Scaled-up biodiesel synthesis from Chinese Tallow Kernel oil catalyzed by *Burkholderia cepacia* lipase through ultrasonic assisted technology: A non-edible and alternative source of bio energy

Gabriel Murillo[a,b,1], Yaojia He[c,2], Yunjun Yan[c,*], Jianzhong Sun[b,*], Pietro Bartocci[d], Sameh S. Ali[b,e], Francesco Fantozzi[d]

[a] *China-EU Institute for Clean and Renewable Energy, Huazhong University of Science and Technology, Wuhan 430074, PR China*
[b] *Biofuels Institute, School of Environmental Science and Safety Engineering, Jiangsu University, 212013 Zhenjiang, PR China*
[c] *Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, PR China*
[d] *Department of Engineering, University of Perugia, Via G. Duranti 67, 06125 Perugia, Italy*
[e] *Botany Department, Faculty of Science, Tanta University, 31527 Tanta, Egypt*

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ABSTRACT

In East Asia, for thousands of years, the fruit of Chinese tallow tree (*Sapium sebiferum*) has been used for multiple purposes because of its chemical composition; the presence of high amounts of lipids is remarkable, showing potential to be used as substrate for biodiesel synthesis. Previously have been reported the use of alkaline and enzymatic catalysts, microwave technology and the use of ionic liquids as co-solvents with the lipids of this tree species to produce biodiesel. This study shows the results of the use of *Burkholderia cepacia* lipase as enzymatic catalyst for transesterification of Chinese Tallow Kernel oil (CTK), extracted from the fruit of Chinese tallow tree, into biodiesel, with the use of ultrasonic assisted technology and without the usage of solvents. The optimal operational parameters were determined and the reactions were developed in a batch reactor with the use of ultrasonic irradiation and emulsification to enhance the mass transfer. The scaled-up experiments, in an especially designed 3 L capacity reactor, showed promising results, obtaining 55.20% biodiesel and a kinematic viscosity of 10.31 mm^2.s^{-1} in only 4 h, in comparison with previously published (*in vitro*) methods. The valorization of this non-edible source of oil represents an opportunity to use as an alternative source for bioenergy and also to tackle the uncontrolled expansion of this oleaginous tree species in some ecologically fragile ecosystems.

1. Introduction

The use of lipids in the ancient times has been mostly focused in the production of candles and soap. In recent years have been especially used in alternative and more advanced applications, such as the synthesis of bio-based polymers [1] and biofuels [2,3]. In the case of biofuels, particularly biodiesel, it is important to use non-edible lipids to avoid the competition between energy vs. food. One of the most well-known sources of non-edible lipids is waste vegetable oil, whith has an annual worldwide generation estimated in 29 million tons, which still has many potential for its exploitation [4]. Moreover, Table 1 shows the potential oil yield of some non-edible oil plant species [5]. An

alternative source of non-edible lipids is also the oil extracted from Chinese tallow tree (*Sapium sebiferum*), known as *Stillingia* oil, also known as Chinese tallow kernel oil (hereafter CTK). Chinese tallow tree belongs to the Euphorbiaceae Spurge Family [6]; it has been cultivated for approximately 1400 years as a seed crop for multiple uses and is present in several provinces in Central China and Japan. Only in China there are approximately 2000 km^2 [7]. CTK is a fast-growing, medium-sized tree that can reach a height of approximately 15 m; in recent times has been mostly used for the production of candles, soap and as wood varnish, thanks to its quick-drying properties. Almost 250 years ago, Chinese tallow tree was planted in other regions of the world. Some of the reported regions are the southern part of France, India, Sudan,

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Table 1
Optimal reaction parameters for biodiesel synthesis according to the lipid source.

Item/Lipid source	Soybean Oil	Chinese Tallow Kernel Oil
Immobilized Enzyme Concentration	3%	5%
Water Concentration	3%	3%
Methanol:oil molar ratio	4:1	5:1

Martinique, Algeria and the southern area of the United States.

Particularly in the the United States, this tree species has been subject of study due to several environmental impacts in the ecosystems where has been grown, even it has created the risk of extinction to some fauna species. The most important factors that make this tree so invasive are its adaptability to different environmental conditions, its capacity to resprouting after being cut and even after being torched. The main mechanisms for its expansion are through bird excretion of its seeds and its transportation along currents of water bodies [8]. Several strategies have been proposed to contain its uncontrolled expansion, among them, the use of herbicides, torching and others, and still the challenges persist [9]. Another option is to make an energetic exploitation of the chemical energy contained in its seeds as a measure to reduce the invasiveness potential of the trees and fostering job creation. It was reported that under optimal conditions there is the capacity to extract approximately 35% of CTK oil based on a dry mass weight basis, with the use of microwave technology, that represents an equivalent for lipid extraction of 4700 $L.ha^{-1}.y^{-1}$, which makes it very competitive compared to other energy crop species [10,11].

