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In Situ Transesterification from Oleaginous Yeast Biomass

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Near-infrared spectroscopy (FT-IR) is an useful tool for the study of biological molecules. The application of this technique is continually expanding mainly for the characterization of proteins and lipids. Quantitative analysis relies on a calibration model which can be semi-quantitative by multiple linear regression analysis. The advantage of this technique is that substances are analysed without chemical treatment, avoiding secondary reactions. In addition, all compounds are measured simultaneously, speeding analysis. Accordingly, two techniques were tested: i) FT-IR , based on rapid determination of trans geometric isomers isolated in fats and oils at ATR (Attenuated Total Reflection) by infrared spectroscopy (Method Cd 14d- 99, AOCS), ii) Gas Chromatography Coupled with Mass Spectrometry, which analysis were made in Fourier Transform Infrared Spectrometer (FT-IR). A calibration curve was constructed using a B100 biodiesel, to estimate the percentage of ester in the samples. The area was obtained by integration of the absorption region at 1750 cm^{-1} and the slope and intercept determined in the linear fit. The FT-IR analysis indicated the major structural features of the product, the estimate ester percentage in the samples and the percentage of reactions conversion. The identification of the ethyl ester was confirmed by gas chromatography with mass detector. The samples obtained by different methods of methyl and ethyl transesterification reaction in situ yielded 75.1 % and 93.4 % of ester, respectively, while the transesterification reaction of the extracted oil yielded 95.5 L% of esters. The spectras of the crude oil and biomass were also obtained for comparison and identification of the compounds. Besides the identification of the components and the estimation of esters in the sample, the use of FT-IR as a primary or auxiliary technique can contribute to the process control quickly with good response. However, this technique requires a validation for the construction of a mathematical model using a chemometrics method for making it reliable and effective.

1. Introduction

Infrared spectroscopy is a powerful tool for the study of biological molecules and its. It is used for characterization of biological molecules, particularly proteins and lipids. The infrared bands for the study of lipids are the vibrations of the ester group, particularly the elongation of the C = O band in the region of 1750-1700 cm^{-1} (Stuart, 2004)

Several biotechnological applications require further development of analytical methods for biomass characterization in order to monitor the batch-to- batch reproducibility (Pistorius et al., 2009) The success of this technique in quantitative analysis depends on the availability of a suitable system for model calibration, that may require relatively large data sets (Cen and He, 2007).

One of the most important advantages of FT-IR is that the samples need no chemical pretreatment thus avoiding secondary reactions. In addition, all compounds are measured simultaneously, reducing the analysis time (Grube et al., 2002b).

Fermentation processes were carried out under different conditions by GRUBE et al., (2002a) establishing a quantitative analysis of multiple cellular components, based on the average of the coefficients. The composition may be determined semi-quantitatively (Pistorius et al., 2009).

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FT-IR analytical methods are also suitable for controlling transesterification reactions and quality paramenters of biodiesel, since vibrational techniques allow probes for monitoring (online) real-time (Trevisan et al., 2008).
Accordingly, two techniques were tested: i) FT-IR based on Rapid Determination of Trans Geometrical Isomers Isolated on Fats and Oils in ATR Infrared Spectroscopy in the AOCS method Cd 14d- 99 ii) Gas Chromatography coupled with mass spectrometry.

2. Materials and methods

2.1 Methyl In SituTransesterification

The in situ transesterification protocol was adapted from Lewis, Nichols et al. (2000). Samples of 200 mg of lyophilized yeast biomass were transesterified by adding 30 mL of methanol / hydrochloric acid / chloroform (10:1:1, v / v) and keeping in a thermostatic bath at 90 °C for one hour. The esters produced were extracted three times by adding 20 mL of hexane: chloroform (4:1, v / v) and 10 mL of water. The mixture was centrifuged at 2000 rpm for 10 minutes to complete phase separation. The light phase (hexane + methyl esters) was separated, excess solvent was removed and recovered by evaporation under reduced pressure in a Rotary vacuum evaporator RV - 10 IKA (Germany) and samples were dried with anhydrous sodium sulphate. The fraction of the methyl esters was dried in an oven until constant weight and gravimetrically measured as a percentage of dry weight.

