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Comparative study between microwave and ultrasonication aided *in situ* transesterification of microbial lipids

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Abstract

Recent trends have focused on the development of a rapid method to convert microbial lipids to biodiesel. *In situ* transesterification allowed minimizing the requirement of solvents by combining the two steps (extraction of lipid and conversion to biodiesel) to a single step. Box–Behnken design was used for optimization of the variables to optimize the biodiesel yield and conversion. Microwave and ultrasonication assisted *in-situ* transesterification methods were compared based on the conversion efficiencies and their performance. Microwave approach revealed that around 99±0.5% of conversion of FAMEs (w lipid conversion/w total lipids) was obtained in the presence of methanol to lipid molar ratio above 183:1 and NaOH addition of 2% (w/w) lipid in 20 min at 100 ^{o}C. Meanwhile, the ultrasonication yielded around 95.1±0.2% (w/w total lipids) in the presence of methanol to lipid molar ratio 183:1 and NaOH addition 3% (w/w) lipid in 20 min at 25 ^{o}C. The final profile of FAMEs was fully compatible with that of the conventional process based on chloroform and methanol extraction and required 12 hours for extraction.

Keywords

In situ transesterification; Ultrasonication; Microwave; Fatty acids methyl esters (FAMEs)

1 Introduction

The gradual depletion of fossil fuel reserves and the continued use of petroleum-based fuels have encouraged researchers to seek viable, sustainable, and environmental friendly alternative sources of energy. [1-3] The exploitation of vegetable oils for biodiesel production had created numerous problems of food supplies and arable lands. Therefore, microbial oils called single cells oils (SCO) are considered to be a viable alternative since they do not have an impact on food supply and they do not require arable lands and could replace fossil fuels. [4] Many technical hurdles limit the use of these renewable source on large scale, especially, harvesting and extraction processes. Lipid extraction from oleaginous microorganisms required large amounts of organic solvents. Commonly, Folch method or its variant, the Bligh and Dyer method, have been used extensively for lipid extraction and quantitation. [5] However, owing to the hazardous nature of extraction using flammable organic solvents, and the adverse impact of solvent on the environment, it is strongly recommended to reduce the organic solvents and time of the extraction process. Terpenes, green solvents obtained from plants have been investigated as a replacement of organic solvents, although their efficiency and high costs limit their potential uses. [6] An ideal solution was to perform both extraction and transesterification processes simultaneously in one step thereby eliminating the solvent extraction step required to obtain the oil feedstock. In-situ transesterification refers to the direct transesterification of lipids in a biomass matrix without prior lipid extraction and offers the advantage of reducing processing units, lowering the fuel product costs and later quantifying fatty acids. Besides, process wastes and eventual pollution could also be reduced by this method. [7] Moreover, several methods are listed in literature (e.g. solvent,

68 enzymatic, mechanical, alkali, acid); however, not all were applicable due to their
69 relatively high cost and equipment corrosion. Besides, there is no definitive standard
70 method for neither lipid extraction nor quantification, nor for process development.[8]
71 Current works were based essentially on lipid extraction from algal species. [9-11]

72 Consequently, choosing a relevant method and optimizing its parameters was the main
73 challenge. Microwave-assisted *in situ* transesterification could be an alternative to
74 address the above concerns. This method allowed cell disruption and enhanced mass
75 transfer rates [12], which may result in high oils and lipids recovery.

76 Microwave irradiation has been reported to extract oil derived biomass, soils and
77 vegetable feedstock. [13-17] Besides, this method allowed good quality of extracts with
78 better target compound recovery.

79 A process that enables simultaneous oil extraction and transesterification is thus
80 worthwhile to develop. Response surface methodology (RSM), a multivariate technique,
81 was used in this work to optimize the levels of different variables (e.g. temperature,
82 reaction time, catalyst concentration, and different methanol to lipid molar ratios.)
83 reported highly critical in the *in situ* transesterification process. An optimum yield of
84 FAMEs was envisaged. The analyses were performed on lyophilized biomass. Several
85 trials were conducted to optimize the parameters related to this study. Besides, the impact
86 of ultra-sonication aided in-situ transesterification on FAMEs composition was also
87 investigated.

88

2 Material and Methods

2.1 Biological method

2.1.1 Crude glycerol, reagents and analyses

All the reagents were of analytical grade and used without further purification. Methanol, hexane and NaOH were purchased from Fisher Scientific, Canada. Crude glycerol was obtained from Rothsay in Canada. Ultra-sonication experiments were conducted with ultrasonic processor CPX 750 (Cole-Parmer Instrument, IL) at 24 kHz. Microwave trials were carried out with MARS microwave extractor, CEM Corporation, North, 155 Carolina, USA) equipped with Teflon tubes irradiated simultaneously. FAMEs were analyzed using a Gas Chromatograph linked with Mass Spectroscopy (GC–MS) (Perkin Elmer, Clarus 500). The dimensions of the column used are 30 m, 0.25 mm, with a phase thickness of 0.2 l/m. 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 at 50 ppm.

2.1.2 Strain, culture and harvesting conditions

The strain, *Trichosporon oleaginosus* (ATCC20509) was grown in a glycerol based medium containing (per liter): 1 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.2 g yeast extract, 50 g glycerol, and minerals 0.04 g $CaCl_2.2H_2O$, 0.0055 g $FeSO_4.7H_2O$, 0.0052 g citric acid·H_2O, 0.001 g $ZnSO_4.7H_2O$, and 0.00076 g $MnSO_4.H_2O$ were added. [18] Experiment was performed in 5 L fermenter at pH 6.5 and 28°C. pH was controlled by the addition of 4 N (NaOH and H_2SO_4). After 70 h, the biomass was harvested by

centrifugation at 5000 *x* g for 15 min. Biomass was washed twice with distilled water to remove the residual nutrients and glycerol. The experimental method is shown in Fig. 1.