It was reported the synthesis of biodiesel from CTK oil with the use of NaOH as catalyst, methanol and hexane as solvent under the assistance of microwave irradiation. It was found that the optimal amount of catalyst was 1.74 wt%, solvent ratio of 3 v/w%, reaction time of 20 min and reaction temperature of 58.1 °C, meeting ASTM biodiesel quality specifications [12]. Moreover, reference [13] reported biodiesel production from CTK oil catalyzed by *Candida rugosa* lipase (CRL) in 20 wt % as optimal concentration with nineteen different kinds of ionic liquids; [Hmim][PF6] showed the best performance at improving biodiesel conversion efficiency from 35 to 95.4%.

In the above mentioned approaches, co-solvents were used to enhance the catalytic reactions. Nevertheless the experiments were not scaled-up, keeping the limitation of *in vitro* conditions. Additionally, to the best of our knowledge, there are no studies regarding to the synthesis of enzymatic biodiesel with CTK oil at using ultrasound assisted technologies, which have proven promising results.

Therefore, the objective of our study is to determine the effects of ultrasound assisted technologies, particularly irradiation and emulsification, in the synthesis of enzymatic biodiesel in scaled-up conditions with the use CTK oil, without the incorporation of co-solvents. Biodiesel was produced in a batch reactor especially designed and that we recently reported its performance with the use of waste vegetable oil as the main substrate under the effects of ultrasonic irradiation [14]. A comparison with the synthesis of biodiesel based on edible soybean oil (hereafter SBO) was made under the same conditions. In parallel to biodiesel yield analyses, the determination of kinematic viscosity evolution as a complementary indicator of the biofuel quality was made.

2. Materials and methods

2.1. Materials

Burkholderia cepacia (PS lipase) was bought from Amano (Japan). Edible soybean oil with was purchased from a local market in Wuhan. Chinese kernel oil, was obtained from from a local company in Dawu County (Hubei, China). Macroporous resins NKA was purchased from Tianjin Nankai Sci. & Tech. Co. Ltd., (Tianjin, China). Other reagents such as methanol, ethanol, n-hexane, Na_2HPO_4, NaH_2PO_4; 2,2,4-tri-methylpentane, produced by Sinopharm Chemical Reagent Co. Ltd., (Shanghai, China) were of analytical grade and used without further purification.

2.2. Resin preparation and enzyme immobilization

NKA resin preparation and enzyme immobilization were made according to references [17,18].

2.3. Gas chromatography (GC) analysis

One sample per flask was taken to be tested in the GC instrument (GC-9790 Fuli, China), which is equipped with HP-INNOWAX (19091-133) capillary column (25 m × 0.32 mm × 0.3 μm, Agilent Technologies, USA). Samples were prepared according to references [19,20], 1 μL was injected into the capillary column for analysis.

The fatty acid methyl ester yield (FAME) was calculated as a percentage value with the formula:

$$\eta_{FAME} = 100 * (W_{FAME}/(3_* MW_{FAME}))/(W_{SBO}/MW_{SBO}) \tag{1}$$

W_{FAME}: weight of biodiesel sample, g. W_{SBO}: weight of soybean oil sample, g. MW_{FAME}: molecular weight of fatty acid methyl ester, $g.mol^{-1}$. MW_{SBO}: molecular weight of soybean oil, $g.mol^{-1}$.

2.4. Kinematic viscosity measures

A Pinkevitch glass viscometer was used for the measurement of kinematic viscosity.

The formula used for the determination of the kinematic viscosity was

$$n = C * t \tag{2}$$

ν: kinematic viscosity of the sample, $mm^2.s^{-1}$; C: viscometer constant, $mm^2.s^{-2}$ (calibrated at 20 °C, and adjusted to 40 °C); t: flowing time of liquid by moving through two marks along the viscometer, s.

2.5. Chinese Tallow Kernel oil filtering

CTK was poured into a ceramic cup with perforations. A paper filter of pore size of 10 μm was collocated above the ceramic base; the cup was installed on a 500 mL glass bottle and then connected with a hose to the vacuum pump that forced the oil to enter into the bottle at a vacuum pressure of −0.1 MPa.

