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ORIGINAL ARTICLE

Analyses of Lipid accumulation in oleaginous yeast through Fourier transform infrared spectroscopy and gas Chromatography

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ABSTRACT
Lipid accumulation in oleaginous microorganisms need to be monitored for the development and validation of new approaches for biodiesel production. The available techniques such as flow cytometry, gravimetric, and microscopic staining are time-consuming, invasive, costly and can also be influenced by fatty acids deterioration during lipid extraction. In the present study, the oleaginous yeast Candida tropicalis ASY2 (Accession number: MH011502) took as a model organism to study its lipid profile using advantaged techniques via FTIR and GC-FID analyses. FTIR results showed that (C=O) stretches of esters from lipids, particularly triacylglycerides (TAGs) was observed at a wave number of 1740cm^{-1}. Similarly, results of GC-FID analyses revealed that, at alternative days, a wide range of FAME from short-chain (5 carbons) to very-long-chain fatty acids (>22 carbons) was observed. In conclusion, the FTIR spectroscopic and GC-FIDmethod identified the most represented classes of the produced lipids and its FAME distribution.
Keywords: *Fourier transform infrared spectroscopy (FTIR), Candida tropicalis ASY2, Oleaginous yeasts, Fatty acids (FA), Biodiesel*

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INTRODUCTION

The interest in biodiesel as an alternative biofuel has been growing over the past two decades. The use of edible vegetable oils (soybean and rapeseed) and non-edible oils (Jatrophacurcas) needs improvement with emerging technologies and renewable sources of oil, which are currently being explored and developed, in the perspective of feasible production. Microbial lipids from oleaginous microorganisms known as single-cell oils (SCO) can aggregate up to 70% or more of their biomass while growing in high carbon / nitrogen (C / N) media are considered to be a possible alternative for biodiesel production [1,2]. Monitoring the lipid accumulation in these olegenous microbes becomes an important criteria in deciding the quality of biodiesel. Various lipid detection and quantification techniques such as fluorescent microscopy, spectrophotometry or flow cytometry [3]are available for current use.

Fourier transform infrared (FTIR) spectroscopy, on the contrary, is a non-invasive and label-free methodology that enables the fast acquisition of a biochemical fingerprint of the substance under investigation, providing information on its main content of biomolecule. Nonetheless, this spectroscopic technique is used successfully not only to characterize the structural properties of biomolecules such as proteins, lipids, nucleic acids and carbohydrates [4,5], but also to examine complex biological structures *in situ*, including intact cells, tissues and whole-model organisms[6,7,8].It is noteworthy that the FTIR approach has recently been used to assess the concentration of lipids in microalgae and marine yeasts and protists [9,10].

The FAMEs composition should be accurately determined to assess the feasibility of oleaginous species for biofuel production. GC-FID is more commonly use dowing to the fact that cheaper instrumentation and FID is more accurate for quantitative analysis, resulting in lower standard deviations [11].Often, FAME profiling plays a vital role to check its suitability for biodiesel production. Here we propose FTIR and GC-FIDas an alternative method for *in situ* lipid detection, which not only offers a glimpse of FA output but also provides the relative abundance of the individual fatty acid species.

MATERIAL AND METHODS

Strains and culture conditions

The yeast strain *Candida tropicalis* ASY2 (Accesion number: MH011502)isolated from sago processing wastewater (SWW) was used in the present study. The culture is routinely maintained at YEME slants at 4ºC.

Cultivation of oleaginous yeast in synthetic medium

The yeast culture was prepared 24 h before the experiment with a concentration of 10^6CFU.mL^{-1}. The active seed inoculum was inoculated into synthetic medium (Composition per litre: Starch, 10 g; Ammonium sulphate, 0.5 g; Potassium dihydrogen phosphate, 7 g; Disodium hydrogen phosphate, 2.5 g; Magnesium sulphate, 1.5 g; Ferric chloride, 0.15 g; Calcium chloride, 0.15 g; Zinc sulphate, 0.02 g; and Manganese sulphate, 0.06 g, pH 6.5) and incubated in a mechanical shaker (Orbitek, Scigensis Biotech) at 150 rpm for 5 d (30±2°C). Microbial biomass was harvested by centrifugation at 3000 rpm for 10 min and washed with distilled water twice, and dried in a hot air oven at 50 °C until constant weight.

