaneous recovery of lipids and carotenoids and protein should be investigated.

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APPENDIX

1. Chemical Oxygen Demand (COD) by Open Reflux Method (APHA, AWWA and WPCF

Wash culture tube and caps with 20% H2SO4 before first use to prevent contamination. Place sample in culture tube or ampule and add digestion solution. Carefully run sulfuric acid reagent down inside of vessel so an acid layer is formed under the sample-digestion solution layer. Tightly cap tubes or seal ampoules and invert each several times to mix completely. Place tubes or ampoules in block digester or oven preheated to 150 ºC and reflux for 2 h. Cool to room temperature and place vessels in a test tube rack. Remove culture tube caps and add small TFE-covered magnetic stirring bar. If ampoules are used, transfer contents to a larger container for titrating. Add 0.05 to 0.10 mL (1-2 drop) ferroin indicator and stir rapidly on magnetic stirrer while titrating with 0.10 M ferrous ammonium sulfate (FAS). The end point is a sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample

$$COD = \frac{(A-B) \times M \times 8000}{mL\ sample}$$

where :

A = mL FAS used for blank,

B= mL FAS used for sample

M = molarity of FAS

2. Standard curve of sugar

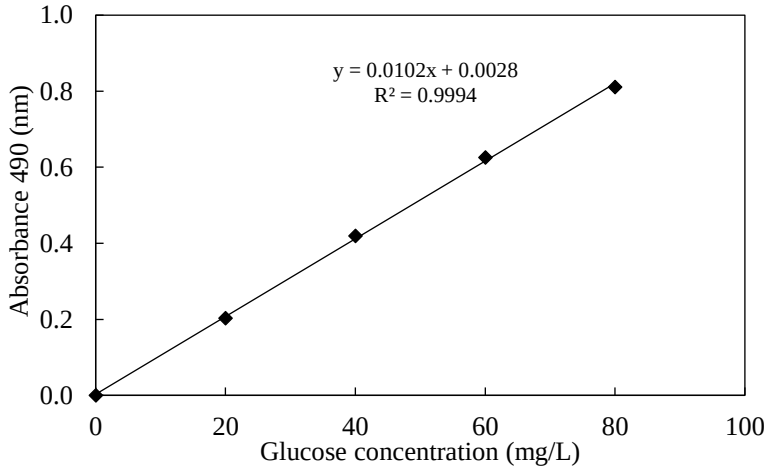

Figure 37 Standard cure of sugar

2. Standard curve of glycerol

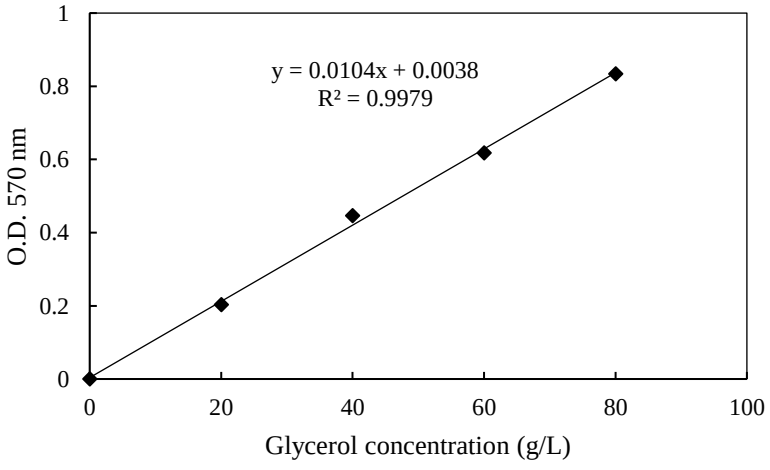

Figure 38 Standard cure of glycerol

3. Standard of furfural

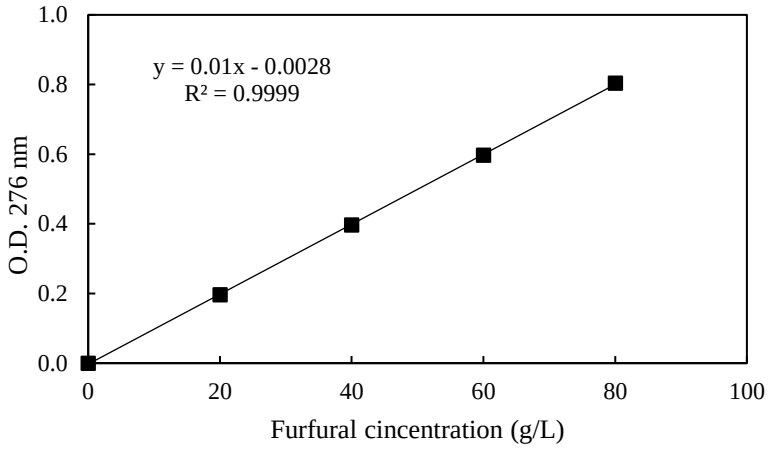

Figure 39 Standard cure of furfural

4. Standard of HMF

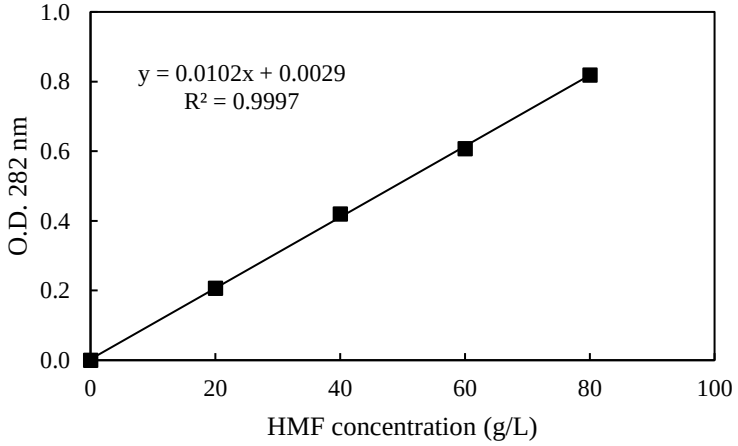

Figure 40 Standard cure of HMF

5. Determination of fatty acid composition by GC analysis

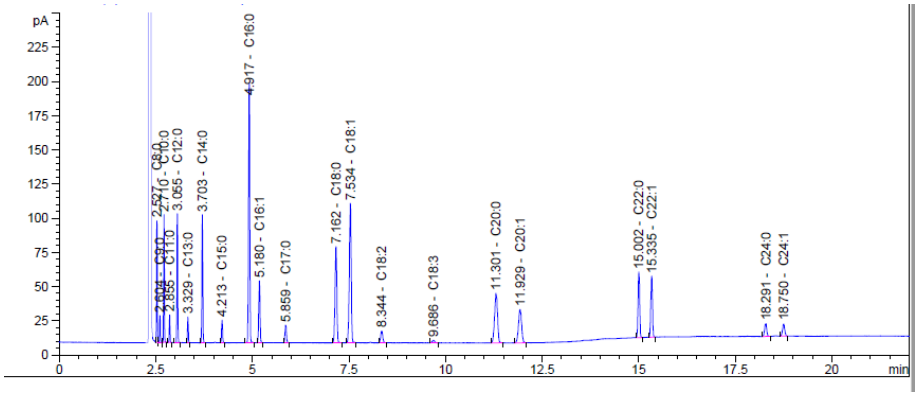

Figure 41 GC chromatogram of fatty acid composition.

6. Determination of sugar composition by HPLC analysis

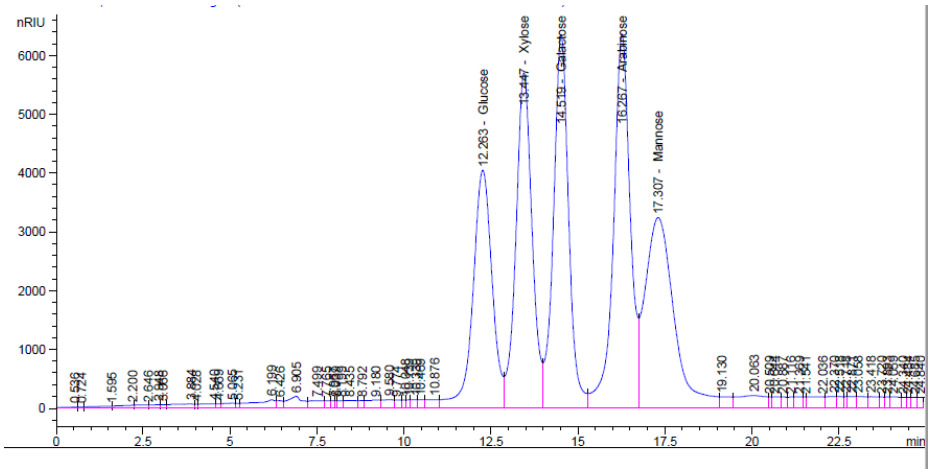

Figure 42 HPLC chromatogram of sugar composition.