2.6. Apparatus

Ultrasonic emulsifier (200 W, model EA200-H, Ouhor, Germany). Incubator shaker (model HNY-2102C, Honor Instruments, China). Centrifuge (model 5418, Eppendorf, Germany). GC analyzer (model GC-9790, Fuli Instruments, China). Vacuum filter (model SHZ-D (III), Keer Instruments, China). Water purifier (model UPH-IV-10T, Ulupure, China). Water bath (model DKB-501A, Senxin, China). Constant speed stirrer (90 W, model S212-90C, Wuhan KE'ER, China). Peristaltic pump (8 W, model BQ50S, Lead Fluid, China). A 3 L batch reactor especially designed (unpublished design) with a sonotrode probe (2.2 kW, serial number WH15417, Ouhor, Germany). The batch reactor from China, the sonotrode from Germany, asked under special request to be used as for biodiesel synthesis.

2.7. Experiment set-up

Several experiments in flask were made to set the operational parameters to produce biodiesel from SBO and CTK oil in the reactor. The parameters analyzed were methanol to SBO and CTK molar ratio, water concentration and immobilized lipase concentration, the last two

parameters as percentage relative to the weight of the lipid sources. The reaction temperature was taken from previous experiments (data not shown). All the results in flask were made in triplicate, the measurements of kinematic viscosities were made in duplicate. The results of GC for biodiesel samples from the batch reactor were made in triplicate.

The batch reactor of 3 L capacity was equipped with a sonotrode probe of 1200 W of power that can be set from 0 to 100% in intervals of 5%, it was used to irradiate the substrates in the emulsion and the lipases submerged inside the mixed media. The batch reactor also includes a temperature controller based on heat oil and a heat exchanger that uses water to cool down the temperature when necessary. The optimal ultrasonic power and the decoupling power were determined in previous experiments as 15% and 50%, respectively [14].

The amount of immobilized catalyst on NKA was 40.50 g, 3% in weight respect to the weight of lipid sources. The emulsion of the total volume inside the reactor was approximately 1660 mL, prepared in a small in 3 small batches (1L glass Erlenmeyer flask), taking care of the proportions of each substrate (500 mL of CTK oil, 41.0 mL of methanol, and 12.95 mL of purified water, respectively). The ultrasonic emulsification was applied during 3 min per batch at 8000 rpm under room temperature to get a better mixed before batch reaction. Substrate in each batch were mixed into emulsion with particle size between 0.2 and 20 μm after pre-emulsification. The 3 batches were collected in a 3L glass Erlenmeyer flask. Finally, the whole volume was poured at once inside the batch reactor, the metallic lid was closed and then the sonotrode started to work at a constant frequency of 20 kHz for 240 min for each test. Another group was treated without ultrasonic emulsifier and lid, instead only with conventional stirring (1000 rpm) under the same conditions. Samples were collected for analyses at 2.5, 15, 30, 60, 120, 180 and 240 min, respectively. The temperature inside the reactor was set at 40 °C.

After the mix was poured into the batch reactor, a peristaltic pump started to inject 120.4 mL of methanol into the reaction mixture at an angular velocity of 10.0 rpm inside the reactor (the total volume of methanol was introduced in the reactor at approximately 60 min). In order to verify biodiesel quality, besides measuring the yield of biofuel through GC analyses results, kinematic viscosities also were measured. A summary of the main steps of our experimental setup are shown in Fig. 1.

Graphs of the results of this study were made in Origin Software with average values. Standard deviation values (± σ) were added as error bars.

3. Results and discussion

Our comparison between the performance of CTK oil with respect to SBO starts with the optimal operational parameters (the operational parameters of SBO were already published in [14]). The experiments were made *in vitro* and the first question to answer was if PS lipase could catalyze transesterification reactions with CTK oil, given that some enzymes have limitations to perform these reactions in front of some lipid substrates, or in some cases only can develop reactions under pretreatment with solvents. After preliminary tests we found that PS lipase can catalyze the synthesis of biodiesel without the use of organic solvents, it is just required a small amount of water to optimize its yield. Then we determined the optimal operational parameters for both lipids. The optimal concentration of water was 3 wt% with respect to the weight of each lipid, which is a relative small amount. With regard to the immobilized enzyme concentration, SBO showed its optimal performance with a concentration of 3 wt%; in contrast, CTK oil required 5 wt%. For CTK oil, the biodiesel yield between a concentration of 3 and 5% was not dramatically different. Finally, for the case of molar concentration of methanol:lipid, SBO required only a proportion of 4:1. In the case of CTK oil its optimal catalytic performance was obtained with a molar proportion of 5:1, which is not far from the doses used for the case of SBO. For both lipid substrates it was used 40 °C as reaction

temperature. The summary of these results are shown in Table 1. Fig. 2 shows the of the optimal parameters for CTK oil more in detail.

The study of reference [13] found that CTK oil has a broad variety of molecular species of fatty acid alkyl esters, the biggest percentage of its composition is concentrated in the species comprehended between C16 and C18 chains (in this case C17 is a standard species), this is clearly shown in the results of gas chromatography. We made a comparison between SBO and CTK oil biodiesel species from our samples. Fig. 3 shows the most important differences for both lipid sources.

