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PII: S0960-8524(16)31542-5
DOI: http://dx.doi.org/10.1016/j.biortech.2016.11.037
Reference: BITE 17283

To appear in: *Bioresource Technology*

Received Date: 22 August 2016
Revised Date: 31 October 2016
Accepted Date: 7 November 2016

Please cite this article as: Yellapu, S.K., Kaur, R., Tyagi, R.D., Detergent assisted ultrasonication aided *in situ* transesterification for biodiesel production from oleaginous yeast wet biomass, *Bioresource Technology* (2016), doi: http://dx.doi.org/10.1016/j.biortech.2016.11.037

Detergent assisted ultrasonication aided *in situ* transesterification for biodiesel production from oleaginous yeast wet biomass

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ABSTRACT

In situ transesterification of oleaginous yeast wet biomass for fatty acid methyl esters (FAMEs) production using acid catalyst, methanol with or without N-Lauroyl sarcosine (N-LS) treatment was performed. The maximum FAMEs yield obtained with or without N-LS treatment in 24 h reaction time was 96.1 ± 1.9 and 71 ± 1.4 % w/w, respectively. The N-LS treatment of biomass followed by with or without ultrasonication revealed maximum FAMEs yield of 94.3 ± 1.9 % and 82.9 ± 1.8 % w/w using methanol to lipid molar ratio 360:1 and catalyst concentration 360 mM (64 µL H_2SO_4/g lipid) within 5 and 25 minutes reaction time, respectively. The FAMEs composition obtained in *in situ* transesterification was similar to that obtained with conventional two step lipid extraction and transesterification process. Biodiesel fuel properties (density, kinematic viscocity, cetane number and total glycerol) were in accordance with international standard (ASTM D6751),which suggests the suitability of biodiesel as a fuel.

Keywords : Microbial lipid; N-Lauroyl sarcosine; *In situ* transesterification; Ultrasonication; Biodiesel

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Highlights:

- The *in situ* transesterification process was used to convert lipid to biodiesel.
- N-Lauroyl sarcosine was used for wet biomass pretreatment.
- The ultrasonication was used to enhance FAMEs yield with low reaction time.
- There is no impact of N-LS pretreatment and ultrasonication on fatty acid profile.

1.Introduction

The global demand towards renewable energy is increasing due to the major problem of greenhouse gas emissions (GHG) (Piemonte *et al.*, 2016). There are two different possible approaches to decrease GHG: a) the use of microalgae to convert CO_2 into potential biomass by photosynthesis (ElMekawy *et al.*, 2014) and b) use of the biofuel is another alternative to decrease GHG emissions. Thus, the environment friendly and renewable fuel like biodiesel from oleaginous microorganisms (microalgae, yeast, fungi and bacteria) are found to be attractive in replacing fossil based fuels (Alvarez *et al.*, 2002, Lunin *et al.*, 2012, Medeiros *et al.*, 2015, Zhang *et al.*, 2014a). However, microbial lipid production using oleaginous microorganism is highly expensive process than first and second generation fuels,which is the most important barrier for commercialization of the process (Rathore *et al.*, 2016).

The biodiesel production process includes microbial oil production, harvesting, lipid extraction and transesterification. The lipid extraction followed by transesterification (two step transesterification) is high energy consumption process (approximately 2.3 to 40.5 Mj/kg) depending upon different cell disruption methods applied to disrupt the oil bearing cells (Dong *et al.*, 2015, Praveenkumar *et al.*, 2015a, Praveenkumar *et al.*, 2015b). To make the biodiesel production economical, it is a challenge for researchers to decrease the energy consumption for biodiesel production.

In situ transesterification (one step process or simultaneous cell disruption and transesterification process) is considered as an emerging alternative to two step transesterification, which has its obvious advantage. *In situ* transesterification involves the direct contact of reactants (methanol, catalyst and co-solvent) with biomass instead of lipids. This could reduce the energy

consumption used for extraction of lipids and hence cut down the cost of biodiesel production. In the previous studies, high FAMEs yield up to 92.1% w/w has been accomplished within 20 min reaction time using dry (lyophilized) oleaginous yeast biomass with ultrasonication assisted in-situ transesterification (Zhang *et al.*, 2014b). Another study by Zhang *et al.,*(2016) also reported high FAMEs yield of 95% w/w within 50 min reaction time using dry (lyophilized) sludge biomass with high concentration of sludge solids by ultrasonication assisted in-situ transesterification. However, biodiesel production by *in situ* transesterification of dry (or) lyophilized biomass is a time effective process, but due to high energy consumption in drying operation, it is not feasible for industrial scale biodiesel production.

In situ transesterification using wet biomass is also highly questionable process due to the presence of high water content in the biomass, which may interfere with the transesterification process by enhancing the hydrolysis of FAMEs. It is reported that in the case of alkali based *in situ* transesterification using wet biomass, more than 6% water content reduces the lipid to FAMEs conversion efficiency due to saponification (Suter *et al.*, 1997). Whereas, in case of acid catalyzed *in situ* transesterification using wet algal biomass, more than 20% moisture content affected the lipid to FAMEs conversion efficiency. However, by increasing reactant concentration (sulfuric acid and methanol), the FAMEs yield was improved (Sathish *et al.*, 2012). Nagle *et al.* (1990) also studied the effect of acid and alkali catalysts on conversion of microbial oil to FAMEs and found that acid catalysts resulted in consistently higher yield due to total conversion of free fatty acids (FFA) into FAMEs and there was no soap formation even in the presence of moisture (wet biomass).

Few authors reported the use of surfactants along with the catalysts to enhance the FAMEs yield. The surfactant has high water tolerance and therefore have ability to disrupt the cell wall as well

as the phospholipid membrane layer (Brown *et al.*, 2008). Haas *et al.* (2011) described the use of (CTAB) cetyltrimethylammonium bromide (a cationic surfactant) along with an alkali catalyst during *in situ* transesterification of *Jatropha curcas* and observed increased FAME yield as well as a reduction in the catalyst concentration. Sodium dodecyl benzene sulfonate (SDBS) along with the catalyst H_2SO_4 also enhanced extraction of FFA and lipids from microalgae. SDBS significantly reduced the catalyst concentration required to convert the oil to FAMEs (Park *et al.*, 2014). N-Lauroyl sarcosine (N-LS), an anionic detergent is non toxic and biodegradable. It can disrupt the cell wall by formation of micelle at specific concentrations with yeast wet biomass with high moisture content (83.8 %) without effecting fatty acid profile (Yellapu *et al.*, 2016). On the other hand, according to Zhang *et al.,* (2014b) ultrasonication can decrease methanol requirement (*in situ* transesterification) as well as the reaction time. Thus, combining N-LS (N-Lauroyl sarcosine) treatment of oil bearing cells with ultrasonication could further improve the cell disruption and transesterification process. Therefore, the objective of this work is to investigate the effect of N-LS treatment along with the ultrasonication on oleaginous wet yeast oil bearing biomass for *in situ* transesterification. The different parameters such as ultrasonication time, H_2SO_4 (catalyst) concentration and methanol to lipid molar ratios were optimized to enhance the efficiency of conversion of oil to FAMEs. The *in situ* transesterification without ultrasonication as well as without N-LS treatment was also conducted to compare the results.