7. Techno-eco and environmental analysis of BSG

Table 21 Lists of techno-eco and environmental analysis of BSG

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
1. Reaction in reactor					
1.1 Acid hydrolysis of BSG into fermentable sugars (1 m^3)					
-BSG 150 kg with conversion yield to sugars of 0.22	33	22.60	0.000	-	24.41
-Sulfuric acid 3 M 18 kg	4.81	3.29	0.31	0.1219	0.40
1.2. Other nutrients for yeast cultivation (1 m^3)					
-$MgSO_4.7H_2O$ 0.2 kg	0.2	0.14	0.082	0.3385	0.05
-KH_2PO_4 0.5 kg	0.5	0.34	0.204	1.9272	0.66
-$CaCl_2.2H_2O$ 0.1 kg	0.1	0.07	0.041	0.8729	0.06
1.3 Electricity for 1 m^3 reactor					
-Electricity acid hydrolysis	35	23.97	2.16	0.6093	14.61
-Electricity for agitation and aeration	13.3	9.11	0.82	0.6093	5.550

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
2. Harvesting					
-Electricity for centrifugation (kW.h)	1.87	1.281	0.115	0.6093	0.78
3. Direct transesterification					
3.1 Direct transesterification (30 L)					
-Methanol to biomass at 4:1 (5% Loss during reaction)	1.5	1.03	0.36	0.7212	0.741
-Sulfuric acid 0.6 L	0.176	0.12	0.01	0.6093	0.073
3.2 Electricity for 30 L reactor					
-Vigorous mixing for 1 h	0.74	0.51	0.046	0.6093	0.309
-Heating at 50 degree celcius	0.58	0.40	0.036	0.6093	0.242
-Centrifugation (kW.h)	1.87	1.28	0.115	0.6093	0.780
-Evaporation for 2 h	0.72	0.4932	0.044	0.6093	0.300

8. Techno-eco and environmental analysis of SYC

Table 22 Lists of techno-eco and environmental analysis of SYC

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
1. Reaction in reactor					
1.1 Acid hydrolysis of SYC into fermentable sugars (1 m^3)					
- SYC 72 kg with conversion yield to sugars of 0.55	39.6	58.24	0.000	-	62.89
-Sulfuric acid 3 M 18 kg	4.81	7.07	0.66	0.1219	0.86
1.2. Other nutrients for yeast cultivation (1 m^3)					
-$MgSO_4.7H_2O$ 0.2 kg					
-KH_2PO_4 0.5 kg	0.2	0.29	0.175	0.3385	0.10
-$CaCl_2.2H_2O$ 0.1 kg	0.5	0.74	0.438	1.9272	1.42
	0.1	0.15	0.088	0.8729	0.13
1.3 Electricity for 1 m^3 reactor					
-Electricity acid hydrolysis	35	51.47	4.63	0.6093	31.36
-Electricity for agitation and aeration	13.32	19.6	1.76	0.6093	11.935

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
2. Harvesting					
-Electricity for centrifugation (kW.h)	1.87	2.750	0.248	0.6093	1.68
3. Direct transesterification					
3.1 Direct transesterification (30 L)					
-Methanol to biomass at 4:1 (5% Loss during reaction)	1.5	2.21	0.77	0.7212	1.591
-Sulfuric acid 0.6 L	0.176	0.26	0.02	0.6093	0.158
3.2 Electricity for 30 L reactor					
-Vigorous mixing for 1 h	0.74	1.09	0.098	0.6093	0.663
-Heating at 50ºC	0.70	1.03	0.093	0.6093	0.627
-Centrifugation (kW.h)	1.87	2.75	0.248	0.6093	1.676
-Evaporation for 2 h	0.72	1.0588	0.095	0.6093	0.645

9. Techno-eco and environmental analysis of ES

Table 23 Lists of techno-eco and environmental analysis of ES

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
1. Cultivation					
1.1 nutrients for yeast cultivation (1 m^3)					
Expired soft drink (40 kg/m3)	40	62.5	0	-	67.50
$MgSO_4$.7H2O 0.2 kg	0.2	0.31	0.186	0.3385	0.11
KH_2PO_4 0.5 kg	0.5	0.78	0.465	1.9272	1.51
$CaCl_2.2H_2O$ 0.1 kg	0.1	0.16	0.093	0.8729	0.14
1.2 Electricity for 1 m^3 reactor					
-Electricity for agitation and aeration	13.32	20.8	1.87	0.6093	12.681
2. Harvesting					
-Electricity for centrifugation (kW.h)	1.87	2.922	0.263	0.6093	1.78
3. Direct transesterification					
3.1 Direct transesterification (30 L)					
-Methanol to biomass at 4:1 (5% Loss during reaction)	1.5	2.34	0.82	0.7212	1.690
-Sulfuric acid 0.6 L	0.176	0.28	0.03	0.6093	0.168