According to GC results, both lipid substrates show a predominant amount of two double-bond chains (C18:2, 54.99% for SBO and 49.9% for CTK, respectively), followed by single double-bond chains (C18:1, 25.79% for SBO and 30.42% for CTK, respectively), the third most abundant methyl are saturated molecules of 16 carbon atoms (C16:0, 12.33% for SBO and 16.43% for CTK, respectively). The last species are saturated chain of 18 carbon atoms (C18:0, 3.75% for SBO and 5.81% for CTK, respectively) and triple double-bond chain of 18 carbon atoms (C18:3, 4.66% for SBO and 3.57% for CTK, respectively). Looking more in detail, it can be observed some differences in the chemical composition of both lipid substrates. SBO has a higher proportion composition in the C18:2 (almost 10% higher than CTK) and a tiny higher for C18:3. In contrast, CTK shows a higher proportion of saturated species of C16:0 and C18:0 by approximately 4% and 2%, respectively. In unsaturated species, CTK oil shows a higher amount in C18:1 of near 5%. CTK oil shows a higher composition of saturated species, which must be taken into account for its suitability in cold-weather conditions and its use in sub-tropical regions. These results contrast the findings of the study in reference [13], where the main percentage of ester species corresponded to C18:3, by 42.8% and, in comparison to our study, the rest of the species had considerably less percentages in their composition. Moreover, is important to mention that in reference [7] was discussed about the excess of unsaturated species of biodiesel from CTK oil, (for the case of that reference, getting an amount of 92%), lead to biodiesel oxidation and poor stability of the biofuel, giving complications in their management which also does not coincides with our findings, which showed a percentage of unsaturated species of biodiesel of approximately 79%, which is a reduced amount compared to the value mentioned in that reference and also in the findings published in reference [13], which accounts for 91%. Is also important to mention that our findings show that the composition of unsaturated biodiesel species of CTK oil is even lower to the values of SBO, which accounts for 85.4% putting into question the risks of poor stability of this particular source of CTK oil for its use in biodiesel production.

To the best of our knowledge, the publication that reported the most abundant information of biodiesel production through enzymatic catalysis with CTK oil is in reference [13]. It is worthy to summarize some of its contents for comparison purposes with our study before showing our results of scale-up conditions. It was reported in [13] that biodiesel was produced in small amounts, in flasks of 50 mL; furthermore, the best reaction conditions were achieved with the use of a concentration of 1 mL.g^{-1} of the ionic liquid [Hmin][PF6], which significantly enhanced its yield. The optimal parameters were: MeOH:CTK oil molar ratio: 4:1, water concentration of 10 wt% free enzyme concentration of 20 wt% and optimal reaction duration of 24 h to get as the highest biodiesel yield a value of 95.4%.

In spite of the differences in some optimal amounts between SBO and CTK oil, particularly for the case of immobilized enzyme concentration and MeOH:lipid molar ratio (see Table 1), we decided that in order to make a more practical comparison in operational terms, we set the same amounts for the operational parameters for both lipid sources inside the reactor as 3 wt% for immobilized enzyme concentration and a molar ratio of MeOH:lipid source of 4:1, in principle favoring biodiesel yields for SBO.