Lipid content

The yeast biomass (100 mg) was homogenized with 20 volumes of chloroform: methanol mixture (2:1; v:v) and agitated overnight in an orbital shaker at room temperature. The lipidphase was separated by adding 0.2 volume of distilled water and centrifuged at 3,000 rpm for 10 min. The upper phase was siphoned out, and the lower chloroform phase containing lipids was evaporated under vacuum. The extracted lipids were measured gravimetrically [12].

Fourier-transform infrared spectroscopy (FTIR) analysis

The lipid samples derived from the alternative days are analyzed in FTIR (FTIR–6800 JASCO, Japan).The absorbance spectra were recorded in a wave number range of 4000 to 400 cm^{-1} with a spectral resolution of 4 cm^{-1} and 32 scans per sample.

Extraction of FAME

Direct trans esterification method was employed to extract FAME from yeast strain. In brief, dried cells (100 mg) accumulated with lipids were added to a mixture of 10 ml of methanolic sulphuric acid (0.1% sulphuric acid in methanol) and were mixed vigorously, and then 10 ml of chloroform was added. The mixture was heated at 80 °C for 2 h, and the samples were then cooled down at ambient temperature. One mL of distilled water was added and the suspension was centrifuged at 1500 rpm for 5 min. The lower aqueous phase containing fatty acid methyl esters (FAME) was then transferred into new clean tubes for gas chromatography (GC) analysis.

Analysis of FAME by GC-FID

The FAME esters extracted and analyzed through gas chromatography (Perkin Elmer Clarus 680, USA) equipped with flame ionization detector (FID) and Elite-5 column 30m: 0.25mm Film: 0.25 µm film thickness. The injection temperature was 220 ºC; the initial column temperature was 160 ºC and the final temperature of 190 ºC at a rate of 3 ºC per min; the detector temperature was 270 ºC. The carrier gas helium was used at a flow rate of 1.3 mL.min^{-1}.The FAME composition of lipids was analyzed by comparing the retention time and peak areas of samples with the standard FAME mix (Sigma).

RESULTS AND DISCUSSION

In the present study, the lipid accumulation phase of oleaginous yeast was studied using FTIR and GC-FID. The FTIR analysis and FAME profiling of oleaginous yeast are discussed below.

Monitoring lipid production in oleaginous yeast through FTIR spectroscopy

The spectra of lipid samples of *C. tropicalis* ASY2 at periodical intervals (1,3,5,7 and 9) were obtained using an FTIR. The FTIR absorption spectra of *C.tropicalis* ASY2 lipids in the synthetic medium are presented in Fig 1. The distinct biomolecules were observed in the FTIR spectra of the yeast biomass. The major absorption peaks observed in the ranges between 3030 and 2800 cm^{-1} and 1500 and 1350 cm^{-1}, 1700 and 1500 cm^{-1}, 1250 and 1000 cm^{-1}, 1000 and 800 cm^{-1}. The absorbance peaks between 3030 and 2800 cm^{-1} and 1500 and 1350 cm^{-1} represent the lipid acyl chains. Also, the spectrum between 1700 and 1500 cm^{-1} indicates amide I and amide II bands, respectively, due to C = O stretching and the peptide bond NH bending. The absorption range between 1250 and 1000 cm^{-1} is due to the C-O absorption of

carbohydrates, phospho-di-ester nucleic acid groups, and phospholipids. Finally, the peaks between 1000 and 800 cm^{-1} represent sugars, phosphates, and nucleic acids.