2.2 Ethyl In SituTransesterification

Acid catalysed in situ transesterification reaction (H_2SO_4) was carried out in a 100 mL stainless steel water jacketed vessel with a mechanical Shaker Q-251 D brand IKA Labortechnik (Germany).
Approximately 5 grams of dry yeast biomass was mixed with ethanol and sulphuric acid reacted for 8hour (µmolar ratio of 600:1:100 (ethanol:oil:acid)(Velasquez-Orta et al., 2012, Georgogianni et al., 2008, Ehimen et al., 2010, Ehimen et al., 2011), followed by centrifugation at 2000 rpm and phase separation. The mixture of esters was subsequently purified by addition of hexane and washed with distilled water at 60° C to neutralize pH to 7.0. The excess of solvent was recovered by evaporation in a Rotary vacuum evaporator IKA RV-10 (Germany) and dried with anhydrous sodium sulphate. The fraction of esters was dried until constant weight and quantified as a percentage of dry weight.

2.3 Ethyl Conventional Transesterification of oil Extracted by Bligh & Dyer

Lipids extraction was based on Bligh & Dyer (1959) Manirakiza et al., (2001) modified gravimetric method, which extracts both groups of lipids (polar and nonpolar)in lyophilized yeast biomass with methanol and chloroform.
The extraction was performed with 1 g of freeze-dried yeast biomass and 12 mL of methanol: chloroform (2:1, v / v). The suspension was stirred by vortex for 2 minutes followed by addition of 4 mL of chloroform and 2 minutes of mixing. To generate a two phase system, 7.2 mL of water was added and the system was mixed again for 2 minutes. The phases were separated by centrifugation for 10 minutes at 2000 rpm. The lower phase was transferred to a 50 mL flask using Pasteur pipette. A second extraction was performed with 8 mL of a 10% (v / v) methanol in chloroform mixing for 2 minutes. After centrifugation, the chloroform phase was added to the first extract. The chloroform was evaporated in a rotary evaporator and the residue dried in an oven at 60 °C until constant weight. The lipid obtained was quantified gravimetrically.
The transesterification of the lipids extracted was made using a adapted protocol from Ehimen et al. (2010, 2011). The transesterification reactions catalysed by acid (H_2SO_4) were carried out using the molar ethanol:oil ratio of 6:1 and 2 % sulphuric acid catalyst for 8 hours. The reaction was conducted at as tirring system and apparatus described above. After the transesterification reaction, the sample mixture was centrifuged, purified and dried.

2.4 Identification of the Reaction Products by Infrared Spectroscopy Fourier Transform (FT -IR)

The samples were analysed in the spectrometer with Fourier Transform Infrared (FT -IR) (Thermo - Scientific Nicolet 6700/USA). The spectras were obtained in ATR (Attenuated Total Reflection) mode with SMART OMNI - SAMPLER accessory in the range of 4000-675 cm^{-1} with resolution of 4 cm^{-1} and 32 scans per spectrum. This methodology was based on the rapid determination of trans geometrical isomers isolated on fats and oils in ATR Infrared Spectroscopy by the AOCS method (Cd 14d-99).

Esters samples were transferred to the ATR cell using an automatic pipette. A 100 mL of the sample was sufficient for coating the entire ATR cell. Standards prepared with biodiesel fuels were used to construct the calibration curve, as shown in Table 1. The spectra for each point is presented in Figure 1.

Table 11. Data of the calibration curve construction by FT -IR.