2. Materials and Methods

2.1 Strain, production and lipid harvesting conditions

Yarrowia lipolytica SKY-7, oleaginous yeast (isolated in our lab INRS-ETE Quebec, Canada) (Kuttiraja *et al.*, 2015) was grown in the medium containing 8.5 L starch industry wastewater (SIW) and 500 mL of crude glycerol solution with 11% (w/v) glycerol (byproduct of biodiesel production Quebec, Canada) in a 15L fermenter with working volume 10L (Biogene, Quebec). The fermenter was operated at constant pH 6.8–7.0 and temperature 28 ^{o}C and dissolved oxygen was maintained above 30% of saturation. After fermentation (72 h), the broth was heated in the fermenter at 80 ± 2^{o}C for 10 min to kill cells and to preserve the accumulated lipid inside the cells (Zhang *et al.*, 2015). Thereafter, the biomass was harvested by centrifugation at 8000 rpm for 10 min and the biomass was washed with warm water to remove residual glycerol as well as soap. To estimate dry weight and to perform lipid extraction as well as *in situ* transesterification, 3.1±0.2 g wet biomass (83.8% water) harvested from 25 mL fermented broth was used.

2.2 Two step process (lipid extraction and separation followed by transesterification)

2.2.1 Conventional lipid extraction and separation using choloroform and methanol

The standard chloroform and methanol extraction method was used to determine the lipid content of the biomass (Bligh *et al.*, 1959, Folch *et al.*, 1957, Vicente *et al.*, 2009). The lipid extraction was conducted in the same way as discussed in our previous study (Yellapu *et al.*, 2016). The washed biomass (wet) pellet (3.1 ± 0.2 g having 83.8 moisture content) was mixed with 15 mL solvent mixture of chloroform and methanol (2:1 v/v), and then incubated for 4h in an agitator water bath at 60 ^{o}C and 100 rpm. The mixture was then centrifuged at 4000 rpm for 10 min. After centrifugation, the mixture was separated in three different layers. The residual biomass was in the middle layer, bottom phase was lipid in chloroform and top layer methanol and water. The bottom layer of chloroform containing lipid was pipetted out and transferred into

a pre-weighed glass tube (L_1). The rest of the solution (containing cell debris, methanol) was again fortified with 15 mL solvent mixture of chloroform and methanol (2:1 v/v) and again incubated for 4h at 60 ^{o}C in the agitated water bath. After 4h incubation, the solution was filtered (Fisherbrand^{TM} Qualitative-Grade Filter paper, Particle retention: 5 to10 µm) using vacuum filtration. The filtrate was mixed with previously extracted solution (chloroform solution containing lipid) and the mixed solution was allowed to stand for phase separation. The bottom phase containing lipid in chloroform (the other phase was water and methanol) was collected and subjected to nitrogen sparging until total chloroform evaporated. The samples were further dried in an oven at 60 ^{o}C until constant weight (L_2). The lipid recovery from the biomass was calculated as:

$$CL\ \% = \frac{L2-L1}{DBW} \times 100\% \quad \text{--------- (1)}$$

The obtained lipid was stored for further transesterification study. Equation (1) CL represents the weight obtained from conventional lipid extraction, L_1 expresses the pre-weighed glass tube and L_2 denotes the oven dried microbial lipid in a pre-weighed glass tube and DBW denotes dry biomass weight.

2.2.2 Lipid transesterification

The lipid obtained from solvent extraction (as described above in section 2.2.1) was first dissolved in hexane (25 mL hexane per gram lipid), then mixed with methanol (methanol to lipid ratio was 6:1 or 0.08 mL methanol per gram lipid) containing sulfuric acid as a catalyst with concentration of 180 mM (4 µL H_2SO_4/g lipid) (Zhang *et al.*, 2016). Acid catalyst was used because alkali catalyst will form soap if biomass consists of more than 6% water content (Suter *et al.*, 1997). The mixture was then heated to 60^{o}C for 12 h. After the reaction, 50 mL of NaCl

solution (5% w/v) per gram of lipid was added and the solution was allowed to stand for 15 min. FAMEs were extracted in hexane (top) phase. The bottom phase was again treated with hexane (25 mL per gram lipid) to recover the remaining FAMEs. The FAMEs were separated and mixed with the fraction separated earlier. The FAMEs in hexane was washed with 2% sodium bicarbonate solution (10 mL per gram lipid), to remove excess water and the top hexane layer was then dried at 60^{o}C in an oven (Halim *et al.*, 2011).

The FAMEs were re-dissolved in hexane (0.01 g FAMEs/10 mL hexane) and analyzed using a Gas Chromatograph linked with FID (GC-FID) (Perkin Elmer, Clarus 500). The dimensions of the column used are 30m x 0.25 mm, with a phase thickness of 0.2 µm. Helium was the carrier gas at a flow rate of 1.18 ml/min with the oven temperature 230 ^{o}C. Transesterified sample of 1µl was injected with an automated sample injector and the sample analysis was performed with Agilent chem Station module software from Agilent Technologies.The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1,3-Dichlorobenzene was used as internal standard with concentration of 50 ppm.

2.3 In situ transesterification (with or without N-LS treatment)

The oil bearing biomass pellet (3.1±0.2g wet biomass containing 83.8% water) was obtained after centrifuging the fermented and heat treated broth at 8000 rpm for 10 min. The biomass pellet was washed with hot water. To disrupt the cells and release the lipids, the biomass pellet was treated with N-LS solution (2 mL solution containing 12.5 g/L N-LS, which is equivalent to 48 mg dry N-LS/g dry biomass) for 8 minutes at 30^{0}C and 180 rpm in an incubator shaker (Yellapu *et al.*, 2016b). For *in situ* transesterification, sulphuric acid solution in methanol of

different strength (180, 360, 540mM) was prepared and added to the reaction mixture (after 8 minutes of N-LS treatment) so as to obtain different molar ratio of methanol to lipid (methanol to lipid molar ratio of 60:1, 120:1 and 240:1). Ten mL of co-solvent (hexane, which aids in the separation of FAMEs) was also added. For control (N-LS non-treated biomass), methanol acid solution and 10 mL hexane was directly added to the washed wet biomass pellet. The reaction mixture was incubated in a water bath at 60^{o}C, 100 rpm and 24h. A similar expirment was conducted without adding hexane to check the effect of co-solvent on *in situ* transesterification and concluded that hexane did not affect the either cell disruption or transestetrification process. Six tubes were kept for each reaction parameters and one tube was withdrawn at 2, 4, 6, 10, 16 and 24h to determine FAMEs concentration. After 24h reaction, 5mL hexane was added again in all the tubes to extract FAMEs. For each sample drawn, the reaction mixture was allowed to stand for 15 min for phase separation. The top phase, FAMEs in hexane, was collected and the bottom phase (containing biomass, water, methanol glycerol, catalyst) was again treated with hexane (25mL/g lipid) and allowed for phase separation. The top layer (FAMEs in hexane) was collected and mixed with the previous fraction of FAMEs in hexane. FAMEs in hexane was purified by adding 5% w/v NaCl solution (50 mL NaCl solution per gram lipid) and the solution was allowed to stand for 15 min. FAMEs were extracted in hexane phase (top layer) and the bottom phase was again treated with hexane (25 mL per gram lipid) to recover the residual FAMEs. Further, the FAMEs in hexane were washed with 2% w/v sodium bicarbonate solution (10 mL per gram lipid) to remove the excess water and the top layer containing FAMEs in hexane was then collected and dried at 60^{o}C in an oven (Halim *et al.*, 2011). The FAMEs concentration was determined and analyzed with GC-FID as described in section 2.2.2. The

FAME yield (%w/w) was calculated based on concentration of FAMEs measured by GC-FID divided by the total lipids concentration × 100%).