2.2 Chemical method

2.2.1 Conventional extraction and transesterification method

Extraction was carried out at room temperature using the standard chloroform and methanol extraction procedure. [19-20] About 0.2 g dry biomass resulting from the fermentation of *T. oleaginosus* after 72 hours was mixed with 4 mL solvent mixture of chloroform and methanol (2:1 (v/v)), and then subjected to 60 °C for 4 hours. The mixture was then centrifuged at 5000 *x* g for 15 min and the solvent phase was withdrawn and transferred into a pre-weighed glass vial (W1). The extraction procedure was repeated two times. Afterwards, the vial containing the total volume of the supernatant collected from each extraction was subjected to 60°C in an oven to evaporate the solvents and was then weighed (W2). The lipid amount was calculated by the difference of W2 and W1. The lipid content in the biomass is calculated as (W2-W1)/200 mg × 100%. The obtained lipid was first dissolved in hexane (25 mL hexane per gram lipid), then mixed with methanol. Lipid to methanol molar ratio is 1:6 (0.3 mL methanol for per gram lipid). Sodium hydroxide was used as catalyst with addition of 1% (w/w) (NaOH/oil). The mixture was then subjected to 55 ^{o}C for 2 hours. After reaction, 5% (w/v) NaCl solution was added (100 mL NaCl solution per gram lipid), and then FAMEs was extracted by two times washing with hexane (100 mL per gram lipid). After phase separation by settling, the hexane phase (upper layer) was collected. The FAMEs in hexane was washed with 2% sodium bicarbonate solution (20 mL per gram lipid) and the mixture was

allowed to stand for 15 min for phase separation, and the top layer was collected and dried at 60 ± 1 ^{o}C in an oven. [21]

2.2.2 Ultrasonication aided transesterification

Amounts of methanol and NaOH catalysts corresponding to ml equivalent of methanol/oil ratio (6:1, 183:1, 360:1) relating to 0.08, 2.45, 6.4 mL were added to 0.2 g of dry biomass and then reacted with a sonication probe immersed directly in the solution in a beaker placed in a water bath to control temperature at around 25^{o}C for 20 min. Thermal meter was inserted to the bath to check the temperature. The sonication time was fixed at 20 min with one pause (2 min) at every 5 min sonication, and methanol to oil ratio was set at 60:1 - 360:1 (v/w). Amount of catalyst was varied from NaOH catalyst at 1 to 5% (w/w).

2.2.3 Transesterification aided by microwave heating

Transesterification reactions were carried out in the presence of NaOH catalyst (1 to 5% (w/w) at various reaction temperatures (40 -100 ^{o}C). The catalyst was dissolved in methanol (6:1 – 360:1) (v/w) and the resulting solution was added to the oil. This reaction was then irradiated by microwave field under reflux and heated to the desired transesterification temperature in desired time. Power output of microwave was 400 W. An aliquot of 25 mL of the hexane was added to each vessel. This reaction was then irradiated by microwave field under reflux and heated to the desired transesterification temperature.

2.3 *In situ* transesterification with microwave

A pre-determined mass of 0.2 g of biomass was weighed accurately into each teflon vessels. Corresponding percent of methanol and NaOH was added separately to each vessel. The microwave power was set to 400 W. The temperature was kept at ambient (± 25ºC and the time was set to an initial 15 min ramp with 15 min hold time and a final 15 min cooling time). After the transesterification, vessels were removed, 5% w/v NaCl solution was added (1 mL per gram biomass) and all samples filtered using Whatman filter paper to remove the residual biomass and the solvent was evaporated. The collected samples were allowed to stand overnight or (centrifugation (5000 *x* g, 20 min). A small aliquot of the supernatant was siphoned off and transferred to a vial for gas chromatographic analysis.

2.4 Two-stage process

The extractive-transesterification experiments were conducted using microwave radiation. In the two-step production, transesterification was carried out on the lipid previously extracted from dry biomass with chloroform/methanol (e.c. conventional method), then using microwave and ultrasonicator, following the transesterification (described in section **2.2.2** and **2.2.3** and presented in Fig. 1).

2.5 Optimization of *in situ* transesterification by Box–Behnken design (BBD)

A 4-level 4-factor Box–Behnken design was adopted to evaluate the effects of temperature (X1), reaction time (X2), methanol to lipid molar ratios (X3), catalyst concentration (X4), and lipid conversion efficiency of *T. oleaginosus* on crude glycerol based medium. In this regard, the experimental plan contained 29 trials and the

independent variables were studied at three different levels, namely low (-1), medium (0) and high (+1), whose values are shown in Table 1.

The effect of the three factors and their interactions were studied using the response surface methodology. [22] Based on experience and economic feasibility, a three factorial subset design was employed. [23] The total number of experimental runs was 29 with replications as shown in Table 2. The temperature, time, methanol to oil ratio and catalyst were varied in the ranges of 40 - 100^{o}C, 20-60 min, 6:1 - 360:1 (v/w), 1 - 5% (w/w) respectively. The lipid conversion efficiency was taken as the response variable (Y). The experimental design used in this work is shown in Table 2. The response variable was fitted by a second order model to correlate the response variables to the independent variables. The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software (Version 7.0, Stat-Ease Inc., Minneapolis, USA). The general form of the second degree polynomial equation is:

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

Y: the predicted lipid conversion efficiency (% w/w lipid)

β_0: the intercept

β_i: the linear coefficient

β_{ij}: the quadratic coefficient

β_{ii}: the linear-by-linear interaction between Xi and Xj regression coefficients

Xi, Xj: input variables

Statistical analysis of the model was utilized to evaluate the analysis of variance (ANOVA). This analysis englobed Fisher's F test (overall model significance), associated probability *p (F),* correlation coefficient R and determination coefficient R^2. All parameters play role in measuring the goodness of fit of regression model. Quadratic models were used for each variable and were represented as contour plots (3D). Response surface curves were generated using Design Expert software.

