Identification of Oil Producing Yeast and Characterization of Its Oil

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ABSTRACT

Single cell oil which is also known as microbial oil are produced by oleaginous microorganisms which have ability to accumulate more than 20% lipid per dry biomass, having potential applications in food, feed and pharmaceutical industry. Most of the lipids in this oil are triglycerides characterized with long chain fatty acids similar to that of vegetable oils. This study investigated the growth and identification of oil producing yeasts. Furthermore, the characterizations of the oil fatty acids composition were analyzed using gas chromatography mass spectroscopy (GSMS). The oleaginous yeast Rhodotorula mucilaginosa RIT389 and Pichia kudriavzevii 10526S from different sources were used for the investigation the lipid accumulation was 30.8% and 38.5% of the dry cell weight (DCW) respectively.

From the analysis, saturated fatty acids (SFA) have highest percentage i.e. 60.82% and 78.60% respectively in the two microbial oil studied in which palmitic acid, stearic acid, myristic and lauric acid are the major SFA. Others include pentadecnoic acid and shorter chains fatty acids such as acetic acid, propanoic acid and butanoic acid. Also, monounsaturated fatty acids (MUFA) contain 32.32% and 19.03% respectively and polyunsaturated fatty acids (PUFA) recorded the lowest percentage of 6.86% and 2.37% respectively in the two oil studied. These findings underscore the potential of oleaginous yeasts as alternative lipid sources. The compositional similarity of the microbial oils to conventional edible oils such as soybean and palm oil supports their feasibility for sustainable industrial applications.

Keywords: oleaginous yeast, Single-cell oil, Lipid accumulation, MUFA, PUFA, Microbial oil

INTRODUCTION

Lipids are vital biomolecules produced by all living organisms—both macro and microorganisms—for essential structural and functional roles. One of their most fundamental roles is the formation of selectively permeable membranes in cells and organelles through lipid bilayers (Dowhan and Bogdanov, 2013). Among lipid-producing organisms, a special group known as oleaginous microorganisms stands out for their ability to accumulate more than 20%—and in some cases up to 70%—of their dry cell weight as intracellular lipids (Meng et al., 2009). These lipids, referred to as single-cell oils (SCOs), are synthesized by certain bacteria, fungi, yeasts, and microalgae. While prokaryotic bacteria produce specific lipids, eukaryotic organisms such as fungi, yeasts, and microalgae are capable of synthesizing triacylglycerol (TAGs) with compositions comparable to vegetable oils.

Lipids serve as a dense energy reservoir, providing approximately 39 kJ/g (9 kcal/g). In the form of TAGs and sterol esters, they are sequestered in specialized organelles such as lipid bodies in oilseeds, oleaginous microorganisms, and mammalian adipose tissues. Besides energy storage, lipids contribute essential fatty acids and sterols necessary for the biosynthesis and integrity of cellular membranes. Lipid metabolism is ubiquitous among microorganisms, and the fatty acid profiles of oleaginous microbes often resemble those found in plant and animal-derived oils (Dewapriya and Kim, 2014). SCOs are gaining interest for their applications in human and animal nutrition, pharmaceutical formulations, and as renewable feedstock for biofuel production (Ratledge, 2013).

Among oleaginous microorganisms, yeasts are particularly promising candidates for industrial lipid production due to their advantageous physiological traits. These include high lipid accumulation—sometimes up to 80% of

their dry cell weight—rapid growth rates, independence from light, the ability to thrive on a variety of substrates and carbon sources, and tolerance to low pH environments (Sitepu et al., 2014). However, only about 5% of known yeast species are considered oleaginous.

Well-studied oleaginous yeasts include species from the genera Candida, Rhodosporidium, Yarrowia, Cryptococcus, Rhodotorula, Lipomyces, and Trichosporon, many of which have been reported to accumulate lipids up to 80% of their biomass (Signori et al., 2016; Patel et al., 2016). Their lipid metabolism is relatively well understood (Dourou et al., 2018), and current research is expanding to identify and engineer new strains with improved lipid productivity (Yamada et al., 2017; Park et al., 2017. These strains should meet some certain criteria which include the ability to grow into high cell densities in addition to high lipid content on various carbon sources and robust process conditions (Matsakas et al., 2014, Dien et al., 2016 and Maina et al., 2017). In other to improve economic feasibility, oleaginous yeast strains have accordingly been cultivated on various non-food competing carbon sources, such as lignocellulosic materials (Patel et al., 2015; Poontawee et al., 2017)