2.4 Ultrasonication assisted in situ transesterification (with or without N-LS treatment)

All experiments were conducted as outlined in the previous section (section 2.3). However, in this case the reaction mixture was treated with ultrasonication. Ultrasonication was conducted with ultrasonic processor CPX 750 (Cole-parmer Instrument, IL) at 20 kHz. Methanol and H_2SO_4 (catalyst) was added to N-LS treated (as outlined in section 2.3) or without N-LS treated wet biomass (3.1±0.2g) along with 25 mL of co-solvent hexane. The ultrasonic horn was directly immersed (5mm) in the solution in a 50 mL glass tube surrounded with ice to control temperature less than 25 ^{o}C for a desired time. A plastic cover was wrapped around the glass tube in order to minimize the loss of methanol and hexane. The sonication time was varied from 1 to 35 min and on/off cycle was set 50/10 sec respectively, in order to minimize the heat generated. The samples were treated and FAMEs concentration was determined and analyzed as described in sections 2.2.2 and 2.3. All experiments were conducted in triplicate and the calculated standard deviation was less than 5%.

2.5 Biodiesel fuel properties

The purified biodiesel obtained from above study was tested for estimation and evaluation of its fuel properties and compared with recent literature, using the prediction models for biodiesel (ASTM standard : D664, D613, D445 and D2075 respectively). The physiochemical characteristics of biodiesel such as cetane number, density, Kinematic viscosity and glycerol concentraion were determined (Kakkad *et al.*, 2015). Biodiesel was further blended with

petroleum diesel (5%, 10% and 20% v/v or B-5, B-10 and B-20) and their physico chemical characteristics were also defined.

2.6 Statistical Analysis

The data value presented in this article were obtained in triplicate experiments. The statistical analysis was performed using Sigma plot 11.0 (Systat Software, Inc. Chicago, IL, USA), mean values were compared and analyzed using one-way analysis of variance (ANOVA). Differences were considered statistically significant for $p<0.05$.

3. Results and discussion

3.1 Two step process

The FAMEs yield obtained from two-step (lipid extraction followed by transesterification) transesterification process was found to be 94.6 ± 1.5 % (w/w). The wet biomass used in these experiments consisted of 83.8 % moisture content. According to Laurens *et al.,* (2012), the use of alkaline catalyst will form soap by saponification reaction in the presense of 6 % or higher moisture content of biomass. Therefore, in the present study (two-step and *in situ* transesterification), acid catalyst was used to avoid saponification due to high moisture content and conversion of total lipid to FAMEs.

3.2 In situ transesterification without N-LS preatreated biomass

The FAMEs yield obtained during *in situ* transesterification at a constant concentration of catalyst and with varying methanol to lipid (lipid in biomass) molar ratio of 60:1 to 360:1 is presented in Fig.1. The FAMEs yield increased with reaction time in 24h. Methanol concentration and H_2SO_4 (Catalyst) concentration affects the oil conversion efficiency to FAMEs

(Ehimen *et al.*, 2010). Increasing the H_2SO_4 concentration from 180 to 360 mM at a specific methanol to lipid molar ratio (360:1), there is an increase in FAMEs yield from 50.1 ± 1.1 to 71 ± 1.4 % (w/w) (increased upto 1.44 times) (Fig.1). However, with further increase in catalyst concentration from 360 to 540 mM, FAMEs yield did not appreciably increase. The reaction time required to reach equilibrium (or maximum FAMEs yield) was decreased from 16 to 10h (Fig.1b and 1c), when the catalyst concentration was increased from 180 to 540 mM. An increase in FAMEs yield with an increase in catalyst concentration during *in situ* transesterification was also observed by (Velasquez-Orta *et al.*, 2012). The acid catalyst could involve in reactions other than transesterification, such as hydrolysis of carbohydrates. Therefore, higher concentration of catalyst may be required to achieve high FAMEs yield.

In *in situ* transesterification process, according to equilibrium reaction, 1kg of lipids will produce 1kg of FAMEs. One triglyceride molecule (891 g) requires three methanol molecules (38.04 g) to produce three FAMEs molecules (298 g) and 1 glycerol molecule (92 g). The methanol requirement is very high (360:1 molar ratio, which is equal to 6.4 mL methanol/g lipid) as compared to the two-step transesterification process (0.08 mL/g lipid). FAMEs yield also decreased from 94.6 ± 1.5 (two step) to 50.2 ± 1.1% (w/w) (*in situ*) in 16h reaction time by using similar catalyst concentration (180 mM) (Fig.1a). According to Zhang *et al.*,(2014b) FAMEs yield obtained was 90.4 ± 1.3 % (w/w) using lyophilized biomass and 360:1 methanol to lipid molar ratio. It explains that high moisture content present in the biomass (lipid content) is a severe obstacle for methanol and catalyst to react with lipids (Laurens *et al.*, 2012). High methanol concentration during *in situ* transesterification process is required to weaken and disrupt the outer cell wall and finally reactants can react with lipids to convert them into FAMEs. According to these results (Fig.1) an increase in catalyst concentration and methanol to lipid

molar ratio is not a solution to achieve the maximum FAMEs yield. Moreover, consumption of high quantity of methanol will not be economical for biodiesel production.

3.2 In situ transesterification with N-LS preatreated biomass

Biomass (wet) pretreatment with N-LS can disrupt cell wall (Yellapu *et al.*, 2016b). After N-LS treatment, FAMEs yield obtained was 80.3 ± 1.5% w/w with methanol to lipid molar ratio of 60:1 using a 180mM catalyst (H_2SO_4) concentration. However, under similar conditions, the FAMEs yield obtained from non-treated biomass was only 12.6 ± 0.4% w/w (Fig.1 and 2), which is 6.3 times lower than the yield obtained from N-LS treated biomass. Thus, after N-LS treatment of biomass, the reactants methanol and catalyst (H_2SO_4) react easily with lipid in the solution and enhance the lipids conversion efficiency to FAMEs.

An increase in methanol to lipid molar ratio (from 60:1 to 360:1) and catalyst concentration (180 to 540 mM) increased the FAMEs yield (Fig.2). The maximum FAMEs yield obtained was 96.2 ± 2% (w/w) with 240:1 methanol to lipid molar ratio and 540 mM H_2SO_4 concentration (Fig.2c). However, under similar conditions, the FAMEs yield obtained from non-treated biomass was only 72.2 ± 1.4% (w/w).

In situ transesterification with N-LS non-treated biomass using constant methanol to lipid molar ratio (60:1) and by increasing the catalyst concentration of 180, 360 and 540mM, the FAMEs yield obtained was 12.6 ± 0.4, 14 ± 0.3 and 20.3 ± 0.8, respectively (Fig.1). However, under similar conditions, the FAMEs yield obtained with N-LS treated biomass was 80.3 ± 1.5, 80.9 ± 1.4 and 82.5 ± 1.7, respectively. The increase in FAMEs yield was due to cell wall disruption by N-LS, which lead to a direct contact of lipids with the reactants. Thus, requiring a lower concentration of methanol and catalyst. However, the reaction time to approach the maximum

FAMEs yield of 95.3 and 96.2 ± 2% (w/w) was 10h and 16 h, respectively. Thus, due to long reaction time, it may not be practically feasible to use this process for large scale biodiesel production. The similar results were reported by Salam *et al.,* (2016), where pre-treatment of *Nannochloropsis* (wet biomass with 20% moisture content) with sodium dodecyl sulphate (SDS) and *in situ* transesterification resulted in maximum FAMEs yield of 98 % w/w within 24h reaction time (Salam *et al.*, 2016). Therefore, in this study ultrasonication aided *in situ* transesterification was conducted to decrease reaction time and is presented below.