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
3.2 Electricity for 30 L reactor					
-Vigorous mixing for 1 h	0.74	1.16	0.104	0.6093	0.705
-Heating at 50ºC	0.70	1.09	0.098	0.6093	0.666
-Centrifugation (kW.h)	1.87	2.92	0.263	0.6093	1.780
-Evaporation for 2 h	0.72	1.1250	0.101	0.6093	0.685

9. Techno-eco and environmental analysis of CG

Table 24 Lists of techno-eco and environmental analysis of CG

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
1. Cultivation					
1.1 Nutrients for yeast cultivation (1 m^3)					
Crude glycerol (40% glycerol)	100	49.50	0	-	49.49
$MgSO_4$.7H2O 0.2 kg	0.2	0.10	0.059	0.3385	0.03
KH_2PO_4 0.5 kg	0.5	0.25	0.147	1.9272	0.48
$CaCl_2.2H_2O$ 0.1 kg	0.1	0.05	0.029	0.8729	0.04
1.2 Electricity for 1 m^3 reactor					
-Electricity for agitation and aeration	13.32	6.6	0.59	0.6093	4.018
2. Harvesting					
-Electricity for centrifugation (kW.h)	1.87	0.926	0.083	0.6093	0.56
3. Direct transesterification					
3.1 Direct transesterification (30 L)					
-Methanol to biomass at 4:1 (5% Loss during reaction)	1.5	0.74	0.26	0.7212	0.536
-Sulfuric acid 0.6 L	0.176	0.09	0.01	0.6093	0.053

Lists	Quantity (kg/m^3)	Quantity per 1 kg-biodiesel	Cost (US$/kg-biodiesel)	CO_2 factor	kg CO_2
3.2 Electricity for 30 L reactor					
-Vigorous mixing for 1 h	0.74	0.37	0.033	0.6093	0.223
-Heating at 50ºC	0.70	0.35	0.031	0.6093	0.211
-Centrifugation (kW.h)	1.87	0.93	0.083	0.6093	0.564
-Evaporation for 2 h	0.72	0.3564	0.032	0.6093	0.217

10. Emissions factor

Table 25 Lists of emissions factor environmental analysis

Lists	Unit	Emissions factor ($kgCO_2eq$/unit)	Source
Electricity	kWh	0.6093	Thai National LCI Database/MTEC
Glycerin	kg	1.2372	Thai National LCI Database/MTEC
Sugar	kg	1.08	Thailand greenhouse gas management organization (TGO)
Sulfuric acid	kg	0.1219	Ecoinvent 2.2, IPCC 2007 GWP 100a
$MgSO_4.7H_2O$	kg	0.3385	Ecoinvent 2.2, IPCC 2007 GWP 100a
KH_2PO_4	kg	1.9272	Ecoinvent 2.2, IPCC 2007 GWP 100a
$CaCl_2.2H_2O$	kg	0.8729	Ecoinvent 2.2, IPCC 2007 GWP 100a
Methanol	kg	0.7212	Ecoinvent 2.2, IPCC 2007 GWP 100a

VITAE

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Bachelor of Science (Biology Science)	Prince of Songkla University	2010
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Publications

Saengae, S., Cheirsilp, B., Tachapattawearakul, T. and Intharapat, P. Impact of agro-industrial wastes on production of single cell oils and their potential use as biodiesel feedstocks (Submitted).

Saengae, S., Cheirsilp, B., Tachapattawearakul, T. and Intharapat, P. Techno-economic feasibility and environmental impact analysis for production of biodiesel feedstocks from agro-industrial wastes by oleaginous yeasts (In preparation).

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Saengae, S., Cheirsilp, B., Tachapattawearakul, T. and Intharapat, P. 2016. Screening of Oleaginous Yeasts for Lipid Production from Industrial Wastes under Nitrogen rich and Nitrogen-Limitation. The 28th Annual Meeting of the Thai Society for Biotechnology and International Conference (Oral presentation).

Saengae, S., Cheirsilp, B., Tachapattawearakul, T. and Intharapat, P. 2018. Acid Hydrolysis of Brewers' Industrial Wastes and Their Use for Lipid Production by Oleaginous Yeasts. Water and Environment Technology Conference 14th-15th July 2018 Ehime University, Johoku campus (Oral and poster presentation).