From Fig. 4 can be noted that, even not applying its optimal operational parameters, CTK oil shows a higher biodiesel yield in the same conditions compared to SBO, the differences are especially important at
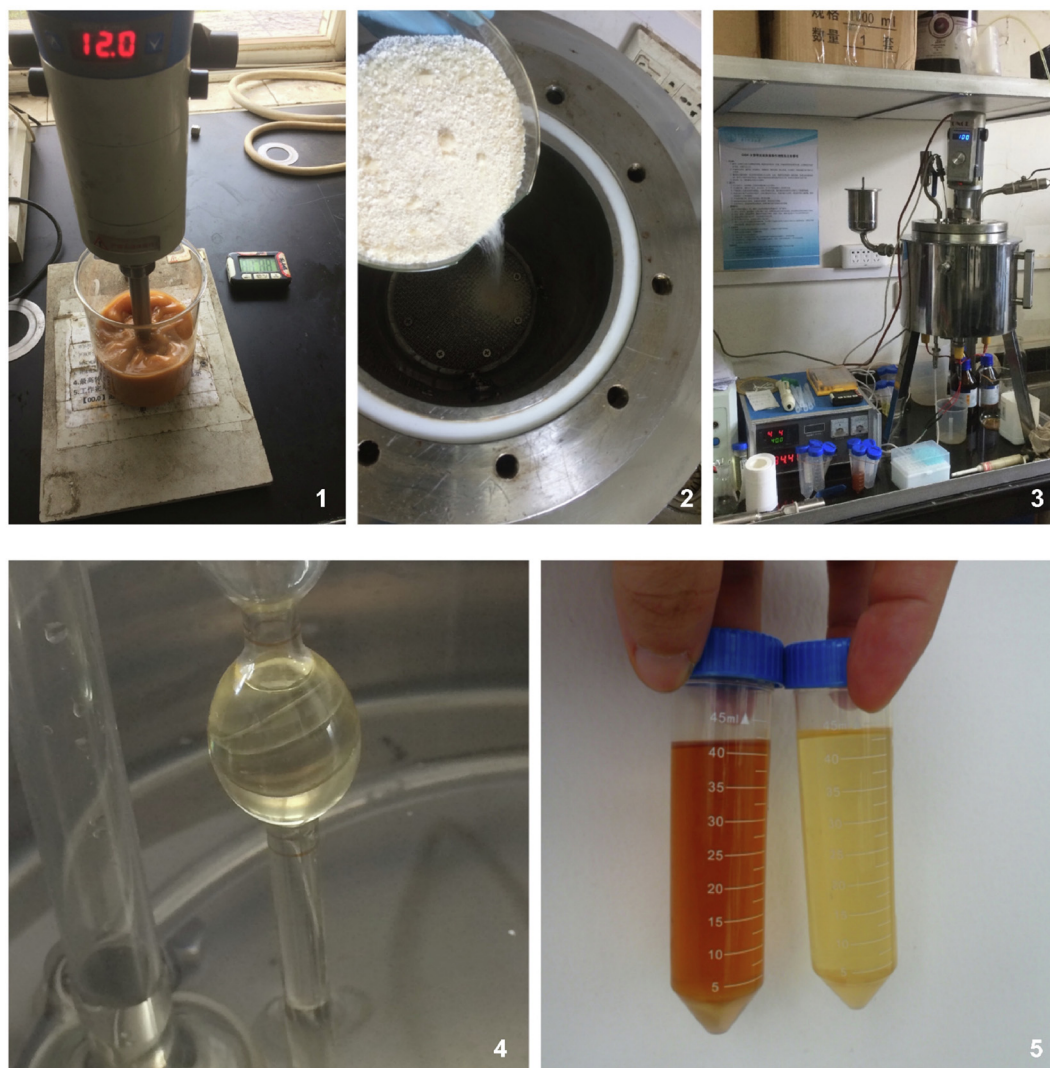
Fig. 1. Description of some of the main experiments. 1: pre-emulsification of reaction mix; 2: pouring immobilized enzymes inside the batch reactor; 3: biodiesel preparation and sampling; 4: reading of kinematic viscosity; 5: biodiesel production (from CTK oil in the left, from SBO in the right).

the beginning of the reactions, in the first minutes where the reactions start to occur. Interestingly, for the case of CTK oil, it can be observed that at just 2.5 min after the reactions reactions started with the application of the optimal ultrasonic irradiation plus ultrasonic emulsification (1000 rpm) biodiesel yield reached almost 38.78%, keeping the highest yields since the beginning. Most of the initial reactions were achieved thanks to the initial emulsion conditions – that corresponds to approximately 40–50% of the total potential biodiesel yield – and after the first hour of reaction the remanant part of the methanol pumped inside the reactor started to be transesterified with the lipid particles, assisted by the ultrasonic technologies.

For the case of the application of only ultrasonication, there is a more limiting extent of reaction, especially due to the total amount of volume that was set to undergo the catalytic reactions. In general we can say that the difference compared with and without ultrasonic emulsification is approximately between 5 and 15% along the reactions, getting as final yields 35.03% and 50.24% for SBO, and 40.50% and 55.22% for CTK oil, respectively.

In Fig. 4 it can be noted that, CTK oil and SBO both show higher biodiesel yields with emulsification treatment compared with conventional stirring. For the case of using pre-emulsification, it can be observed that biodiesel yields of CTK oil and SBO both can reach over 30% within 15 min with the application of ultrasonic irradiation plus

ultrasonic emulsification (1000 rpm), while for conventional stirring, in more than 180 min the final biodiesel yield were only 35.15% and 30.43% for CTK oil and SBO, respectively. As can be seen from Fig. 4, CTK oil and SBO show faster starting reaction speed with pre-emulsification compared with the group only with conventional stirring. That may be due to it that the pre-emulsification provided a better mixed substrate for batch reaction, resulting in a relative reduced concentration of methanol (which is harmful to lipase) in reaction system compared with the conventional stirring.