3.3 Ultrasonication assisted in situ transesterification (without N-LS)

Using ultrasonication, the FAMEs yield of 80.2 ± 1.5% w/w (Fig.3) was obtained with lower requirement of methanol to lipid molar ratio (120:1) and catalyst concentration (180 mM) within a reaction time of 25 min. The similar FAMEs yield of 80.1 ± 1.1% w/w (Fig. 2a) was obtained at 10h reaction time using similar reactant conditions [methanol to lipid molar ratio of 120:1 and catalyst (H_2SO_4) 180mM concentration] and N-LS assisted cell disruption process without ultrasonication. The ultrasonic wave creates violent shear forces (based on amplitude) upon cell wall, which results in quick cell rupture and resulting in release of lipids. The rapid reaction of methanol with the released lipids for transesterification leads to lower requirement of methanol and shorter reaction time compared to that without ultrasonication.

Increasing the catalyst concentration from 180 to 540 mM at a constant methanol and a lipid molar ratio (240:1), the FAMEs yield increased from 80.2 ± 1.5 to 82.3 ± 0.9% (w/w), which is only 2% (w/w) enhanced (Fig.3). Further increase in catalyst (H_2SO_4) concentration did not show an appreciable impact on FAMEs yield.

According to Zhang *et al.,*(2014b), high conversion of lipid to FAMEs (92.1% w/w) for lyophilized biomass was achieved with molar ratio of 60:1 using 1% NaOH catalyst within 20 min reaction time. However, in the present study the maximum lipid conversion or FAMEs yield of 82.9 ± 1.6 % w/w was obtained with methanol to lipid molar ratio of 360:1 using 180mM H_2SO_4 within 25 min reaction time. Thus, high biomass moisture content requires high concentration of reactants (methanol and catalyst), which is also observed by other researchers (Laurens *et al.*, 2012).

3.4 N-LS assisted and ultrasonication aided in situ transesterification

N-LS treatment followed by ultrasonication process leads to a high FAMEs yield of 94.3 ± 1.9% w/w with methanol to lipid molar ratio of 360:1 using 360mM catalyst concentration within 5 min. reaction time (Fig. 4b). However, under similar conditions, the FAMEs yield of 82.9 ± 1.8% w/w (Fig. 3b) was obtained within reaction time of 25 min using ultrasonication treated biomass (without N-LS). As per the equilibrium reaction, the methanol requirement is 0.08 mL methanol/g lipid, but in this study 6.4 mL/g methanol gave maximum FAMEs yield. The process ccost can be decreased if the residual methanol with glycerol solution is reutilized for lipid production.

The various researchers have reported different FAMEs yield during *in situ* transesterification of oleaginous wet biomass (Table 1). Different chemicals (such as hydrochloric acid, supercritical methanol and sodium dodecyl sulphate) have been applied for pre-treatment of lipid containing wet biomass (moisture content 20 to 80.4 Wt%). However, low FAMEs yield was obtained after *in situ* transesterification, when high moisture content was present in the biomass (Jazzar *et al.*, 2015, Laurens *et al.*, 2012). Another study by Salam *et al.,*(2016) reported high FAMEs yield of 98% w/w within 24h reaction time, when pre-treated

(Sodium dodecylsulfate) wet biomass with moisture content 20% was used for *in situ* transesterification.

In the present study, the maximum FAMEs yield of 94.3 % w/w was obtained using wet biomass (with 83.8% moisture content) in only 5 min reaction time, which is the highest yield at high moisture content and lowest reaction time (Table 1). In comparison with the two-step transesterification process, the methanol requirment is high (methanol:lipid ratio 360:1), but the time of reaction decreased from 16 h to 5min using N-LS assisted ultrasonication aided *in situ* transesterification. It indicates that the mild surfactant (N-LS) effectively disrupted the cell wall and released lipids, thereby increasing accessibility of methanol to the internal body lipids and hence further exposed the lipid to direct contact with reactants within a short time. The less reaction time can offset the cost of high reactants volume required at industrial scale biodiesel production. The results obtained in this study are statistically significant for $p<0.05$.

Moreover, pre-treatment cost for cell (wet) wall disruption using N-Lauroyl sarcosine is expected to be low (0.48$ N-LS/kg dry biomass) compared to the conventional process used to release the intracellular lipids without drying and application of organic solvents (chloroform, methanol and isoproponol). In case of the conventional process (centrifuge, drying) high quantity of organic solvents are used for lipid extraction and transesterification, which adds the cost up to >4 $/kg dry biomass to the whole process (Table 2). These organic solvents can be recovered by using distillation, but it is highly energy intensive process.

3.6 Comparison of FAMEs composition from different transesterification processes

The FAMEs obtained from *in situ* transesterification with (or) with out N-LS pretreatment and ultrasonication assisted *in situ* transesterification was presented in Table 3. Increasing the

methanol to lipid molar ratio from 60:1 to 360:1, there is an increase in C16:0, C18:1 and C18:2 during *in situ* transesterification. High methanol to lipid molar ratio (360:1) is required to release the neutral lipid granules, which are surrounded by phospholipids membrane. These neutral lipids are then converted into FAMEs (Giroud *et al.*, 2013). It explains that when the lipids granules are released out of the cell, H_2SO_4 and methanol are able to react well with them and improve FAMEs yield. The GC-FID data indicates that microbial FAMEs was mainly composed of oleic acid and linolelaidic acid (82% of total FAMEs). It has been reported that microbial lipids were characterized as highly unsaturated fatty acids content feed stock for biodiesel (Patil *et al.*, 2011). The saturation rate ~48.2 % w/w total lipids (Table 3) indicates that the FAMEs obtained from microbial oil will have very high oxidation stability than the biodiesel obtained from plant oil (*Jatropha*) (Jain *et al.*, 2011). Comparing the fatty acid profiles, the results revealed that N-LS followed by ultrasonic aided *in situ* transesterification did not cause any impact on the FAMEs composition.

3.7 *Biodiesel fuel properties*

The measurement of biodiesel fuel properties is quite complex due to high cost and the requirement of a considerable amount of fuel sample. Therefore, the researchers have developed prediction models and mathematical equations to predict the biodiesel properties from the FAMEs for *Aspergillus candidus*, *Aspergillus terreus* IBB M1 and *Yarrowia lipolytica* strains (Kakkad *et al.*, 2015). In order to certify the biodiesel for commercial sale, well defined standards have been set for FAMEs (ASTM D6751). In the present study, density, cetane number, acid value number, kinematic viscocity and total glycerol percentage were experimentally determined and the results were summarized in Table 4. The density, cetane number, acid number, kinematic viscocity and total glycerol percentage of the biodiesel

produced by N-LS pretreatment of the biomass followed by ultrasonication assisted i*n situ* transesterification was 0.8602 g/cm^3 , 65.2, 1.15, 8.08 mm^2/s (at 40^{o}C) and 0.095 (%wt), respectively. The fuel properties of pure biodiesel and blended biodiesel (B100, B20, B10 and B5) were almost similar to ASTM standard biodiesel. Moreover, biodiesel obtained from *Yarrowia lipolytica* (N-LS pre-treated and ultrasonication assisted *in situ* transesterification) has fuel properties comparable to the biodiesel obtained from microalgae and fungi (Kakkad *et al.*, 2015, Mostafa *et al.*, 2013). Therefore, the biodiesel obtained by N-LS pretreatment followed by ultrasonication assisted *in situ* transesterification and having properties similar to the standard fuel will be suitable for commercial production and for application as transport fuel as well as electricity generation.