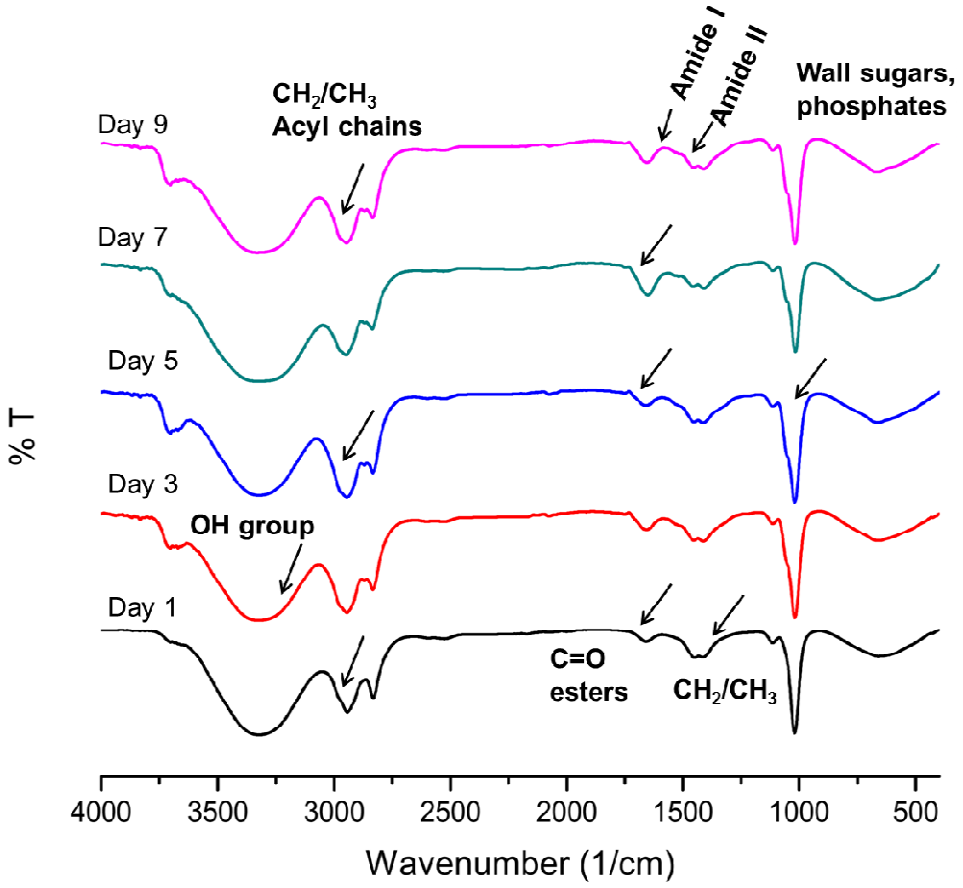

Fig. 1 FTIR profiling of fatty acid from *Candida tropicalis* ASY2 grown in synthetic medium

Prominent changes are found in the main absorption bands when analyzing the spectral time dependence of *C.tropicalis* ASY2 in alternative days.In particular, there was a substantial increase in the intensity of the CH_2/CH_3 bands was observed between 3,000 and 2,800 $cm^{-1}$from 1st day to 5th day of growth and then decreased on 7th day, which represents (= CH) stretches from unsaturated lipids. Indeed, C = O absorption around 1,740 cm^{-1} recorded a higher peak intensity was observed on 7th day, it symbolizes (C=O) stretches of esters from lipids particularly triacylglycerides (TAGs).Bands in the spectral range 4000 to 3250 cm^{-1}show the presence of hydroxyl groups. The intensity of the hydroxyl group onthe 5th day is comparatively less compared to other days it may be due to cell metabolism. Several wave numbers responsible for lipid accumulation is observed on 5th day. The wave number 3318.89 cm^{-1} represents (OH) stretches from water molecules; 2944.77 and 2869.56 cm-1 indicates (CH_2) asymmetric stretches of acyl chains. The wave number at 1752.01 cm^{-1} shows (C=O) stretches of esters (TAGs). The wave number 1658.48, 1453.1, 1112.73 and 1020.16 cm^{-1} represents amide I group from proteins, (CH_3) asymmetric bend from lipids, C-O-C stretches from carbohydrates and (P=O) symmetric stretches from nucleic acids, respectively.