In comparison to the results of reference [13], we can make some comments. Fig. 2f of that reference shows the biodiesel yield along the time, it reaches approximately 60% after 6 h reaction. For our case, CTK oil produced a similar yield in only 4 h. Is important to highlight the fact that the biodiesel yield of CTK oil was synthesized without the optimal doses of methanol and immobilized enzyme concentrations. With regard to this point is also worthy to mention that biodiesel from CTK oil in our reactor was produced with a considerable less amount of catalyst, without the use of any organic solvent or ionic liquid to enhance the extent of transesterification reactions; this shows that our approach is promising, and reflects the efficacy of the ultrasonic technologies as an alternative in the synthesis of enzymatic biodiesel. If we keep good reaction conditions for biodiesel synthesis with CTK oil, we probably could expect its highest yield in shorter times.
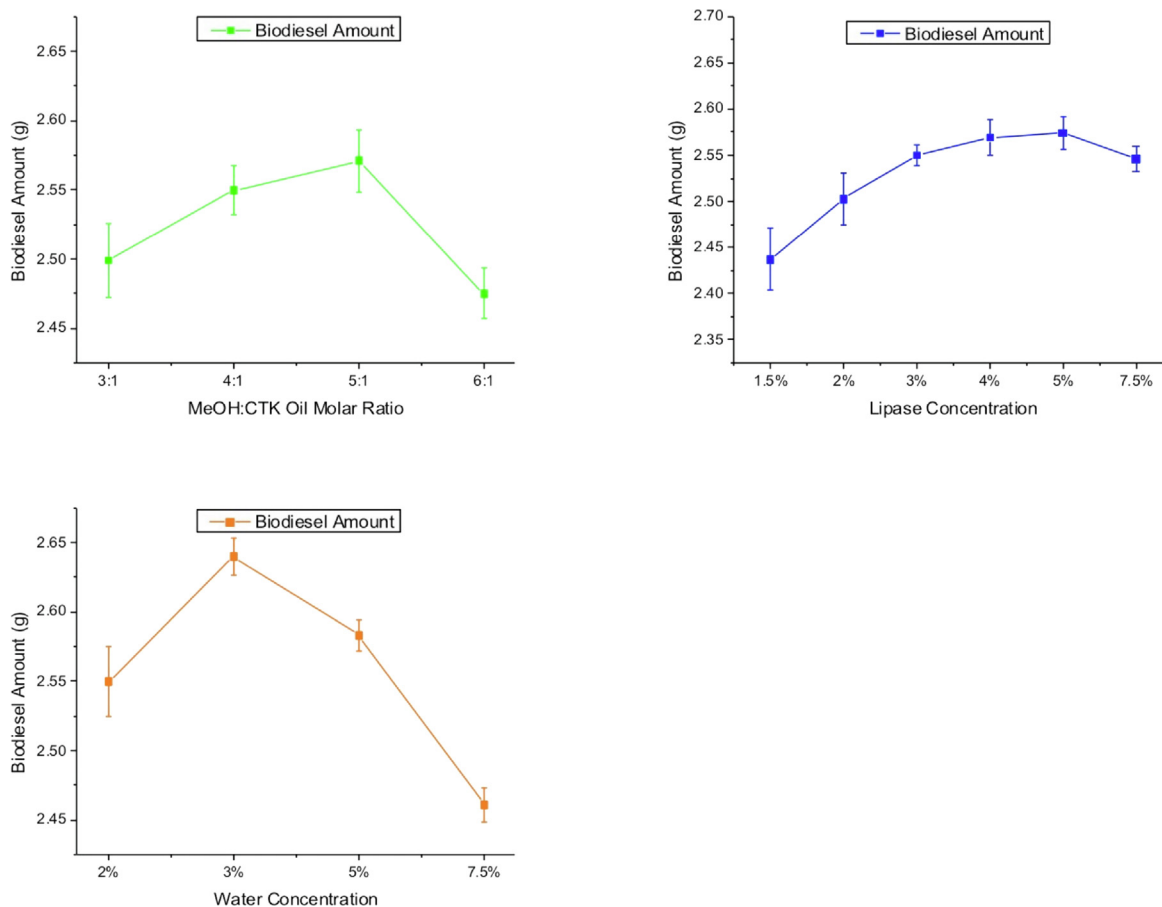
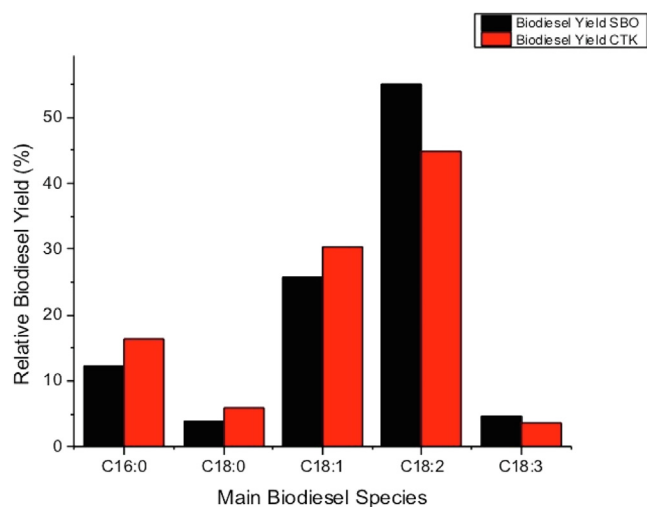**Fig. 2.** Optimal reaction parameters of CTK oil *in vitro*.