SCO production has some advantages over the production from plant and animal based sources with a wide range of applicable oleaginous microorganism species to use, accumulation of large amounts of lipids in the cells, the fast growth rate of the biomass as compared to plants and animals, and reduction in the cost of production (Huang et al; 2013; Garay et al., 2014). Another advantage is that, SCO production is an ecofriendly process when compared to plant-derived oils, less consumption of water, smaller portion of space is required, and it has a minute negative impact on climate change as the case is in oil derived from plant and animal source (Spalvins and Blumberga, 2018), furthermore, the ability to utilize a wide range of biodegradable agricultural by-products in the cultivation of SCO-producing microorganisms.

Materials and Methods

Study Site and Sample Collection

Palm oil mill effluent (POME) and palm oil press fiber (PPF) were collected from palm oil mills located in Ijaye and Olode, Ibadan, Oyo State, Nigeria. Sorghum grains were purchased from Bodija Market, Ibadan. All experimental work was conducted in the Food Microbiology and Chemistry Laboratory, Department of Food Technology, University of Ibadan (Longitude: 3° 54′ 59.99″ E; Latitude: 7° 23′ 28.19″ N).

Preparation of Culture Media

Fermented Sorghum Water (FSW) Medium

Sorghum grains (1 kg) were cleaned, sorted, and soaked in water at ambient temperature for 48 hours, following the method of Akingbala et al. (1981). The soaked grains were wet-milled and allowed to ferment naturally for 24 hours. The resulting fermented water was decanted and used as a nutrient-rich culture medium for yeast growth.

Potato Dextrose Agar (PDA).

PDA medium was prepared using 4 g/L potato infusion, 20 g/L glucose, and 15 g/L agar (Bhosale and Gadre, 2001). The medium was autoclaved at 121°C for 15 minutes and poured into sterile Petri dishes.

Yeast Cultivation and Isolation

One milliliter each of POME and PPF samples was inoculated onto PDA plates. The medium was prepared by dissolving 7.8 g of PDA in 200 mL distilled water, sterilized, and cooled to 45°C before plating. Plates were incubated IN thermostat incubator (DNP-9052-1) at 28°C for 72 hours. Morphologically distinct yeast colonies were isolated by repeated sub culturing AS described by Osorio-Gonzalez et al. Isolates were subsequently inoculated into the fermented sorghum water and incubated at 28°C for 72 hours. Following incubation, cells were harvested by centrifugation and washed once with distilled water. Parallel cultures were also grown on fresh PDA for 96 hours at 28°C, after which the cells were harvested for lipid extraction (Cabral et al., 2011).

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Biomass and Lipid Quantification

Dry cell weight (DCW) was determined by filtering harvested yeast cells through pre-weighed Whatman filter paper, followed by two rinses with distilled water and drying in a hot-air oven at 60°C until a constant weight was achieved. Results were expressed in g/L.

Microbial Oil Extraction

Lipids were extracted from dried biomass (approx. 3.9 g) using a Soxhlet extractor with 350 mL of n-hexane (98% purity) as solvent. The biomass was wrapped in Whatman filter paper and loaded into the Soxhlet chamber. The extraction was carried out for 4 hours. Extracted oil was recovered by solvent evaporation and oven-dried at 60°C for 1 hour to remove residual solvent.

Fatty Acid Composition by GC-MS

Fatty acid methyl esters (FAMEs) were prepared and analyzed using a Varian 3800/4000 Gas Chromatography–Mass Spectrometry (GC-MS) system equipped with a VF-5MS column (30 m × 0.25 mm, 0.25 µm film thickness), following Wild (2009) method.

GC-MS settings included: Initial oven temperature: 100°C for 1 min, Ramp: 30°C/min to 270°C, held for 10 min, Inlet and transfer line temperatures: 270°C and 280°C, Carrier gas: Nitrogen (99.9995%) at 1.0 mL/min and Injection volume: 20 Ml. Methyl heptadecanoate served as internal standard, the free fatty acid content was determined using European standard method EN 14103:2003. Peak identification was performed using the NIST-MS library. Results were expressed as relative area percentages.