4. Conclusion

The results obtained in this study revealed that it is feasible to reduce reaction time employing N-LS treatment of wet biomass followed by ultrasonication aided *in situ* transesterification. Maximum FAMEs yield was obtained within 5 min of reaction time using N-LS assisted ultrasonication aided *in situ* transesterification, which is very low as compared to 12 h of reaction time used in the two-step transesterification process. The composition of FAMEs obtained in *in situ* transesterification with (or) without N-LS treatment was similar to that obtained in two step transesterification. The lipid bearing wet biomass (83.8% moisture) conversion to FAMEs using N-LS followed by ultrasonication aided *in situ* transesterification could be a promising approach as it eliminates the use of toxic solvents for lipid extraction and obviates the lyophilization or drying the wet biomass, which helps in reducing the energy consumption for an industrial scale biodiesel production.

Acknowledgements

Sincere thanks are given to the Natural Sciences and Engineering Research Council of Canada (Grant A 4984 and Canada Research Chair) for their financial support. The views and opinions expressed in this paper are those of the authors.

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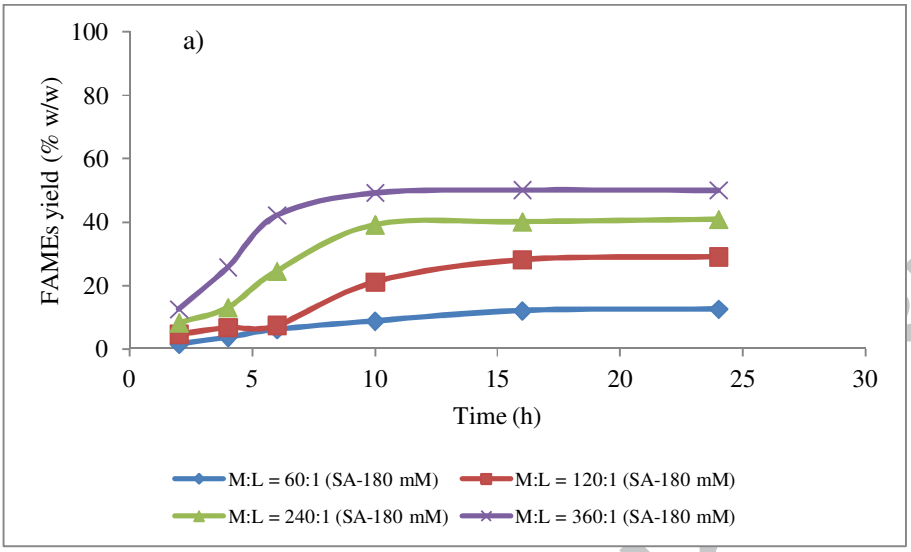

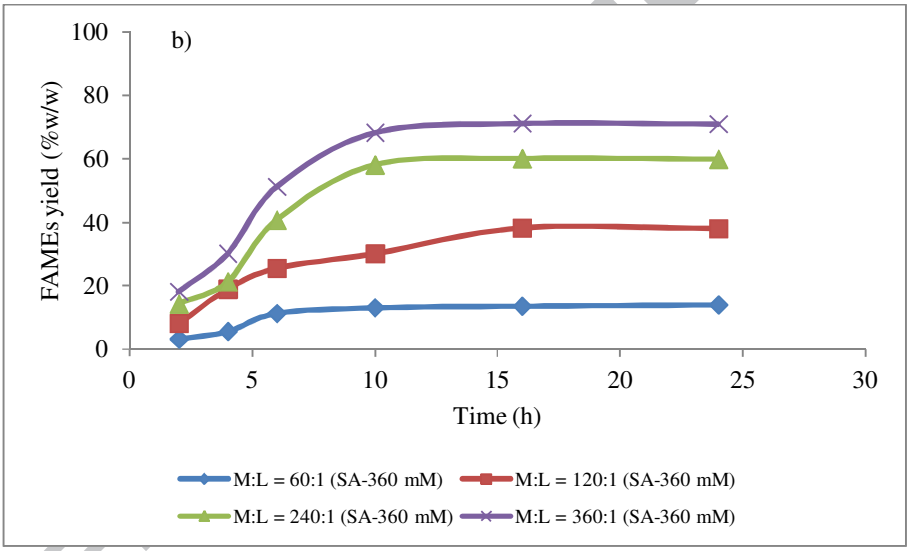

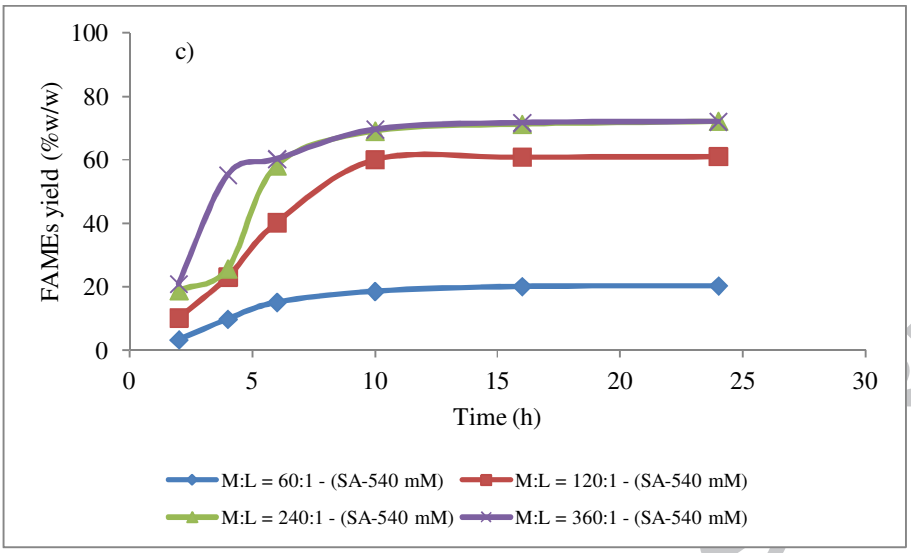

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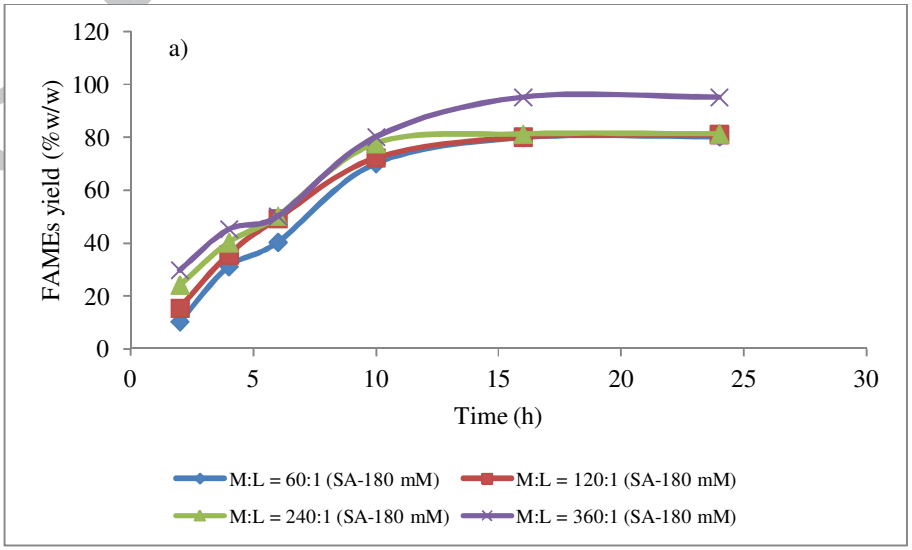