Therefore, significant changes were found in the absorption region between 1,250 and 1,000 cm-1.Surprisingly, there are several bands of absorption noticed in this spectral region due to beta-glucans, which are significant components of the yeast cell wall [13].Therefore, it indicates that the essential cell wall modifications that are known to occur in oleaginous yeasts during the development of fatty acids.

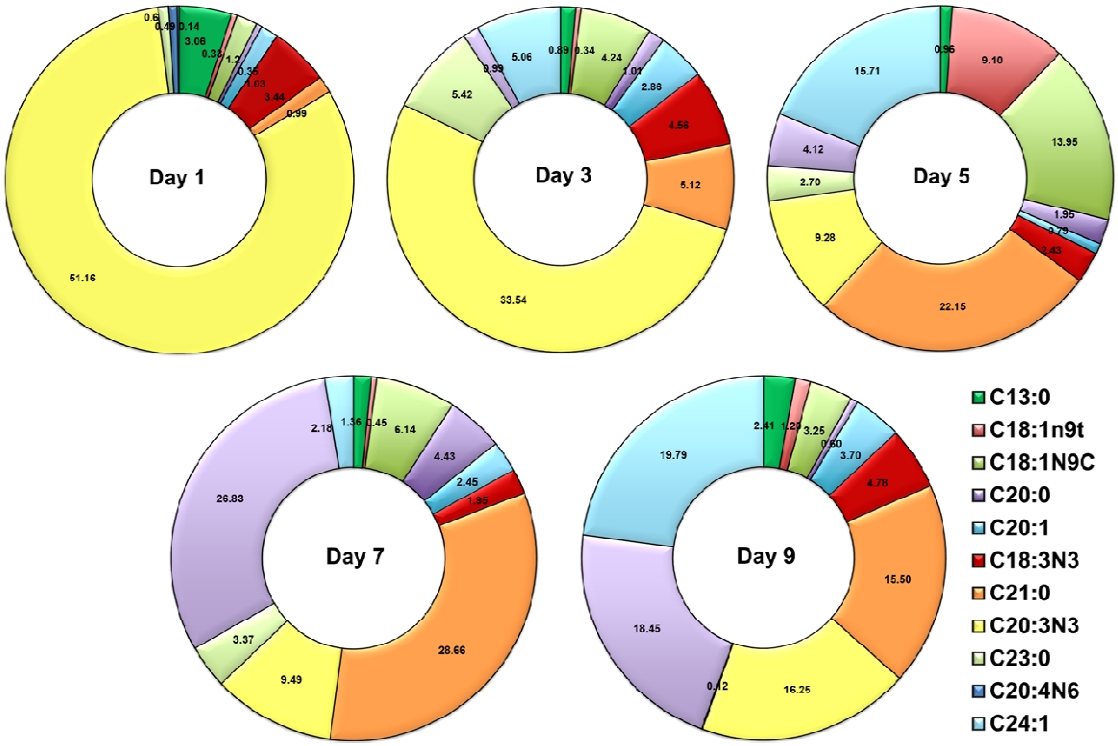

Fig.2. Fatty acid composition (%)of yeast *C. tropicalis*ASY2 grown in synthetic medium

Fatty acid profiling of oleaginous yeast *C.tropicalis*ASY2

In order to evaluate the prospective utilities of the extracted total lipids in synthetic medium from the oleaginous yeast, *C.tropicalis* ASY2 as abiodiesel feedstock, its FAME profile was determined by GC-FID. The FAME composition of lipids was determined by comparing the retention time and samples peak area with standard FAME mix (Sigma). Based on the length, fatty acids are categorized as short-chain fatty acids (up to 5 carbons), medium-chain fatty acids (6 to 12 carbons), long-chain fatty acids (13-21 carbons) and very long-chain fatty acids (22 or more carbons) [14]. In the present study, the yeast biomass obtained from different incubation times (1, 3, 5, 7, 10 days) was acid-catalyzed through transesterification. Based on the results, the wide range length of fatty acid methyl esters from short-chain to very long-chain fatty acids was observed on different days. It includes both saturated and unsaturated forms up to 24 carbon chain length.