Genomic DNA Extraction

Yeast isolates were grown overnight and harvested by centrifugation at 14,000 rpm for 2 minutes. DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Derreck Carter-House et al., 2020). The cell pellet was treated with 600 µL of 2× CTAB buffer and incubated at 65°C for 30 minutes. After cooling, chloroform was added and the mixture was centrifuged at 14,000 rpm for 15 minutes. The aqueous layer was transferred to a new tube, mixed with cold isopropanol, and incubated at −20°C for 1 hour. DNA was precipitated by centrifugation, washed with 70% ethanol, air-dried, and resuspended in 100 µL sterile distilled water. DNA quality was assessed spectrophotometrically at 260/280 nm, with purity ratios between 1.8 and 2.0 considered acceptable.

Agarose Gel Electrophoresis

DNA quality and integrity were assessed using 1% agarose gel electrophoresis in 0.5× TBE buffer. Gels were stained with ethidium bromide and run at 80 V for 2 hours. Bands were visualized under UV illumination.

PCR Amplification and Sequencing

Fungal Internal Transcribed Spacer (ITS) regions were amplified using universal primers ITS1 and ITS4. PCR reactions (10 µL) contained 1 µL 10× buffer, 0.4 µL $MgCl_2$ (50 mM), 0.5 µL dNTPs (2.5 mM), 0.5 µL each of ITS1 and ITS4 primers (5 mM), 0.05 µL Taq polymerase (5 U/µL), 2 µL template DNA, and 5.05 µL sterile distilled water. The PCR profile was: 94°C for 3 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min; followed by a final extension at 72°C for 5 min.

PCR Product Purification

Amplicons were purified using 2 M sodium acetate and ethanol precipitation. To 10 µL of PCR product, 1 µL of 2 M sodium acetate (pH 5.2) and 20 µL absolute ethanol were added. The mixture was incubated at −20°C for 1 hour, centrifuged at 10,000 rpm for 10 minutes, washed with 70% ethanol, air-dried, and resuspended in 5 µL sterile distilled water.

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Sequencing Reaction

Sequencing reactions (10 µL) included 0.5 µL BigDye Terminator, 1 µL 5× sequencing buffer, 1 µL M13 forward primer, 1 µL PCR product, and 6.5 µL sterile distilled water. The thermal profile was: 96°C for 1 min; 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min; followed by a hold at 4°C.

Purification of Sequencing Products

Purification was performed using 2 M sodium acetate and ethanol precipitation. To 10 µL of the sequencing reaction, 1 µL of 2 M sodium acetate (pH 5.2) and 20 µL absolute ethanol were added. The mixture was incubated at −20°C for 1 hour, centrifuged at 10,000 rpm for 10 minutes, washed with 70% ethanol, air-dried, and resuspended in 5 µL sterile distilled water.

Sample Preparation for Sequencer

The Cocktail mix is a combination of 9µl of Hi-Di Formamide with 1µl of Purified sequence making a total of 10µl. The samples were loaded on the Analyzer and the sequencing data in form of nucleotide base (A, C, T, and G) were produced.

RESULTS AND DISCUSSION

The methodology above were used to isolate, identify, and evaluate the oil-producing potential of oleaginous yeast Rhodotorula mucilaginosa RIT389 and Pichia kudriavzevii 10526S obtained from two different sources. Each isolate was subjected to characterization and lipid accumulation. The lipid extract were subsequently analyzed using Gas chromatography–mass spectrometry (GC–MS) to determine their fatty acid profiles. The results obtained from these analyses are presented in the following section.

Biomass Growth on Culture Media

Fermented Sorghum Water

There was no significant microbial growth in the fermented sorghum water used which serve as culture media for the cultivation of the yeast. This could be because of a very high C/N (carbon to nitrogen) ratio or a very low amount of available nitrogen in the media, reason being that carbon causes an osmotic shock when its available in large amounts in the media due to a decrease in Krebs cycle activity, which thereby reduce the energy production, and intracellular increase of critic acid (Yaegashi et al., 2017). Although, Liang et al., (2010) use sorghum juice to cultivate Schizochytrium limacinum but obtain a relatively low biomass for the microalgae strain.

Potato Dextrose Agar (PDA)

A significant biomass growth was observed in Potato dextrose agar (PDA) culture media and the cells grow steadily until after 72h, after which no significant growth observed. The maximum biomass yield was 3.9 g/L each for both POME and PPF derived-isolates.