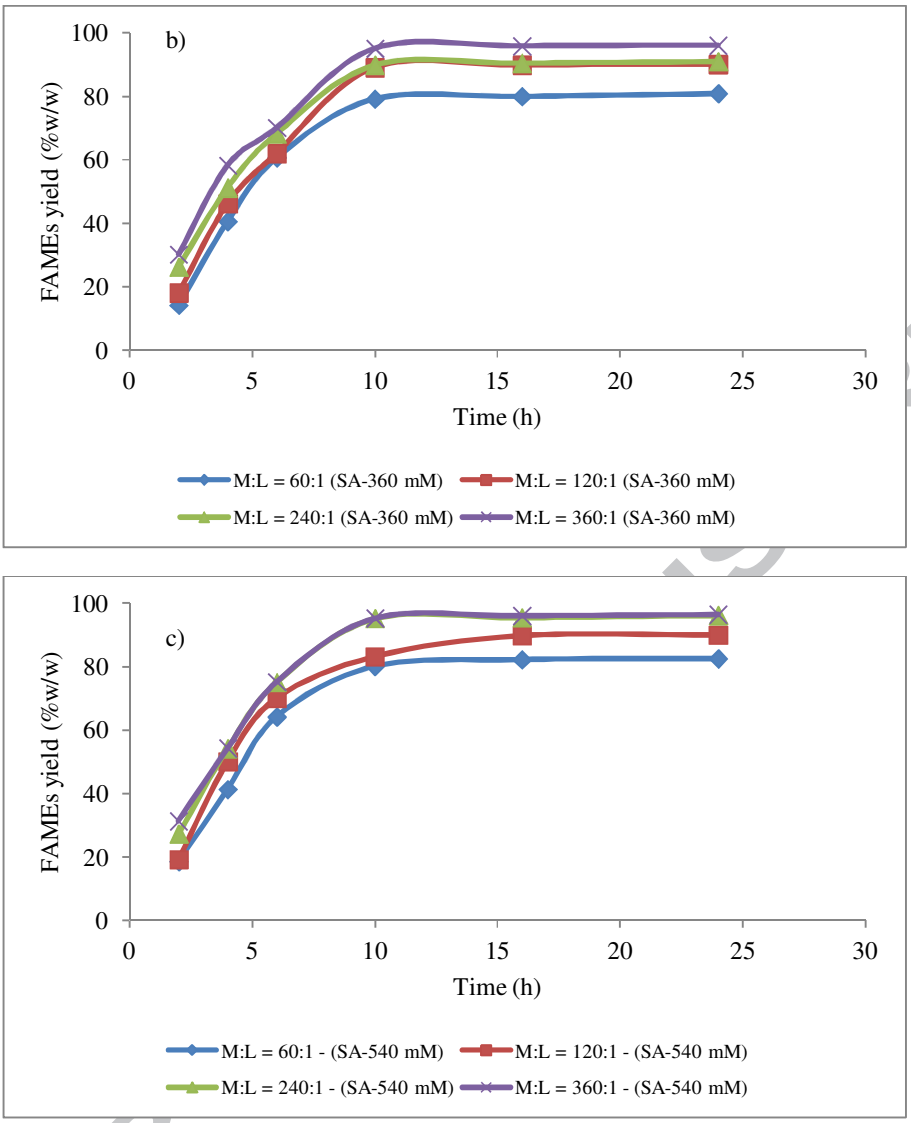

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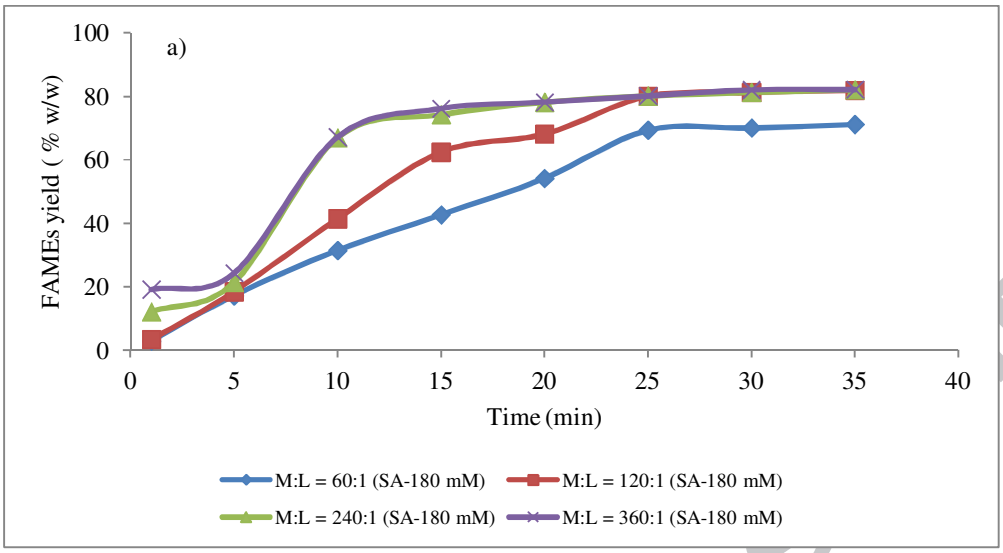