3 Results and discussion

3.1 Statistical analysis of experimental design

The conventional extraction method which consisted of a mixture of chloroform/methanol (2:1 (v/v)) provided lipid content of 47.3±0.9 % (w/w) of dry biomass. This percentage is considered as 100% of conversion of biomass to lipid. The lipid conversion efficiency to fatty acids methyl esters (FAMEs) is calculated by determining amount of FAMEs by GC–MS and dividing this value by total lipids (g FAMEs/g total lipids). Many parameters have been reported to control the lipid efficiency including (e.g. the amount of catalyst added, reaction time, temperature and molar methanol to lipid ratio).

The statistical significance of the designs was determined by F-test for ANOVA (Table 3). As seen from this table, operating parameters had a significant effect on the fatty acid methyl ester content which is confirmed by the p-values of the analysis. Values of "Prob > F" are less than 0.05 which indicated that the model is significant with 98.54% confidence level. Therefore, the P-value of the lack of fit analysis was (< 0.0001) which confirmed that the model was significant and reliable for lipid production in this study. Besides, correlation coefficient, R^2 (0.989) supported the correlation between the *in situ* transesterification process parameters.

The value of adj-R^2 (0.979) suggested that the total variation of 97.99% for the lipid concentration was attributed to the independent variables and only about 3.01% of the total variation could not be explained by the model. Besides, model coefficients for each variable are also shown in Table 3. The larger F-value and smaller P-value suggested higher significance of the corresponding coefficient. Among the model terms, X1

(temperature), X3 (methanol/oil ratio), $X1^2$, $X3^2$ were significant. By contrast, other terms were not significant. The relationship between the response and experimental levels of each variable can be demonstrated by three-dimensional response surface plots which represented the regression equation mentioned below:

$$\mathbf{Y= 82.5 + 17.08333\ X1 + 0.066666\ X2 + 28.316666\ X3 + 0\ X4 - 1.625\ X1X2 + 6.4\ X1X3 - 2.025\ X1X4 - 0.475\ X2X3 + 0.65\ X2X4 - 1.525\ X3X4 - 9\ X1^2 - 0.05\ X2^2 - 27.675\ X3^2 - 0.175\ X4^2} \quad \mathbf{(2)}$$

where Y is the observed response (lipid conversion efficiency) for the microwave in-situ transesterification. X1, X2, X3 and X4 are the coded values of independent factors temperature, reaction time, methanol to oil molar ratio and catalyst amount, respectively.

3.2 Optimization of microwave process parameters with RSM

In conventional method of biodiesel synthesis, the reaction time and temperature are 30 min–12 hours and 55–65 °C, respectively. [24-26] Besides, Melo-Junior et al. (2009) have studied in detail the esterification of oleic acid (C18) under microwave irradiation while varying alcohol type (methanol or ethanol), temperature (150-225°C) and molar ratio of alcohol/fatty acid (3.5-20) , a conversion rate up to 60% was obtained in 60 min of reaction [27]. In this regard, present study was carried out to optimize different parameters in the microwave assisted direct transesterification; reaction temperature, time, methanol to oil molar ratio and catalyst amount were chosen as variables. To compare the temperature effect on the conversion yield, in-situ transesterification was conducted at 40, 80 and 100 ℃. Thus, according to literature, when using a homogeneous catalyst (herein NaOH), harsher condition including high temperature [28] is required to achieve

high FAMEs yields. Besides, preliminary study has showed that only 14.5±1.2% of FAMEs were obtained under low temperature at 25 °C. Conversely, higher conversion efficiency above (> 90%± 1.2 (w/w) was obtained in a lower reaction time 20 min at 100 °C. Microwave effect at 100 °C was four fold compared to 40 °C which confirmed the positive role of temperature (low p value < 0.0001). At 70 °C, around 83±0.6% of FAMEs (w/w) was obtained. Therefore, higher the reaction temperature, the more the reaction can be driven. This is in accordance with Im et al. (2014) who proved the positive effect of temperature on FAMEs yield, around 91.1 % was obtained at 95 °C for 90 min. [29] Moreover, Sunita et al. (2008) have observed that the conversion rate of oil to biodiesel increased significantly with the rise in temperature and was reported to be 73% and 97% at 180 and 200 °C, respectively. [30] Moreover, a complete conversion (100%) of caprylic acid for the esterification was achieved at a higher temperature, 175–200 °C. [31-32] High temperature may lead to the formation of microzones called "hot spots", which lead to an increase in the escalation of chemical reaction rate. [33] The loss of methanol was not seen in this study compared to current studies [25, 34-35], this is mainly due to nature of the closed system that resists higher temperatures. Both high temperature and thermal effect caused by the microwaves enhanced the extractive properties of methanol to extract more lipids in the biomass via diffusive extraction and extended microwave effect caused the penetration through the cell walls and forces out the oils into the solvent mixture through disruptive extraction. Another observation to be taken in advantage from this work is the absence of emulsions and soap formation which is primarily related to the high temperature effect, thus, free fatty acids (FFA) are converted efficiently into FAMEs, which has been proven in previous studies that noted the role of microwave irradiation in