Fig. 3. Relative abundance of biodiesel species of CTK oil and SBO.

The benefits of the use of ultrasonic irradiation are well-known, especially due to the cavitation bubbles and flow streaming generated through acoustic waves, which facilitate the development of high temperatures and pressures when the micro bubbles collapse, allowing an appropriate contact and mixing of the lipid and alcohol molecules at the interface regions between the two surfaces where the catalytic activity occurs. Ultrasonic emulsification can produce stable micro emulsions in the reaction mix that can facilitate the catalytic activity thorugh an abundant amount of micro bubbles with increased interfacial surface area between CTK oil (main phase) and methanol

(embedded phase), provinding an improved and continuous streaming inside the reactor, in our experiment this effect was improved with the pre-emulsification of the reaction mix, instead of conventional stirring which produces less stable emulsions [15,16].

These results show that the combination of ultrasonic irradiation and ultrasonic emulsification generates synergistic effects in transesterification reactions for biodiesel synthesis; by one hand are quite clear the benefits of flow streaming and micro emulsions generated by the collapse of the cavitation bubbles due to the irradiations; by the another hand we demonstrated the positive effect with the addition of a smooth flow streaming with a very low intensity application of ultrasonic emulsification, especially applied to emulsify the additions of methanol droplets along the first hour of reactions through the peristaltic pump into the reactor.

Additionally to the measures of biodiesel yields in the GC, kinematic viscosity measures also were made, which for this case are only temperature-dependent. These values were measured as a confirmation of biodiesel quality. Fig. 5 shows the results.

From the results of Fig. 5 it can be seen that as transesterification reactions progress, the kinematic viscosity in the reaction mix decreaces, which is an expected phenomenon. Also can be observed that the combination of ultrasonic irradiation and emulsification produced the least viscous biodiesel, this is in agreement with the results obtained from GC analyses. Also in Fig. 5, for the case of ultrasonic irradiation and emulsification for biodiesel from SBO, there is a peak that appears in the minute number 60, this could be due to an excessive sampling volume because of the previous necessary drainage of sedimented water in the batch reactor; this effect was also reflected with a slight decrease of biodiesel yield in Fig. 4. The best results of kinematic viscosities are for the cases of ultrasonic irradiation + emulsification of CTK oil and SBO, 10.31 and 13.06 mm^2.s^{-1}, respectively; however these values are
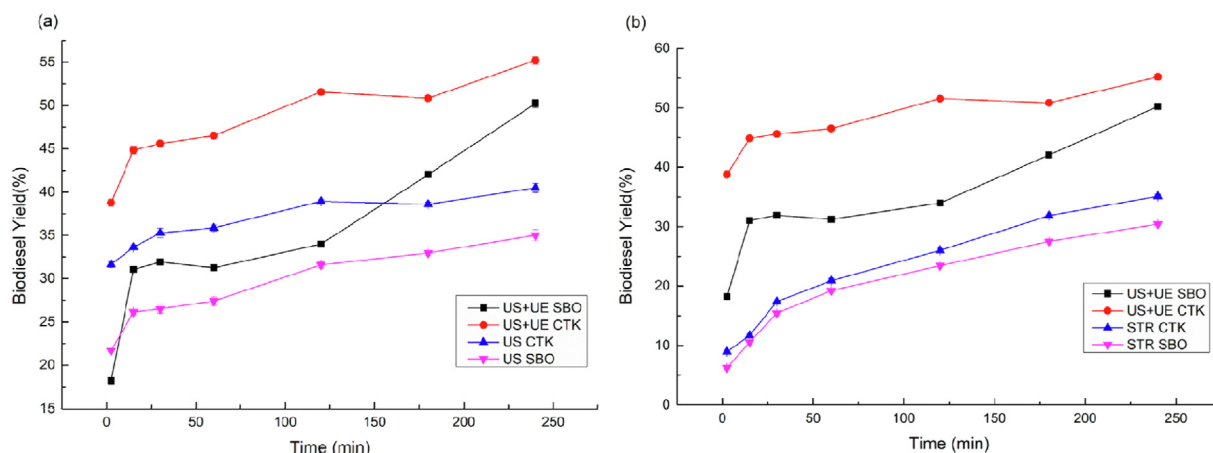
Fig. 4. Biodiesel yield of CTK oil and SBO in the batch reactor. (a) Biodiesel yield of CTK oil and SBO at applying sonication + ultrasonic emulsification and only sonication. (b) Biodiesel yield of CTK oil and SBO at applying sonication + ultrasonic emulsification and only stirring.

still not suitable for their use in diesel engines. International standards such as ASTM D6751 require a maximum value of kinematic viscosity at 40 °C of 6.0 mm^2.s^{-1} [21].