Figure 1: Biomass growth of POME on potato dextrose agar

Figure 2: Biomass growth of PPF on potato dextrose agar

Identification and Characterization of Cultivated Yeast

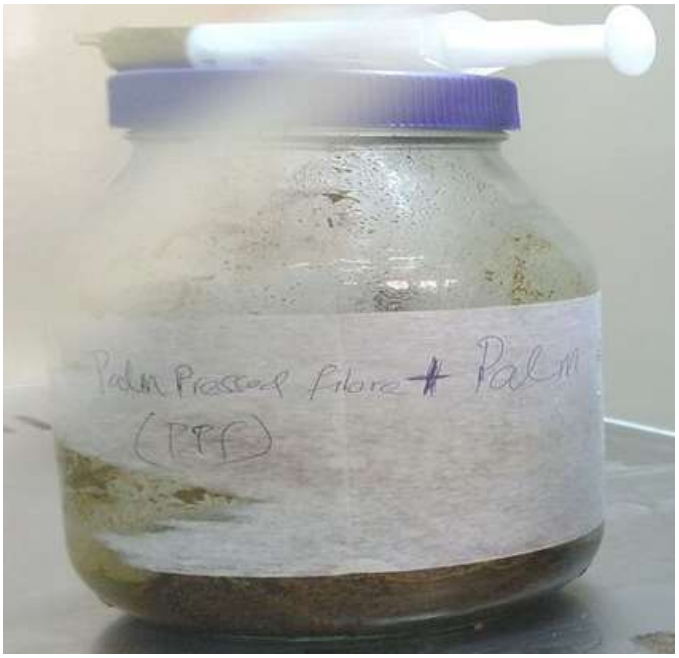
DNA Sequencing of Yeast Isolates

Several species such as of oleaginous yeast from different environments (e.g. soil, waste of palm oil production, plant materials etc.) are well known for their ability to accumulate lipid intracellular when cultivated on various carbon-rich subsrates (Tanimura et al., 2014). In this study, a total number of seven yeast strains from two sources (figure 3 and 4) were isolated and screened and two of the yeast strains were genetically identical. Polymerase Chain Reaction (PCR) amplification carried out on the DNA samples facilitated geometric multiplication of the DNA fragments in billion folds.

The identified yeast strains from POME include Rhodotorula mucilaginosa (RIT389 NC036340.1), Rhodotorula mucilaginosa (RIT389 MF694646.1), Rhodotorula taiwanensis RS1 (HF558455.1), and Pichia kudriavzevii clone test 10526S (JQ419988.1) while strains from PPF include Issatchenkia orientalis N24526S EU268662.1, Pichia kudriavzevii clone test 10526S JQ419988. 1 and Issatchenkia orientalis N24526S EU268662.2.

Figure 3: Palm oil mill effluent

Figure 4: Palm oil press fibre



Lipid Accumulation by Selected Yeast Strains

Two representative strain, Rhodotorula mucilaginosa RIT389 and Pichia kudriavzevii 10526S one from each source used for the investigation were selected and analyzed for lipid accumulation. The result obtained from the two oleaginous yeast Rhodotorula mucilaginosa RIT389 and Pichia kudriavzevii 10526S was 30.8% and 38.5% lipid content respectively. Arising from this result, Rhodotorula mucilaginosa RIT389 and Pichia kudriavzevii 10526S have proving to be a viable source of oil and a short production process is required with less energy utilization making the process a promising one. Usually, nutrient imbalance in the culture media will ultimately triggers lipid accumulation in oleaginous yeast. That is, when the key nutrient such as nitrogen is exhausted, multiplication of the cells ceased and excess carbon is then converted into storage lipids. This implies that the initial Carbon/Nitrogen (C/N) ratio in the culture media play an important role in accumulation lipid. In some literature, lipid production varies and some factors such as the oleaginous strain used, culture media and the ratio of carbon to nitrogen (C/N) of the media determine this.

Papanikolaou et al., 2004**,** in their report reveal the effect of the C/N ratio on Cunninghamella echimulata which show that there is an increase in lipid accumulation from 36% to 47%. Conversely, in this study the lipid production is low (i.e., 1.2-1,5g/L) therefore there is need for further exploration in other to improve the lipid yield. From this study, the timely accumulation of lipids at the early stage, fast microbial growth ultimately decreases the total production time, production cost and the energy consumption. R.toruloides-AS2.1389 reached its maximum lipid accumulation at 120 h when cultivated as reported by Zhao et al., 2021. The cultivation media used (potato dextrose agar), contain only glucose (dextrose) as carbon source and also under stress conditions, some species of yeast (e.g. Rhodotorula spp) tend to accumulate lipids in the early stages of their growth (Osorio-González et al., 2019) this resulted in the rapid consumption of glucose within 72 h of cultivation. When two blends of carbon source such as glucose and glycerol are used, a diauxic growth occurs. Bommareddy et al., 2015, cultivated R. toruloides in a mixed media of glucose and glycerol (10 and 40 g/L respectively) and it was observed that in the cultivation media, the utilization of glycerol only begin after the glucose was exhausted resulting into a double exponential phase at 7 h and 30 h.

