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Biodiesel production of oleaginous yeast isolated from the Mount Makiling Forest Reserve

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ABSTRACT

Microbial oils from oleaginous yeasts are potential alternatives for sustainable biodiesel production. The Mount Makiling Forest Reserve (MMFR) in the Philippines is a tropical rainforest with a megadiverse ecosystem but with limited information on its microbial diversity. This makes it an attractive source of novel and potentially biotechnologically valuable microorganisms. Twenty-two out of 258 yeasts isolated from the barks, roots, canopy leaves, and associated epiphytic plant material of various forest trees in the MMFR, were potentially oleaginous. Using a nitrogen-limited medium containing glucose as a carbon source, BUB8 and NFR6, isolated from the upper bark of a Bagtikan and from the fern root of Narra tree, respectively, showed the highest biomass and lipid production levels. BUB8 accumulated a higher lipid content than NFR6 when grown in the same medium with glycerol as a carbon source. The fatty acid methyl ester profile of the transmethylated microbial oil produced by BUB8 showed that oleic acid is the dominant species (C18:1 >C16:0 >C18:2 >C18:0 >C14 = C16:1). Finally, phylogenetic analysis of the ITS rDNA region classified BUB8 as a member of the *Rhodotorula* genus.

ARTICLE HIGHLIGHTS

- Oleaginous yeasts were isolated from the megadiverse canopies of the Mount Makiling Forest Reserve.
- The lipid production of the isolates was tested in a nitrogen-limiting medium with glucose as the sole carbon source.
- The capabilities of NFR6 and BUB8 for lipid production from glycerol were tested.
- BUB8 belongs to the *Rhodotorula* genera with *R. paludigena* as its closest relative.

1. INTRODUCTION

Alternative sustainable energy resources are actively being explored due to the imminent exhaustion of fossil fuel reserves and global calls to address climate change. Countries have been crafting policies to decrease dependence on petroleum-based fuels and to lessen emissions of greenhouse gases. Biofuels, unlike fossil fuels, are sustainable and renewable [1]. Biodiesel, a first-generation biofuel is produced mostly from the conversion of plant oils into fatty acid methyl esters (FAME) through transesterification (or transmethylation). Oils from crops such as soy, rapeseed, or palm tend to have a high oleic acid content, which provides ideal ignition properties and oxidative stability [2,3]. Biodiesel has a similar combustion property to its traditional counterpart and is considered environmentally sustainable and eco-friendly, due to the reduced carbon dioxide emission and absence of sulfur and aromatic content associated with its production and use [4,5].

The Philippines has begun to explore domestically produced biodiesel to minimize dependency on imported petroleum and to promote energy security. The country has been using coconut methyl ester-blended biodiesel in compliance with Republic Act 9367 also known as Biofuels Act of 2006. However, the utilization of vegetable oils for biodiesel production compromises food security as it directly competes with the utilization of agricultural lands that would otherwise be dedicated to food production. Although the Philippines has targeted higher eventual biodiesel blends, the mandated blends have remained at B2 (2%) and have stalled in recent years [6]. Many countries have difficulty meeting their target blends due to several production bottlenecks in expanding production. Industrial-scale production cannot be realized without diverting the land intended for food production. This shift will be triggered by the high demand for biofuels, which could ultimately lead to high food commodity prices and deforestation [7]. Because of the massive demand in the food sector, large-scale biodiesel production using plant oils will be unsustainable in the long haul [8]. Thus, new bioenergy sources should be explored to promote sustainability.

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Microbial lipids or single-cell oils are now being explored as alternative feedstock for biodiesel production because they are quite similar to plant-based oils [9]. The use of microbial oils has numerous advantages: Short microbial life cycles; high productivities; low labor requirement; reduced dependency on site, season, and climate; amenability to genetic modifications for the specific products; and ease of scale-up [2,10,11]. These lipids can be obtained from oleaginous yeasts, bacteria, and microalga that are able to produce lipids more than 20% of their cell weight in the form of triacylglycerols (TAGs), which is the starting material for biodiesel production [12,13]. A number of these microbes can accumulate ~70–80% w/w lipids per dry cell weight under set cultivation conditions [14]. Among these groups of oleaginous microbes, oleaginous yeasts are perceived to be the most versatile because of their capability to utilize a wide range of substrates including agricultural residues and industrial wastes [15]. They can consume both hexoses and pentoses from various sources, along with non-sugar carbon substrates [16]. Lipid production starts when the yeast proliferates in a medium lacking key non-carbon nutrients such as nitrogen. Nitrogen-limited conditions prevent cell proliferation by inhibiting protein and nucleic acid synthesis, forcing the yeast to alternatively amass intracellular lipids which are then stored in cytoplasmic inclusion bodies in the form of triglycerides [2]. Hydrophilic substrates can be readily metabolized by oleaginous yeasts into lipids through the *de novo* lipid biosynthetic route whereas only a few strains can accumulate lipids from hydrophobic substrates through the *ex novo* route [Figure 1] [8].