Standard points	Biodiesel B100 (g)	Ethanol (g)	B100 (%)
1	0.1	1.9	10
2	0.5	1.5	25
3	1	1	50
4	1.5	0.5	75
5	2	0	100

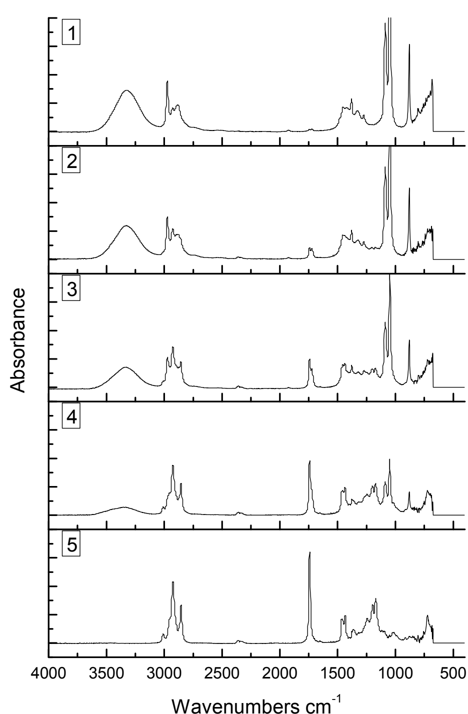

Figure1. FTIR spectra for the standard curve

The integrated areas (absorbance at 1750 cm $^{-1}$ by FT-IR) of the standard analysis were plotted using the slope and intercept generated by a first order linear regression, with, the area percent versus the standard biodiesel. Was obtained a linear fit of 0.99086 by the standardization and slope and intercept values of -0.1163 and 0.01575, respectively. Ester percentage of the samples was estimated by Eq (1). In the spectra from Figure 1 it is noted that the detection limit is higher than 10%.

$$Ester\ (\%) = \frac{(area - slope)}{intercept} \tag{1}$$

where the integration is obtained from the area of the region of absorption of 1750 cm $^{-1}$ and the slope and intercept were determined in the fitting process. The FT-IR analysis indicates the major structural features of the product and the estimated ester fractions of the samples.

2.5 Identification of ethyl esters obtained by Gas Chromatography with Mass Detector.

The identification of ethyl esters was confirmed by gas chromatography with mass detector GC- 2010 (Shimatzu) using a Stabilwax capillary column, 30 m, 0.25 mm ID, 0.25 micrometers. For electroionization70 eV was used with helium as the mobile phase at pressure of 15 psi, Split 1/50, 250 ° C to 300 ° C injector and the detector. The temperature ramp of the column was 50 ° C for 2 min gradient of 10 ° C / minute up to 180 ° C, 5 minutes on hold to 180 ° C, gradient of 5 ° C / minute up to 240 ° C totalling 32 minutes of the chromatographic run. The volume injected for each analysis was 1μL. GS Solution software (Shimadzu, Kyoto, Japan) was used to analyse the chromatograms along with Nist08 and Nist08s libraries.

3. Results and Discussion

An alternative to overcome the limitations of conventional extraction and transesterification process is the in situ transesterification method in which lipids are simultaneously extracted from the cell and esterified. In this approach the extraction and conversion take place in a single step without the need of isolating and refining the lipid before conversion to biodiesel, which may possibly reduce the costs (Dong et al., 2013) . The final results of each reaction employed in this method for conventional versus *in situ* transesterification are shown in Table 2.

Table 2. Results obtained by Spectroscopy Fourier Transform Infrared (FT -IR).

Conventional transesterification (oil extracted from yeast)		In situ transesterification with oleaginous yeast matrix	
Sample	Ester (%)	Sample	Ester (%)
Extracted by Bligh & Dyer	95.5	in situ methyl	75.1
		in situ ethyl	93.4

The acid-catalysed transesterification is retarded by the presence of polar compounds, as they act competing for hydrogen ions and reducing the availability of these ions for catalysis. Acid catalysts bind preferentially to water, leading to a reversible reaction and deactivating the catalyst. Another factor is the steric hindrance between molecules of fatty acids and alcohol, but there is an increase in hydrophobicity with higher alcohol at elevated temperatures thus demonstrating the importance of temperature on acid catalysis. It is also known that large amounts of acid catalyst can promote the formation of ether by dehydration of alcohol (Edgar Lotero and James G. Goodwin, 2005)

In order to verify if the results of Table 2 are consistent with the chemical structure of the esters the FTIR spectra were analysed. In Figure 2 were identified the absorbance bands characteristic of axial ester deformation C = O (1,741 cm $^{-1}$) and C -O (1168-1243 cm $^{-1}$), and an axial and angular deformation of CH in the regions 2921, 1461, 1377 and 719 cm $^{-1}$, which were measured in wave numbers.