Fatty Acid Composition of Single Cell Oils (SCO)

The GC-MS analysis revealed the presence of various fatty acids in the SCO produced by the two yeast strains. The identified fatty acid composition from the oil synthesized by the two oleaginous yeast Rhodotorula mucilaginosa RIT389 and Pichia kudriavzevii 10526S has a good separation of the fatty acids on the chromatograph. The major fatty acid composition of oil from Rhodotorula mucilaginosa RIT389 include palmitic

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acid (20.09%), oleic acid (30.39), stearic acid (10.26%) and arachidonic acid (6.86%) as shown in Figure 5. Those of Pichia kudriavzevii 10526S include palmitic acid (21.03%), myristic acid (10.80%), oleic acid (16.98%) and nonadecanoic acid (9.74%) as shown in figure 6. The composition of the individual fatty acid of oil from Rhodotorula mucilaginosa and those of Pichia kudriavzevii are shown on tabke 1, these compositions align with those of common edible oils and demonstrate the potential of these yeasts as alternative oil sources.

Lipids are the most important food component which play significant role in human diet being the most substantial source of energy and also supply the essential fatty acids that are necessary for effective functioning of the human body **(**Gustone**,** 2011**).**

Figure 5: GC-MS Spectra of FAME of Rhodotorula mucilaginosa RIT389. (Peak identification: Acetic acid =11.80, Propanoic acid =14.00, Butanoic acid =18.50, Lauric acid =30.02, Myristic acid =23.50, Pentadecanoic acid =34.00, Palmitic acid =24.95, Oleic acid =29.50, Palmitoleic acid =21.48, Stearic acid =25.26 and Arachidonic acid =36.00).

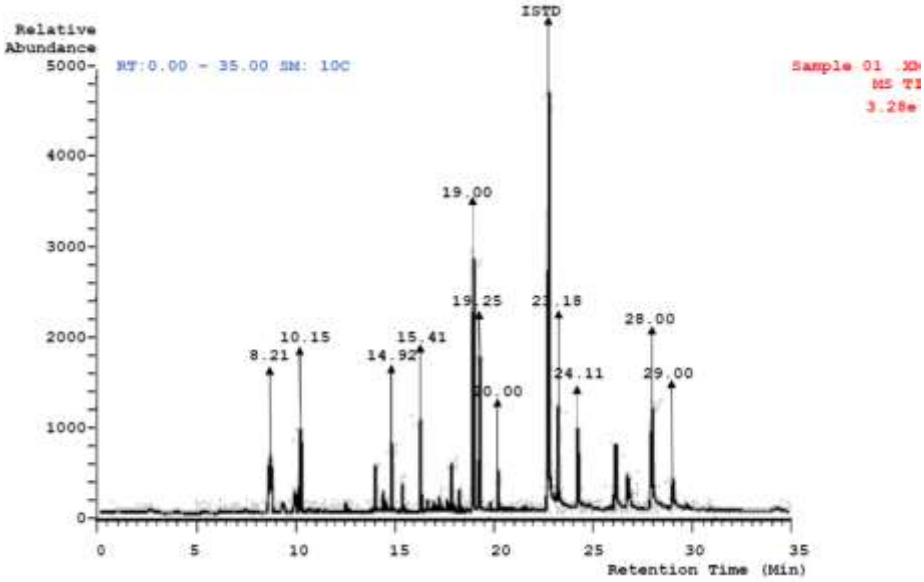

Figure 6: GC-MS Spectra of FAME of Pichia kudriavzevii 10526S. (Peak identification: Caprylic acid =8.21, Capric acid =10.15, Lauric acid at 15.41, Myristic acid at 19.25, Pentadecanoic acid at 23.18, Palmitic acid at 19.00, Oleic acid at 24.11 Palmitoliec acid at 20.00, Stearic acid at 14.92, Nonadecanoic acid at 28.00 and Arachidonic acid at 29.00).