the reduction of FFA content within the first 15 min.[36] Furthermore, Kamath et al. (2011) reported around 87.39% of FFA reduction during the transesterification of crude karanjja oil through microwave irradiation.[37] No soap formation is principally due to absence of the catalytic poisoning by water formed as a result of esterification, so that microwaves and high temperature reduced the free fatty acid content and made it easier to separate biodiesel and alcohol layers. As seen in Fig. 2, catalyst more than 3% (w/w) showed a positive effect on the in situ transesterification reaction. Herein, NaOH is used as a homogeneous, solvent-catalyst; the choice of this catalyst rather than others is related to its higher yield of biodiesel conversion rates, [38] and its ability to break chemically the molecule of the raw renewable oil into methyl or ethyl esters. Highest biodiesel conversion of 93.94± 0.3 % was observed using 3 % (w/w) of NaOH catalyst with methanol to oil ratio of 183:1. Conversely, the lower amount of catalyst (proportional to methanol ratio 6:1) may not efficiently advance the reaction and gave a yield of 24.5 ± 0.1% (w/w) of conversion rate.

Methanol to lipid ratio had a significant effect on the *in situ* transesterification, and this was confirmed with a low P value < 0.0001. Herein, methanol exhibited binary action and acted as a solvent for extraction of the microbial oils/lipids and a reactant for transesterification of esters.[39] Thus, applying microwave irradiation during *in situ* transesterification will serve for dual purpose (e.c. rendering lipids available for reaction as well as intensification of process).

Methanol to oil molar ratio was varied from 6:1 to 360:1 in the microwave direct transesterification reaction. A lower ratio than 6:1 (v/w) does not favor the *in situ* transesterification process and a lower yield is observed. When the methanol to oil molar

ratio was increased to 183:1, the maximum biodiesel conversion observed was 92.3±1.0% because of the increased contact area between methanol and oil/lipid. This is in accordance with Sunita et al. (2008) who found that increasing methanol to oil ration from 10:1 to 20:1 enhance the conversion of sunflower oil to biodiesel from 30% to 90% respectively. [30]

Further increase of molar ratio up to 360:1 did not give significant difference. Generally, a higher amount of methanol may reduce the concentration of the catalyst in the reactant mixture and does not give higher yield during the transesterification reaction. [40] Moreover, with a lower methanol ratio, the downstream cost can be controlled. [41]

The reaction time of around 20 min seemed to be adequate for the complete process. The reaction time had no significant effect (p-value = 0.9510) on the FAMEs content at higher temperature and even time can be further reduced. Generally, extended reaction times allowed higher exposure of microwave irradiations to the reaction mixture which resulted in higher efficiency of extraction and biodiesel conversion.

From the above analysis, the optimum given by the model to achieve a maximum of lipid conversion efficiency was 183:1 of methanol ratio with 2% of catalyst amount (w/w) and at temperature higher than 80°C, around (99% ±0.5% w/w total lipids) in minimum time required 20 min.

3.3 Comparison of microwave vs. ultrasonication for *in situ* transesterification

As discussed earlier, the biggest issue during *in-situ* transesterification is the requirement of large volumes of solvent and longer reaction time. During microwave process, 183:1 (w/w) and 20 min was the optimum condition for lipid extraction and high biodiesel

recovery. For this purpose, ultrasonification has been also tested for its efficiency regarding biodiesel conversion. Accordingly, ultrasonification has been carried out to achieve higher yields of conversion during esterification and transesterification. High conversions yields were reported for converting algal oils and vegetable oils which allowed reduction in the reaction time. [42] This approach was highly dependent on temperature and other operating parameters. Around 97.3% was obtained during conversion of palm oil in 45 min at 60°C with 0.3 % KOH [43] and higher temperature (>60◦C) was less effective during the conversion step. In the present study, ultrasonification is carried out in an open system which results in methanol evaporation. Besides, higher temperatures during ultrasonification were reported to lower FAMEs content [34-35]. Although, higher temperatures are required for harsh extraction in the microwave as reported in the previous section (Section 3.2), Parkar et al. (2012) reported that physical effects of cavitation bubble dynamics in ultrasound assisted transesterification are more pronounced at lower temperature of 15 °C, albeit the low conversion yield of 13.45%. [44] Hence, the temperature was fixed to 25 °C (neither high nor low). Herein, *in situ* transesterification using ultrasound was optimized considering catalyst amount, methanol to oil molar ratio, and reaction time as reaction parameters. The optimisation of different variables is given in Table 4. The model was highly significant ($R^2 = 0.998$). This indicates that model cannot explain only 0.01 % of the total variations which shows that the model fits quite well. Moreover, *p* value for the model was lower than 0.05, which confirms the statistical relation between the response and selected factors. This shows that regression analysis is statistically significant. Therefore

in this model, most significant factors are methanol to oil molar ratio, ($p < 0.0001$) followed by catalyst amount ($p = 0.114$) and reaction time ($p=0.680$)