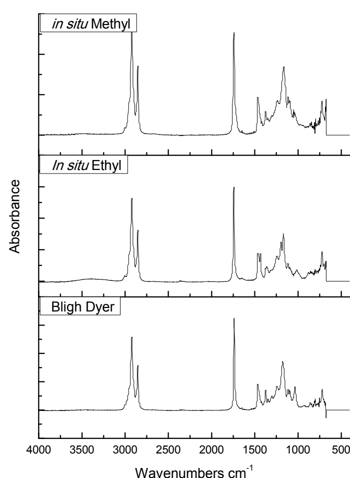

Figure 2. FTIR spectra for the samples.

For the in situ reactions (Figure 2) the spectra show a spectral identifying of both ester and ethylic and methylic samples. The bands are well defined in all spectral regions showing that the longest time and using 100 % of acid catalyst are the major factors for the increase of the reaction yield.
At results obtained by Nascimento et al., (2013), a supercritical ethanolic biodiesel obtained from soybean oil showed a purity of 46 % (w/w) at 300 ºC and pressure from 100 to 150 bar. The analyses used to determine the reaction conversion were: refractive index, density and dynamic viscosity. In this case the oil was free in the reaction system with no impediment of the cell membrane of the microorganisms, but this study still presented a low yield of biodiesel. In another study, Martins et al., (2013) showed that the highest conversion was obtained at a reaction time of 10 hours or more using a molar methanol:oil ratio of (20:1) resulting in 94.8% of esters using a catalyst developed in its study (hydrotalcite).
Unlike the studies presented above, in the
In in situ reactions, the lipids are inside the cell membrane making it less available to the reaction and further hindering the reaction time and conversion. However, in our study, all reactions were performed in milder conditions demonstrating that this reaction occurs efficiently, but more drastic conditions are required for maximum conversion.
Besides the components identification and esters estimation from the samples, the use of FT-IR technique can contribute as an aid in identifying oil as well as mixtures of oil/ester transesterification reactions or oil/ester adulterated samples. However, this technique requires a validation to build a mathematical model by chemometrics to make it a reliable and effective method.
The GC-MS was used to identify the ethyl esters obtained and to compare the profile. Table 3 depicts the profile of the ethyl fatty acids. The fatty acids identified in the oil of *L. starkeyi* are basically composed of C16 to C18:0 and C18: 1 carbons, with a low degree of unsaturation, desirable for the production of biodiesel fuel.

Table 3. Profile of ethyl fatty acids.

Sample	C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C24:0
in situ	0.47	0.07	33.40	3.77	0.42	12.46	43.28	4.18	1.94
Bligh Dyer	0.33	-	35.95	2.54	0.77	12.26	45.07	2.35	0.72

Several factors can affect directly the quantitative results such as incomplete conversion of lipids in to methyl esters of fatty acids, changes in the composition of fatty acids for esterification and formation of unidentified compounds. The catalyst used can also contaminate the chromatographic column, promoting incomplete extraction of the methyl esters and loss of methyl esters with volatile short chain (Brondz, 2002, Milinsk, 2007).
The in situ transesterification reaction from oleaginous microorganisms needs to be investigated on the type of microorganism used and the cell membrane. Biochemical pathways for the lipids formation should be also explored for better understanding and determination of lipid classes formed.

4. Conclusion

Biodiesel produced by microorganisms is still under research and development on laboratory scale, aiming to provide useful information to assist the future development of biodiesel production processes. Cell mass transesterification processes still need to be addressed in the same way of the reaction products identification and the amount determination using chromatography techniques for example. The FTIR with ATR was shown as an auxiliary tool for rapid determination of the reaction products. In this work it was observed that the FTIR provided a good response to the expected products. However, is required to assess the feasibility of using a line or in-line on analysis to follow the reaction kinetics determines the reaction time and the best molar ratio of the reactants.
The conversions obtained in this work were estimated requiring quantitative analysis to validate the results and methods used.

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