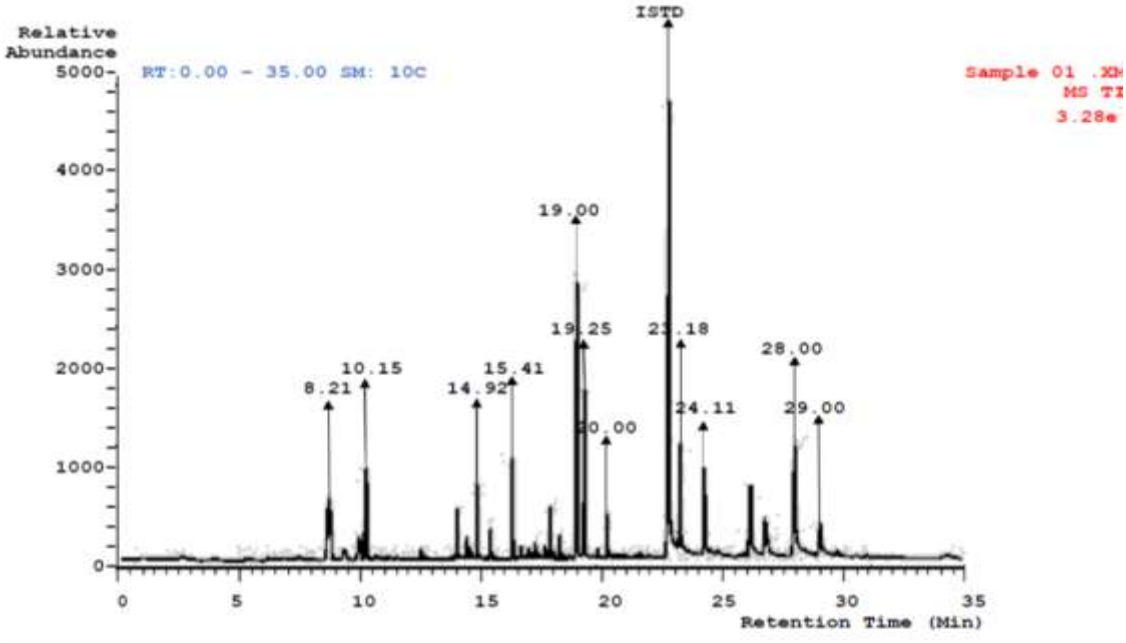

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Table 1: Composition of Individual Fatty acid in Single cell oil (SCO)

	Rhodotorula mucilaginosa RIT389				Pichia kudriavzevii 10526S			
Fatty Acid	Moleculrweight	Peak Area %	Comp. Wt. %	Retention time (min)	Moleculr weight	Peak Area %	CompWt. %	Retention time (min)
Acetic Acid	60	4.39	5.00	11.80	ND	ND	ND	ND
Propanoic acid	74	5.85	5.63	14.00	ND	ND	ND	ND
Butanoic acid	88	3.66	4.48	18.50	ND	ND	ND	ND
Caprylic acid	ND	ND	ND	ND	144	4.38	4.83	8.21
Capric acid	ND	ND	ND	ND	172	6.03	6.74	10.15
Lauric acid	216	6.57	3.75	30.02	216	6.58	5.71	15.41
Myristic acid	228	4.24	5.21	23.50	228	9.86	10.80	19.25
Pentadecanoic acid	242	5.12	6.41	34.00	242	6.85	8.73	23.18
Palmitic acid	256	18.28	20.09	24.95	256	16.44	21.03	19.00
Oleic acid	282	32.16	30.39	29.50	282	6.30	16.98	24.11
Palmitoleic acid	254	2.92	1.93	21.48	254	3.29	2.05	20.00
Stearic acid	284	9.50	10.26	25.26	284	4.66	10.92	14.92
Nonadecanoic acid	ND	ND	ND	ND	298	7.67	9.74	28.00
Arachidonic acid	304	7.31	6.86	36.00	304	1.64	2.37	29.00

ND: Not detected

Characterization of Fatty Acid Classes

Saturated Fatty Acids (SFAs)

SFAs are predominant in the oils produced, with 60.82% in R. mucilaginosa and 78.5% in P. kudriavzevii. Major SFAs include palmitic, stearic, lauric, and myristic acids. These results are consistent with previous findings from L. starkeyi and plant oils (Guerfali et al., 2018; Nainggolan and Sinaga, 2021). While some long-chain SFAs are associated with cardiovascular risk (Keresteš et al., 2011), others such as lauric acid serve important physiological roles.