Akin to microwave approach, catalyst amount of 1, 3 and 5 % (w/w) were considered. Besides, beyond 5 % (w/w) catalyst, no further increase in the conversion of the oil to biodiesel could be achieved as the reaction was limited by mass transfer. Maximum biodiesel conversion of 95± 0.5 % (w/w) was observed using 5 % (w/w), the catalyst in the presence of high methanol ratio 183:1. As seen in Table 4, it can be found that the efficiency of lipid conversion via ultrasonicator equipment (20 kHz, 700 W) increased with the increase of methanol to oil ratio and catalyst amount (%). P values were around (< 0.0001) and (0.1140) for methanol to oil ratio and catalyst amount which justified their positive influence on the lipid conversion. Around 90.1± 2.2% (w/w total lipids) was attained in 20 min with 183:1 methanol to oil ratio (w/w). Higher conversion efficiency shown by ultrasound could be attributed to increased mass and heat transfer provided by the physical and chemical effects during intensification of reaction [45] Another observation to be pointed out by the present study is the formation of emulsions due to the reaction of catalyst with methanol. NaOH leads to water formation which slows the reaction rate and causes soap formation.[46] Thus, the FAMEs mixture remains in emulsion for more than 12 hours. For that purpose, hexane was added and the mixture was filtrated and then allowed to stand for 15 min. Thereafter, the top layer of FAMEs in hexane was collected for quantification. However, at 100°C with microwave irradiation, this problem was resolved since with closed vessels (under controlled pressure and temperature), the solvent can be heated above its normal boiling point, the fact that enhanced extraction

359 efficiency and speed. [47] Therefore, short reaction time, cleaner reaction product, and
360 reduced separation-purification times are the key observations in this the present study.

361 For a conventional method, reaction time for the transesterification was assumed to be
362 12 hours. In contrast, with the microwave and ultrasounds, the time was reduced to
363 20 min. Herein, microwave-assisted reactions may reduce not only the time but also
364 eliminate the need for the catalyst, however, higher reaction temperatures are required.[48-
365 49] During this process, microwaves interacted with triglycerides and methanol present in
366 the mixture which resulted in increased of interfacial polarization (a combination of ionic
367 conduction and dipolar momentum) and ionic conduction. [12, 50-51] These two reactions are
368 the major causes of superheating phenomenon which is observed at elevated temperatures
369 and led to a large reduction of activation energy with a high diffusivity of the solvent into
370 the internal parts of biomass. Thus, methanol is defined to be a strong microwave
371 absorber and the presence of an -OH group attached to biomass matrix behaves as though
372 it was anchored to an immobile raft, so localized rotations result in localized superheating
373 and the reaction may occur rapidly. [52] Consequently, desorption of intracellular
374 components (lipids droplets) from the active sites of the biomass matrix was enhanced.

375 When compared to microwave method, ultrasonic-assisted extraction uses cavitation
376 process to recover oils from microbial cells. Resulting bubbles during this process
377 collapse near cell walls so that the cell contents are released. [53,49-50] The ultrasonic waves
378 had a significant effect on cell disruption. A cavitation process is resulted due to the
379 higher pressure and shear on the cell walls which contributes to the formation of free
380 radicals of reacting species. [54] Accordingly, ultrasound permits the formation of highly
381 reactive radicals through dissociation of entrapped vapor molecules in the bubble, which

382 are subjected to extreme conditions generated at the collapse of the bubble. In ultrasound
383 assisted direct transesterification, cavitational effect caused by turbulence in reaction
384 medium and free radicals are responsible for process intensification. [55]

385 During two-stage of conventional transesterification, around 93.8 ± 1.3 % (w lipid/w total
386 lipids) was achieved with methanol to lipid molar ratio 6:1 in the presence of NaOH
387 amount 1% (w/w) lipid during 2 h, however, under similar conditions, only 3.0±0.2% (w
388 lipid/w total lipids) was obtained in *in-situ* transesterification (one stage). To obtain
389 higher efficiency, the increase of methanol to oil ratio above 360:1 and NaOH above 5%
390 (w/w) were required, thus, more than 90.4%±1.5 was achieved during 12 hours. It is clear
391 that *in-situ* tranesterification required much larger amount of methanol and NaOH
392 catalyst and far longer time to achieve similar lipid conversion yield than two stage
393 transesterification process. These higher requirements during transesterification are due
394 to the nature of cell wall that make barrier to solvent to access and extract lipid droplets
395 from intracellular compartment. So more solvent is required to weaken, disrupt and
396 penetrate into cell walls. In this regard, *in-situ* transesterification is preferable to
397 overcome these hurdles.

398 In the presence of microwave irradiation, transesterification was carried out in two stage
399 and around 98.5± 0.5 % (w/w) was obtained at 100^{o}C in the presence of 1% (w/w)
400 catalyst and 183:1 % (v/w) of methanol ratio. With ultrasonication method, a higher
401 conversion efficiency of 94.1± 0.1 % was achieved under same conditions at 25 ^{o}C.
402 Therefore, transesterification carried in two stages with microwave irradiation or
403 ultrasonication bubbles have the advantage to reduce the longer time and the large
404 amount of catalyst.

In the present study, microwave assisted direct transesterification showed higher efficiency than ultrasound assisted in-situ transesterification. Taken together, both approaches reduce the time, catalyst amount and energy requirements (Table 5). However, main obstacle for commercial application of these intensification methods is their scale up challenges. More research is required for successful implementation of these methods for direct conversion of microbial biomass to biodiesel at commercial scale. Besides, possible recovery of the catalyst from the residual biomass and its reuse needs more attention from the researchers. In this regard, future direction of research ought to focus on the process improvisation, catalyst recovery and reuse.