It is important to recognize the effectiveness of PS lipase immobilized on NKA resin during the experiments, given the fact that we did not renew immobilized enzymes inside the reactor. The enzymes showed their efficacy with CTK oil and SBO under different exposures of ultrasonic technologies. Fig. 6 shows how the bed of immobilized enzymes was affected after the simultaneous application of the sonotrode and the emulsifier inside the reactor; the ultrasonic waves of the sonotrode swept out a small area, exposing the surface of the bottom of the reactor and the continuous rotational flux induced by the emulsifier let a thinner circular surface of an approximate area equivalent to two-thirds of the inner diameter of the reactor. These results demonstrate that exposing the resin with ultrasonic emulsification at low rotational speed does not produce any damage to the polymer particles inside the batch reactor, giving the opportunity to reutilize them for further biodiesel volume cycles.

4. Conclusions

Through our experiments in a batch reactor, were demonstrated the beneficial effects of the combination of ultrasonic irradiation and emulsification, as enhancers of mass transfer in transesterification reactions, through enzymatic catalysis by *Burkholderia cepacia* lipase of Chinese Tallow Kernel oil, without the use of co-solvents.

Compared to previous studies, it was shown that the ultrasonic technology can offer an alternative in the synthesis of enzymatic

Fig. 6. Combined effects of ultrasonic irradiation and emulsification in the bed of immobilized PS lipase inside the batch reactor.

biodiesel of this source of non-edible oil. Our study showed, in comparison to previous publications, important differences in the chemical composition of Chinese Tallow Kernel oil regarding to the amount of double bonds and saturated species in its molecular chains. This is an important issue that must be addressed before producing biodiesel in large-scale. Variations of double bonds could require pretreatments to stabilize biodiesel against oxidation, and to properly manage high viscosities due to cold temperatures in winter.
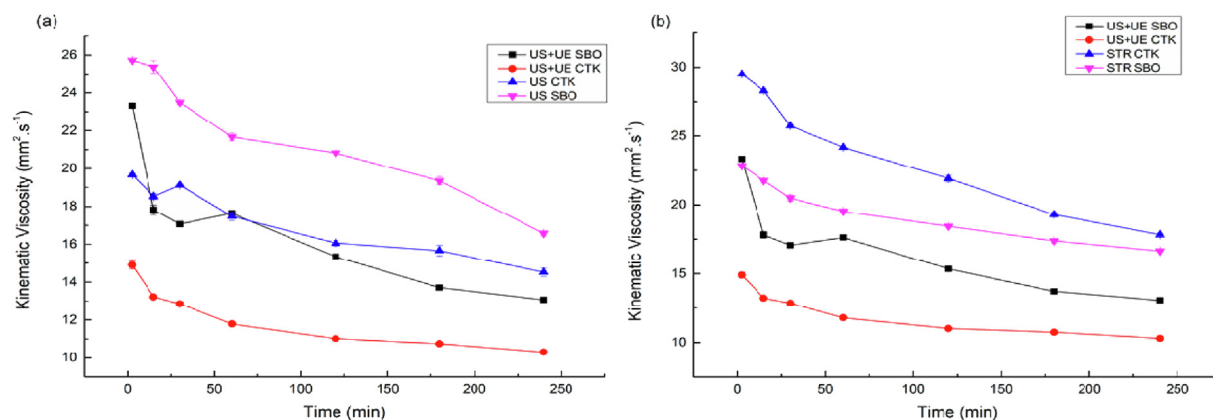

Fig. 5. Kinematic viscosity of CTK oil and SBO in the batch reactor. (a) Kinematic viscosity of CTK oil and SBO at applying sonication + ultrasonic emulsification and only sonication. (b) Kinematic viscosity of CTK oil and SBO at applying sonication + ultrasonic emulsification and only stirring.

CTK oil is a promising substrate for biodiesel production, given its abundance in different countries and the resilience characteristics of the trees of this species against cutting, fire and other destructive actions. Additionally, its exploitation could represent an environmental measure to control its progressive expansion in habitats where native flora and fauna have been negatively impacted.

Funding

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