The similarities between microbial and vegetable oils make it possible to use this microbial biomass as a feedstock for biofuels production. In the present study, the yeast produced the following fatty acids which is similar to vegetable oils such as tridecylic acid (C13:0), elaidic acid (C18:1n9t), oleic (C18:1n9C), α-linolenic acids (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), heneicosanoic acid (C21:0), cis 11, 14, 17 eicosatrienoic acid (C20:3N3), tricosanoic acid (C23:0), arachidonic acid (C20:4N6), nervonic acid (C24:1) and other fatty acids (myristric acid (C14:0), palmitic (C16:0), stearic (C18:0), linoleic (C18:2), margaric acid (C17:0)).

The proportion of FAME obtained in yeast grown in synthetic medium was quantitatively diverse with respect to days. This may be due to the various minerals and compositional changes in the synthetic medium that affected the yeast growth and FAME profile. Our results are in accordance with the earlier researchers [15,16];they reported that the fatty acid profile of oleaginous yeast depends entirely on the conditions under which they were grown especially environmental stress or physical stress.

The FAME profile of synthetic medium revealed the maximum amount of cis 11, 14, 17 eicosatrienoic acid (51.16%) on 1st day itself and decreased up to 7th day (9.49%) and increased on 9th day (16.25%), it may be due to different minerals present in the medium. Eicosatrienoic acid is a unique collection of omega-3 polyunsaturated fatty acids. This accounts for less than 0.25% of serum phospholipid fatty acids in healthy humans (Source: Human Metabolome Database). The oleic acid content increased gradually and recorded the highest content in 5th day (13.95%) and started to decline which is the major fatty acid for biodiesel production. On 7th day, heneicosanoic (21-carbon saturated fatty acid), arachidic and arachidonic acid (polyunsaturated omega-6 fatty acid) recorded a maximum of 28.66, 4.43 and 26.83%,

respectively, compared to other days. Arachidic acid (20 carbon saturated fatty acid) is a minor constituent of cupuacu butter [17].

Nervonic acid is an essential nutrient for the growth and maintenance of the brain [18], a more significant amount of 19.79% was observed on 9th day. A higher content of α-Linolenic acid (4.78%) was observed on 9th day, which is an essential omega-3 fatty acid. On 1st and 3rd day, tridecylic and elaidic acid represent 3.06% and 9.10%, respectively. Based on the findings, various proportions of fatty acids were observed on different days of incubation, which contained a large fraction of essential unsaturated fatty acids. Nevertheless, the major fatty acid (myristic,palmitic, stearic, linoleic, oleic (13.95%) responsible for biodiesel production is present is a negligible amount. So the FAME profile of synthetic medium reveals that optimizing the incubation days/hours helps to recover essential fatty acids of omega 3 and 6 series fatty acids.

CONCLUSION

FTIR is a powerful tool to monitor *in situ*fatty acid accumulation in oleaginous yeasts over time.It is also a quick and non-invasive method not requiring cell disruption and/or extraction of lipids. This FTIR analysis not only eliminates the time and costs but also reduces the risk of lipid loss and degradation. At the same time, GC-FID analysis of oleaginous yeast provides an exact quantification of FAME, and its profiling helps to assess the suitability for biodiesel use.FTIR and FAME can, therefore, be suggested as a powerful tool for initial screening, optimization, and scale-up process, which are all necessary steps to establish a sustainable biodiesel production based on oleaginous microorganisms.Furthermore, FAME profile of synthetic medium reveals that optimizing the incubation days/hours helps to recover essential fatty acids of omega 3 and 6 series fatty acids besides biodiesel.

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