3.4 Comparison of composition of FAMEs from different transesterification processes

The analysis of the FAMEs composition is presented in Table 6. Microwave *in situ* transesterification process with a molar ratio of 183:1 at 100 ^{o}C favored a higher content of C18:2. Similar results were observed during ultrasonication aided *in-situ* transesterification at 25 ^{o}C, in 20 min and with a methanol to oil ratio of 183:1. Meanwhile, a lower C16:0 and C18:1 was observed. In fact, a lower molar ratio favored the production of phospholipids present in cell membrane [53]. On the other hand, higher methanol: oil ratio disrupted cells and allowed more contact with lipid droplets and major FAMEs belonged to intracellular lipids. The composition of FAMEs from two stage transesterification, conventional *in-situ* transesterification, microwave *in-situ* transesterification and ultrasonication *in-situ* transesterification were almost similar.

4 Conclusion

The production of single cell oils and their conversion process to biodiesel are of wide interest in fuel market. Lyophilized biomass of *T. oleaginosus* was utilized for the production of biodiesel using two means of in-situ transesterification: microwave technique and ultrasonication. Among the two methods, microwave was found to give higher conversion efficiency to biodiesel amounting to 99±0.5% w/w total lipids as compared to 95 ±0.2% % w/w total lipids with ultrasonication assisted technique. Another advantage of microwave assisted transesterification is the absence of emulsions during the whole process, the fact that reduce the separation time obtained (> 99% reduction in separation time), and all with a reduced energy consumption, meanwhile, a low reaction temperature (25 ◦C) was required for transesterification during ultrasonication method that will reduce the cost of production of biodiesel. Taken together, both approaches revealed that methanol: hexane efficiently converted FAMEs compared to conventional process which relied on chloroform: methanol 2:1 (v/v) and hexane mixtures and required more catalyst and more time to obtain the desired conversion efficiency. The *in-situ* transesterification process proved to be faster and easier method to produce biodiesel with lower catalyst 1% (w/w) and in short time of 20 min. Overall, microwave *in-situ* transesterification would be a promising alternative of the current two-stage transesterification process and combining the effects of the microwave and ultrasonic energy via hybrid reactor can be innovative and beneficial at large scale.

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Figure Captions List

Fig. 1. Schematic representation of different transesterification methods.

Fig. 2. Response surface plots showing binary interaction of different variables. The interaction between: (A) methanol/oil ratio (% v/w) and temperature (^{o}C); (B) temperature (^{o}C) and time (min); (C) catalyst amount (%) and temperature (^{o}C); (D) methanol/oil ratio (%) and time (min); (E) catalyst amount (%) and time (min); (F) catalyst amount (%) and methanol/oil ratio (% v/w).

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Table 1 Coding and levels of experiment factors

Factor	Parameter	Code level		
		-1	**0**	**+1**
Temperature (^{o}C)	X1	40	70	100
Time (min)	X2	20	40	60
Methanol to oil ratio (v/w)	X3	6:1	183:1	360:1
Catalyst (% w/w)	X4	1	3	5

Table 2 Box–Behnken design arrangement

Run	Parameter			
	X1	X2	X3	X4
1	0	-1	1	0
2	-1	1	0	0
3	1	0	0	1
4	1	0	-1	0
5	0	0	0	0
6	1	-1	0	0
7	0	1	0	1
8	0	1	0	-1
9	-1	-1	0	0
10	0	0	0	0
11	0	0	1	-1
12	0	0	1	1
13	0	0	0	0
14	-1	0	-1	0
15	0	-1	0	-1
16	1	1	0	0
17	-1	0	0	-1
18	0	1	1	0
19	0	0	0	0
20	-1	0	1	0
21	0	0	-1	-1
22	0	-1	0	1
23	1	0	1	0
24	1	0	0	-1
25	0	0	0	0
26	-1	0	0	1
27	0	1	-1	0
28	0	-1	-1	0
29	0	0	-1	1

Table 3 **Analysis of variance (ANOVA) for response surface quadratic model for the FAME content**

Source	Sum of squares	df*	Mean square	F value	p-value (Prob > F)
Model	18788	14	1342	98.545	< 0.0001
X1	3502	1	3502	257.17	< 0.0001
X2	0.05333	1	0.05333	0.00391	0.9510
X3	9622	1	9622	706.57	< 0.0001
X4	0	1	0	0	1.0000
X1X2	10.563	1	10.563	0.77563	0.3933
X1X3	163.84	1	163.84	12.031	0.0038
X1X4	16.403	1	16.403	1.2045	0.2909
X2X3	0.9025	1	0.9025	0.06627	0.8006
X2X4	1.69	1	1.69	0.12410	0.7299
X3X4	9.3025	1	9.3025	0.68311	0.4224
$X1^2$	525.41	1	525.41	38.582	< 0.0001
$X2^2$	0.01622	1	0.01622	0.00119	0.9730
$X3^2$	4968	1	4968	364.82	< 0.0001
$X4^2$	0.19865	1	0.19865	0.01459	0.9056
Residual	190.65	14	13.618		
Lack of Fit	190.21	10	19.021	172.92	< 0.0001
Pure Error	0.44	4	0.11		
Cor Total	18978	28			

** df : degree of freedom*

Table 4 **Box–Behnken model results for ultrasonication assisted direct transesterification.**

Run	Time (min)	Catalyst (%)	Methanol/oil ratio (w/w)	Lipid conversion efficiency (%)
1	60	3	6	25.1
2	40	3	183	92.3
3	40	3	183	93.0
4	40	5	6	25.8
5	20	3	6	28.9
6	40	5	360	95.9
7	40	1	6	25.9
8	40	3	183	93.9
9	60	5	183	94.1
10	40	1	360	93.9
11	20	1	183	90.1
12	40	3	183	92.1
13	60	1	183	93.4
14	20	3	360	92.2
15	20	5	183	95.5
16	60	3	360	92.2
17	40	3	183	94.2

R-Squared = 0.998. Adj R-Squared = 0.997. Pred R-Squared = 0.983.