Unsaturated Fatty Acids (UFAs)

The MUFA content was 32.32% and 19.03% in R. mucilaginosa and P. kudriavzevii, respectively. Oleic acid was the most abundant MUFA in both strains. PUFA contents were relatively low (6.86% and 2.37%). The presence of oleic acid is noteworthy, as it plays a beneficial role in human health and is of increasing interest in food and industrial applications (Tarrago-Trani et al., 2006).

Influence of Culture Medium on Oil Yield

The culture medium significantly affected oil yield. Fermented sorghum water yielded minimal biomass and lipid, likely due to its inadequate nitrogen profile. In contrast, PDA medium supported robust yeast growth and

lipid accumulation. This aligns with previous studies that emphasize the role of nutrient composition, particularly nitrogen limitation, in triggering lipogenesis (Ratledge, 2002).

Influence of Culture Medium on Fatty Acid Profile

The fatty acid profile of microbial oils was also influenced by the culture medium. Both yeast strains predominantly produced fatty acids with 16–18 carbon atoms, similar to vegetable oils as reported by Karatay and Donmez G. (2010) where the microbial oil produced by Candida tropicalis using molasses as carbon source is high in its stearic acid (56.2%) and palmitic acid (29.7%) content, also the compositions of fatty acids accumulated by Rhodotorula glutinis cultivated on xylose yielded 69% saturated fatty acid and on glycerol, yielded 68% (Easterling et al., 2009).

Arising from this study on the two oil samples, Rhodotorula mucilaginosa RIT389 had a highest proportion of SFAs (60.82%) followed by MUFAs (32.32%) but with a small proportion of PUFAs (6.86%), while in Pichia kudriavzevii 10526S, SFAs also recorded the highest proportion (78.50%) followed by MUFAs (19.03%) and PUFAs had the lowest proportion (2.37%) as shown on table 2.

Table 2: Percentage composition of the classes of fatty acid

FATTY ACID			
	Saturated fatty acids (SFAs) %	Monounsaturated fatty acids (MUFAs) %	Polyunsaturated fatty acids (PUFAs) %
R. mucilaginosa RIT389	60.82	32.32	6.86
P. kudriavzevii 10526S	78.50	19.03	2.37

CONCLUSION

The Strains of yeast that was used for this research was cultivated with a remarkable growth on the culture media used (Potato dextrose agar) and were identified for their oil producing ability and the oil produced was characterized. The strain of the oleaginous yeast, the culture media, composition and ratio of the constituent of the culture media all contributed to the accumulation of lipids (yield) and the fatty acid composition of the single cell oil.

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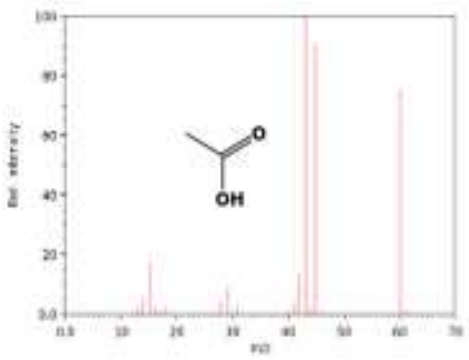
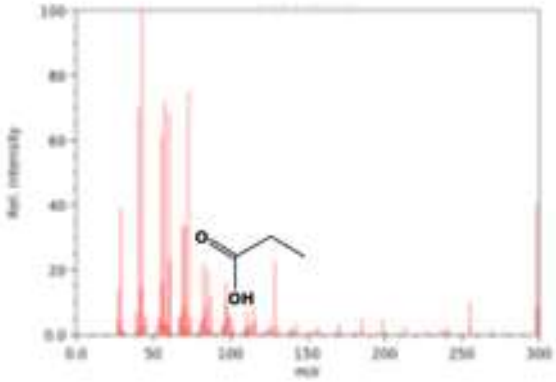
APPENDIX