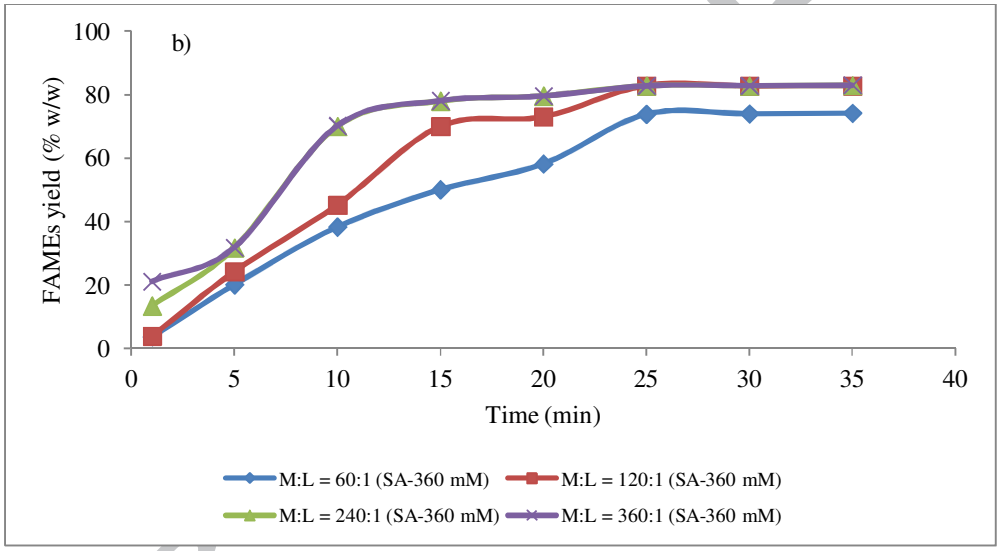

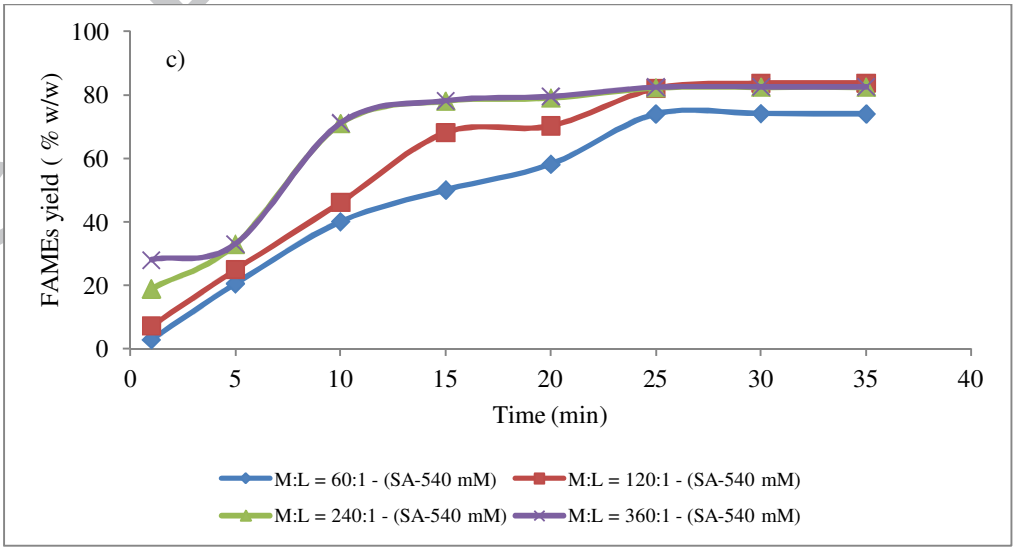

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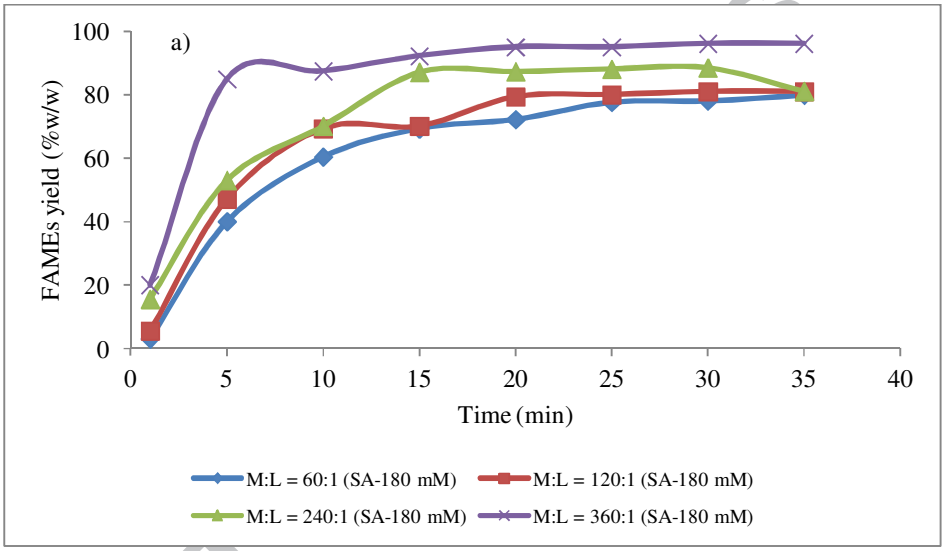

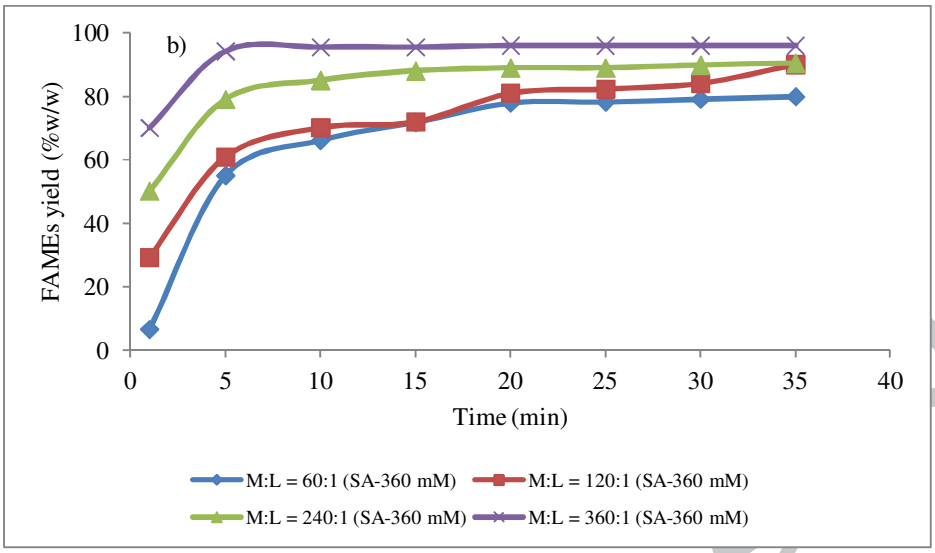

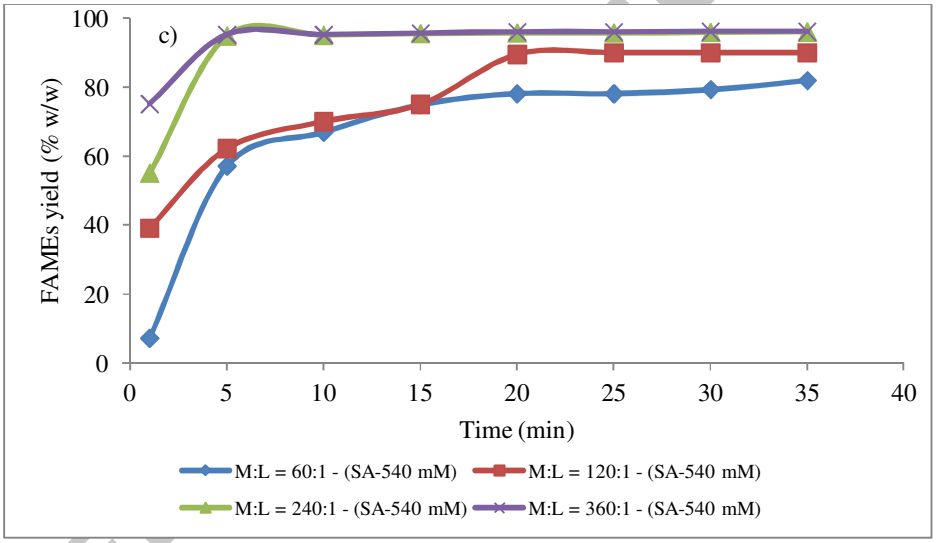

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Table 1.

Comparison of FAMEs yield during different *in-situ* transesterification processes using wet biomass

Oleaginous substance	Moisture Wt %	*In-situ* transesterification conditions	FAMEs yield % w/w	References
Chlorella vulgaris	78.67	HCl- catalyst, for 1h at 85^{o}C	43.05	(Laurens *et al.*, 2012)
Nannochloropsis sp.	80.24	HCl- catalyst, for 1h at 85^{o}C	10.12	(Laurens *et al.*, 2012)
Yarrowia lipolytica	83.8	H_2SO_4 – catalyst, for 10 min, at less than 25^{o}C; N-LS[a]	94.3	This study
Nannochloropsis occulata	20	SDS[b] and H_2SO_4 - catalyst, for 24 h at 60 ^{o}C	98.67	(Salam *et al.*, 2016)
Nannochloropsis sp	20	NaOH – catalyst, for 10 min at 50 ^{o}C; SCMH[c]	75	(Teo *et al.*, 2014)
Nannochloropsis gaditama	80	Supercritical methanol, for 50 min	47.8	(Jazzar *et al.*, 2015)

a- N-Lauroyl sarcosine treated ultrasonication assisted *in-situ* transesterification

b- Sodium dodecyl sulphate

c- Simultaneous cooling and microwave heating

Table 2.