Table 5 Comparative study of *in situ* transesterification methods

	Conventional	**Ultrasonication**	**Microwave**
Time	12 h	20 min	20 min
Temperature (^{o}C)	60	25	100
Power requirements	-	700 W	400 W
Differences	- Easy separation - Longer time - Higher methanol content	- Difficulty of separation (12 h) - Emulsification and saponification - Reduced time	- Separation and purification steps not required (5 min) - No emulsification - Reduced time - Lower catalyst and methanol amount

Table 6 Comparison of fatty acid profiles of biodiesel produced using transesterification methods.

Fatty acids	Conventional transesterification			Microwave *in-situ* transesterification			Ultrasonication *in-situ* transesterification		
	6:1	**183:1**	**360:1**	**6:1**	**183:1**	**360:1**	**6:1**	**183:1**	**360:1**
C14:0	ND	0.5	ND	ND	0.5	0.5	ND	0.5	0.5
C15:0	ND	0.5	0.5	0.5	0.5	0.5	0.4	0.5	0.5
C16:0	22.1	26.5	28.4	25.9	28.2	28.5	25.7	28.5	28.7
C16:1	0.4	0.9	0.7	1.1	1.0	1.1	1.1	1.0	1.0
C18:0	9.0	9.9	10.5	9.2	9.9	10.1	9.3	10.1	10.2
C18:1	39.4	48.0	48.5	44.4	49.3	46.7	44.1	49.2	49.3
C18:2	28.5	11.8	10.3	19.0	8.9	9.0	18.1	8.1	8.9
C20:0	0.6	1.0	1.1	1.2	1.2	0.9	1.3	1.0	1.1
C22:0	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27
C24:0	0.29	0.30	0.31	0.30	0.31	0.30	0.29	0.30	0.30

The fatty acid content is less than 0.5% was not given.

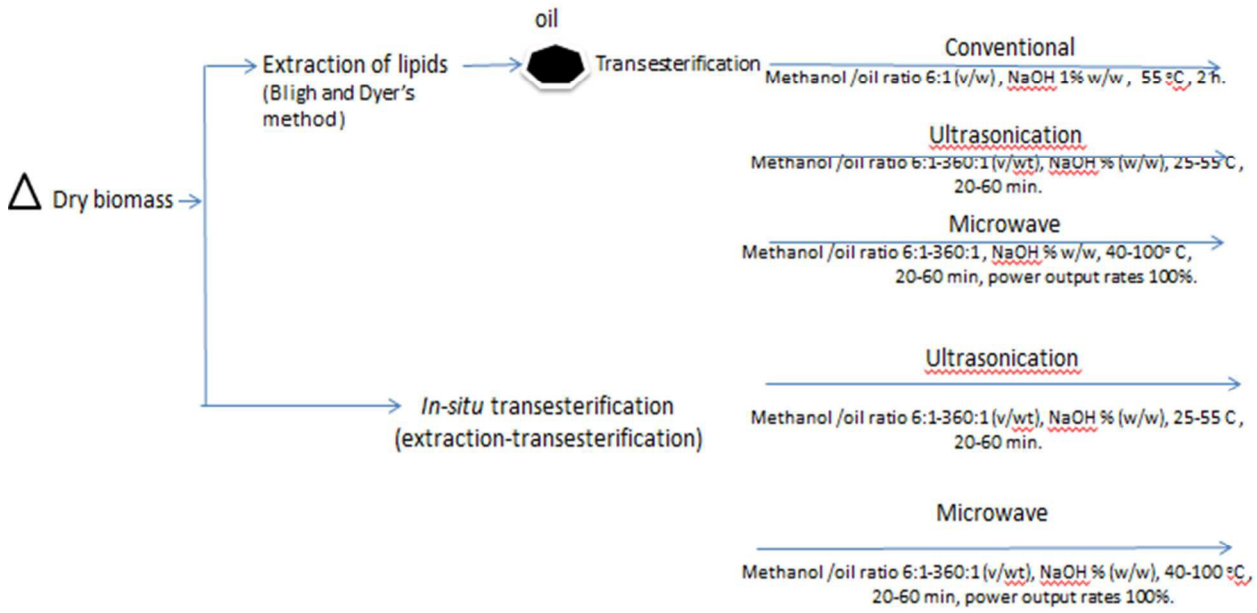
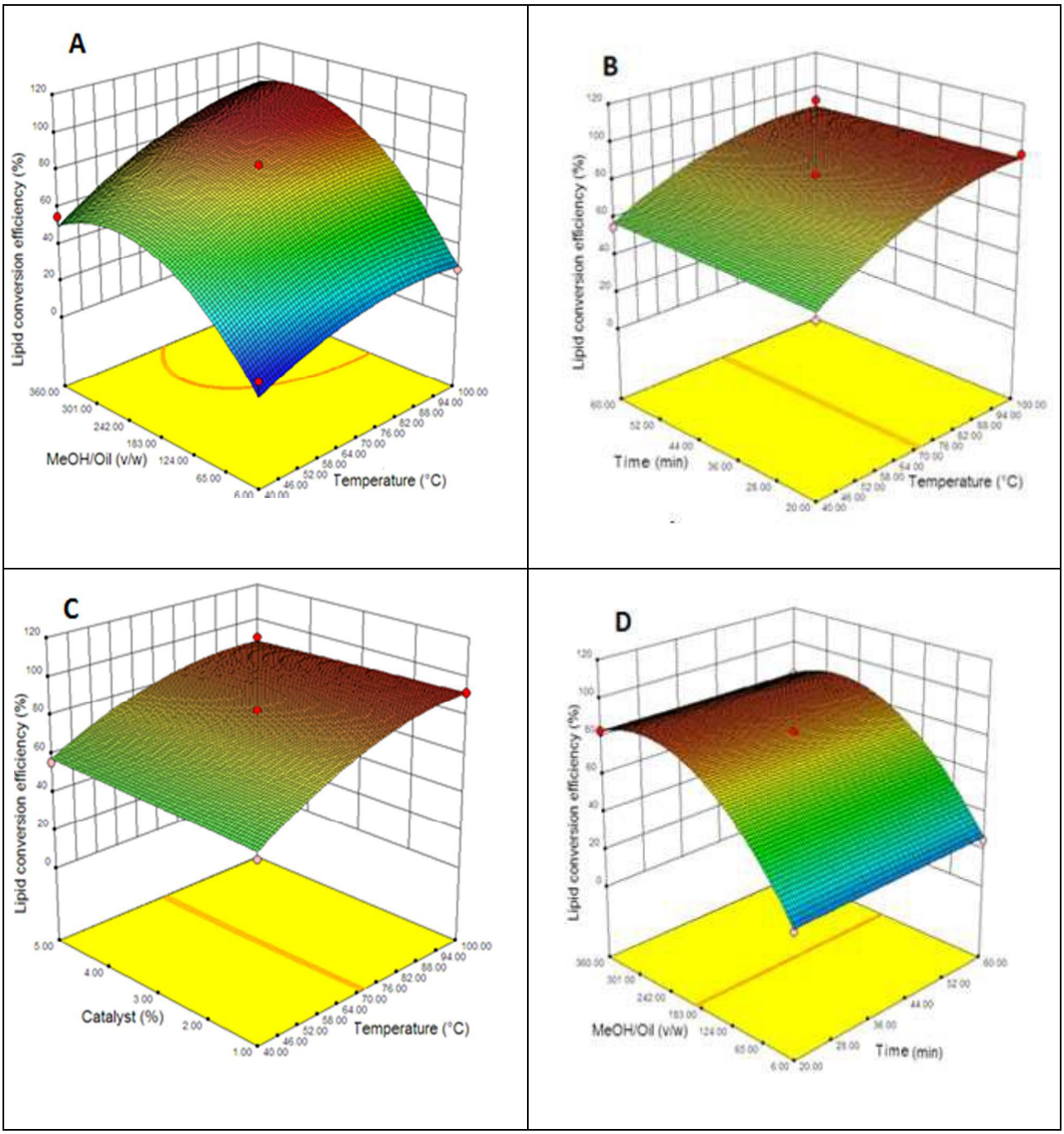