Mass Spectroscopy spectra of FAME's of fatty acids analysis

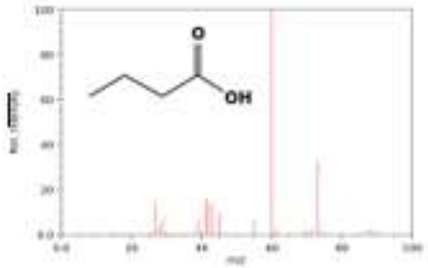
(1). Acetic acid



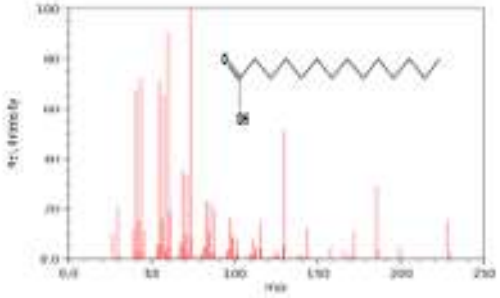
(2). Propanoic acid



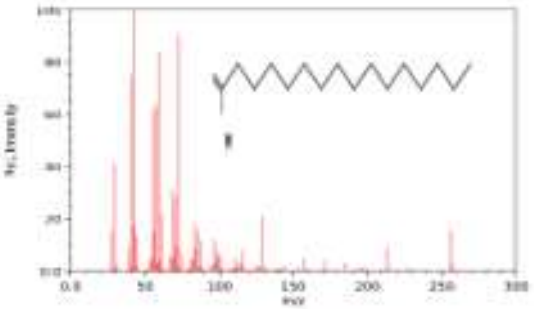
(3). Butanoic acid



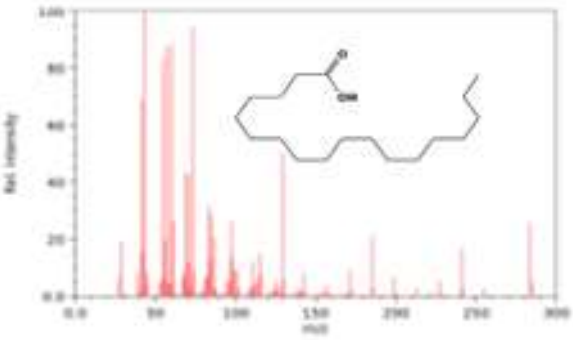
(4). Myristic acid



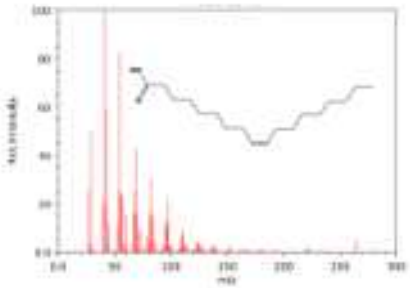
(5) Palmitic acid



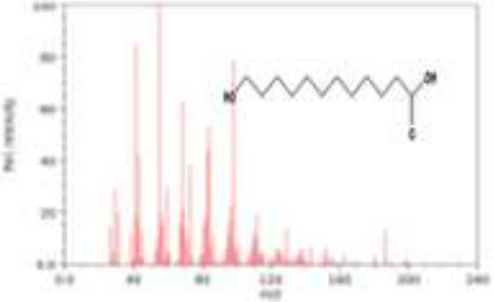
(6). Stearic acid



(7). Oleic acid



(8). Lauric acid



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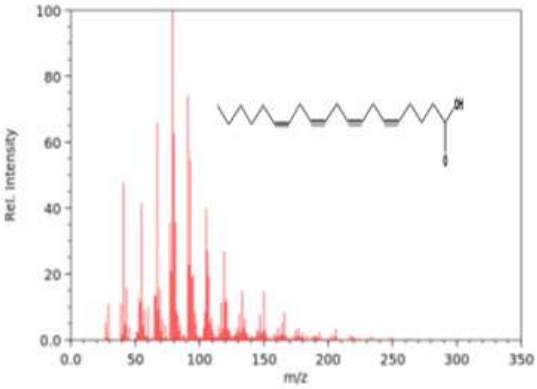
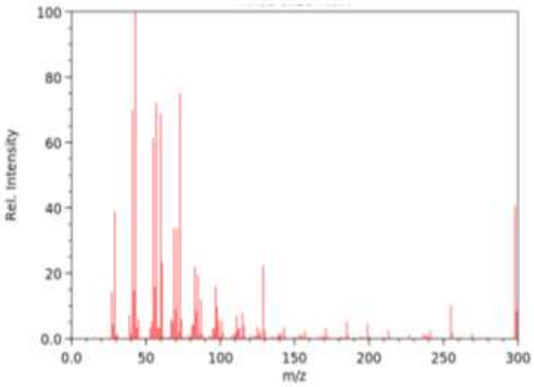
(9). Pentadecanoic acid



(10). Nonadecanoic acid



(11). Arachidonic acid