Comparison of pre-treatment cost for lipid extraction

Method	Biomass harvesting	Pretreatment/Lipid extraction-Cost[a]	Transesterification	References
Conventional process	Centrifugation and freeze drying	C/M[b] cost - > 4 $/kg biomass	NaOH catalyst; 12h; 60°C	(Halim *et al.*, 2011, Zhang *et al.*, 2014)
NUIT[c]	Centrifugation	N-LS cost -0.48 $/kg dry biomass	H_2SO_4 catalyst; Sonication 05 min; 25°C	This study
SDS-IT[d]	Centrifugation and freeze drying	SDS cost - 0.50 $/kg dry biomass	H_2SO_4 catalyst; Incubation at 60°C, for 24h.	(Salam *et al.*, 2016)

a- Cost calculated based upon the market rate of chemical in 2016

b- Chloroform and methanol mixture (2:1) if process equipped with distillation and re-utilize solvent.

c- N-larouyl sarcosine (N-LS) pretreatment followed by ultrasonication assisted *in-situ* transesterification

d- Sodium dodecyl sarcosine (SDS) assisted *in-situ* transesterification

Table 3. Comparison of fatty acid profiles of FAMEs produced through two step transesterification and *in situ* transesterification (with or without N-LS treated biomass); Ultrasonication assisted *in-situ* transesterification (with or without N-LS treated biomass).

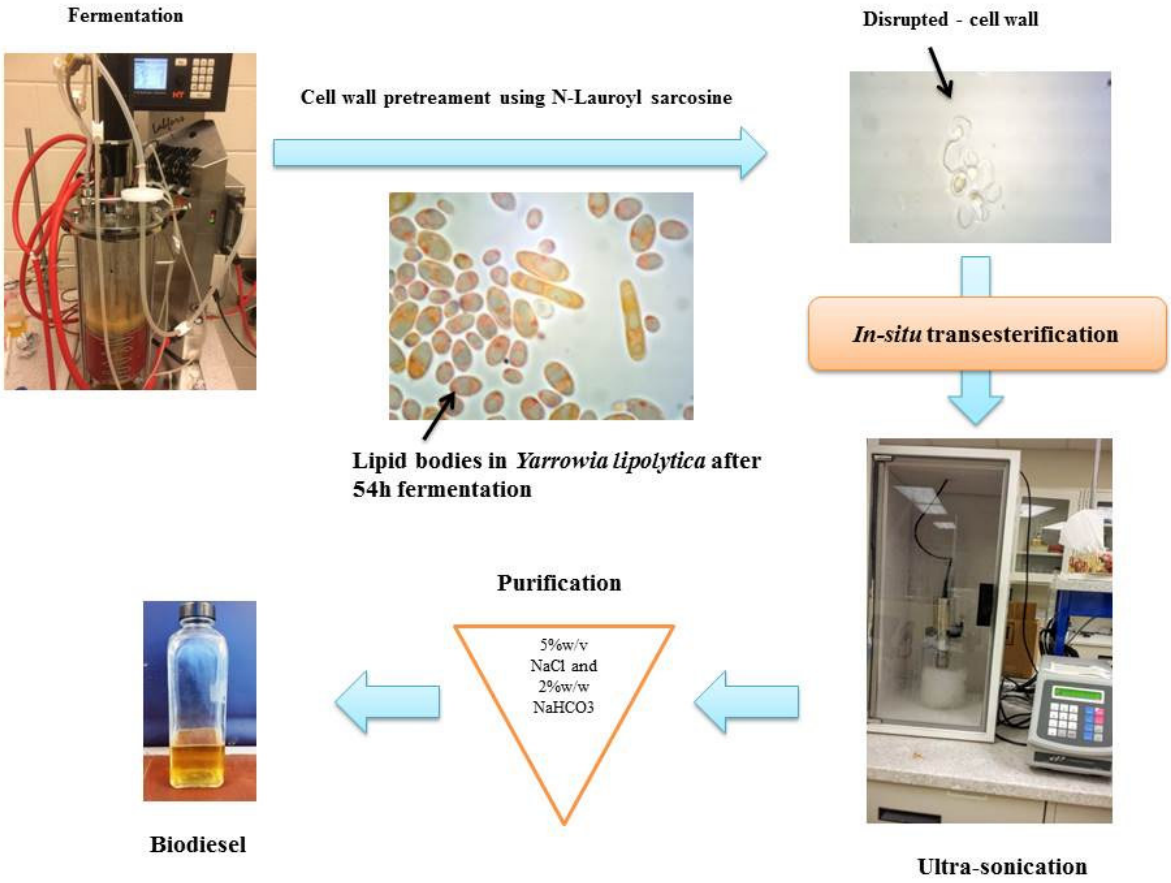
Fatty acids	Relative amount of total fatty acids (%w/w)															
	Two step Tranesterification (Microbial oil to FAMEs)	*In-situ* transesterification (Without N-LS treated biomass)				*In-situ* transesterification (N-LS treated biomass)				Ultrasonication aided *in-situ* transesterification (Without N-LS treated biomass)				Ultrasonication aided *in-situ* transesterification (N-LS treated biomass)			
		60:1	120:1:	240:1	360:01	60:01	120:01	240:1	360:1	60:1	120:1	240:1	360:1	60:1	120:1	240:1	360:01
C16:0	8.5	8.1	8.9	9.3	10.1	8.7	9.1	9.7	9.9	8.9	8.8	9.5	9.8	8.7	9.1	9.3	9.6
C16:1	1.3	0.6	0.8	1.2	0.7	0.8	0.9	1	1	0.9	0.7	0.8	1.0	0.5	0.9	1.1	0.9
C18:0	3.5	3.9	3.8	3.3	3.2	3.2	3.1	3	3.5	3.4	3.2	3.0	2.8	3.5	3.3	3.1	3.3
C18:1	33.1	30.8	31.7	32.9	33.3	31.2	32.2	33	33.2	32.7	31.9	30.6	29.5	32.6	31.6	30.1	33
C18:2	48.9	36.5	46.9	48.2	46.8	39.2	46.2	48.2	48.7	40.8	48.0	48.1	48.8	40.9	48.1	48.2	49
C18:3	4.7	5.0	4.7	5.3	6.4	4.9	5.2	5.9	6.1	5.04	5.3	5.3	5.9	4.9	5.1	5.8	6.09

Table 4. Comparison of biodiesel fuel properties

	Biodiesel fuel properties					
	Density g/cm^3 (D664)	Cetane number	Acid number	Kinematic viscosity; mm^2/s, 40^{o}C (D445)	Total glycerol (% wt)	References
B100	0.8393	47	0.50	1.9-6.0	0.24	ASTM (D6751-15a)
Biodiesel(Microalgae)	0.8637	70	0.75	12.4	Nr	(Mostafa *et al.*, 2013)
Biodiesel (B-100 [a])	0.8602	65.2	1.15	8.08	0.095	This study
Biodiesel (B-20[a])	0.8452	69	0.143	6.12	Nd	This study
Biodiesel (B-10[a])	0.8419	67	0.075	4.99	Nd	This study
Biodiesel (B-5[a])	0.8401	60	0.036	4.01	Nd	This study

a- Biodiesel obtained from N-lauroyl sarcosine pre-treatment of biomass followed by ultrasonication assisted *in-situ* transesterification

Nd- Not determined; Nr- Not reported



Highlights:

- The *in situ* transesterification process was used to convert lipid to biodiesel.
- N-Lauroyl sarcosine was used for wet biomass pretreatment.
- The ultrasonication was used to enhance FAMEs yield with low reaction time.
- There is no impact of N-LS pretreatment and ultrasonication on fatty acid profile.