Fig. 1.



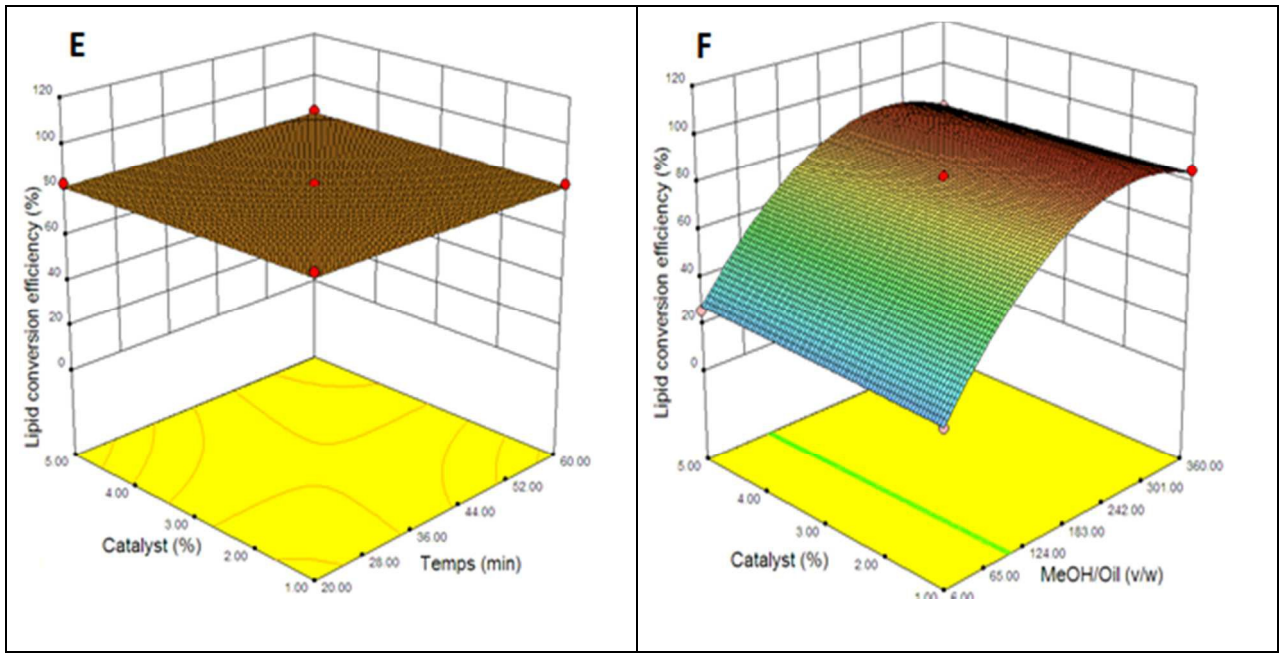


Fig. 2.

Graphical Abstract