Oleaginous yeasts have been discovered from different environments such as soil and plant parts [17]. Forest canopies are home to a diversity of yeast species, making them a suitable location for the discovery of novel microorganisms. For instance, the Mount Makiling Forest Reserve (MMFR) has a rich biological diversity consisting of endemic indigenous and introduced exotic species. In fact, a novel microorganism that produces extracellular lipase was isolated from a fern thriving in a tree canopy in the MMFR [18]. The rich yeast diversity in forest canopies increases the probability to isolate new yeast strains with high-value yeast oils. In this study, the MMFR canopies were explored to isolate and identify oleaginous yeasts which can be potentially used for biodiesel production using nitrogen-limited medium. Succeeding analyses were focused on the selection of the best oleaginous yeast in terms of biomass and lipid production, FAME profile and Fourier transform infrared spectroscopy (FTIR) analyses of the extracted and transmethylated lipids were carried out to establish the suitability of the strain as a biodiesel feedstock. The best-performing isolate was identified through molecular analyses.

2. MATERIALS AND METHODS

2.1. Sample Collection

Samples were collected along the two-hectare long-term ecological plot in the Molawin-Dampalit sub-watershed in MMFR. The forest reserve is geographically located in the southern part of Metro Manila, Luzon Island, Philippines at 14°08'14" N and 121°11'33" E [19]. Soil and various tree materials such as bark, canopy leaves, and epiphytic plants were collected from the identified dominant canopy trees that are distributed across the whole plot area. The set of trees included species of *Pterocarpus indicus* (Narra), *Meliosma pinnata* (Balilang Uak), *Parashorea malaanonan* (Bagtikan), *Ficus benjamina* (Salisi), *Ficus balete* (Balete), and *Syzygium decipiens* (Malaruhat Pula). Samples were aseptically collected and placed in sterile plastics, contained in a jar cooler for transport, and stored in ice until further processing.

After collection, samples were serially diluted in 0.85% sodium chloride solution and 10^{-3} to 10^{-5} dilutions were spread-plated on Dichloran Rose Bengal Chloramphenicol agar (per liter of distilled water: 10.0 glucose; 5.0 g peptone; 1.0 KH_2PO_4; 0.5 g $MgSO_4 \cdot 6H_2O$; 0.5 mL 5% w/v rose bengal solution; 1.0 mL 0.2% w/v dichloran in ethanol solution; 0.1 g chloramphenicol; and 15.0 g agar) [20]. Single unique colonies were selected and streaked onto yeast extract peptone dextrose (YEPD) agar (per liter of distilled water: 10.0 g yeast extract; 20.0 g peptone; 20.0 g glucose; and 16.0 agar) and incubated at 28°C for 24 h. Streaking for isolation was performed thrice to ensure the purity of the isolates.

2.2. Screening for Oleaginous Yeasts

The purified yeasts were grown on YEPD agar plates for 96 h at 30°C. Replica plates were made by transferring the colonies from the initial YEPDA to a ~90-mm round filter paper. The filter paper was

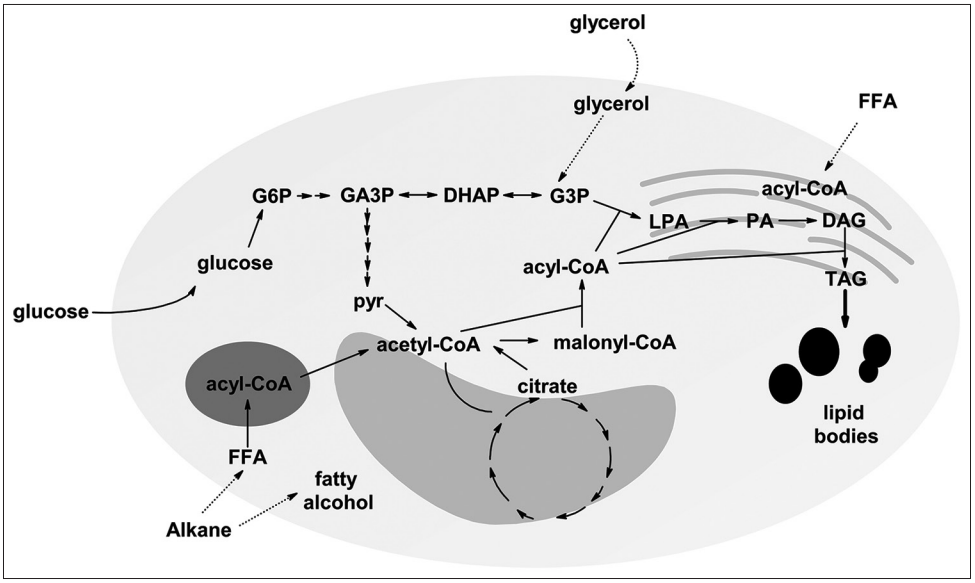

Figure 1: Lipid biosynthesis via the *de novo* and *ex novo* pathways. Dotted lines indicate conversion of the substrates through the *ex novo* route.

dried at 60°C for 15 min and then stained with 0.08% Sudan Black B (in 96% ethanol) with mild shaking (KS 260 Basic Orbital Shaker, IKA® Works, Selangor, Malaysia) for 20 min at room temperature. The filter was then washed 2x with ethanol for 5 min. Blue colonies were selected as potentially oleaginous yeasts [21,22].

2.3. Cultivation of Yeasts for Microbial Oil Production

Selected oleaginous yeasts were cultured in flasks with 30 mL of YEPD broth. Cultures were incubated at 150 rpm at 30°C for 24 h in a Thomas Shaking Incubator (Thomas Kagaku Co., Japan). A volume of the seed culture equivalent to 1.0 absorbance unit at 600 nm was harvested by centrifugation at 2000 × g for 15 min (CD-50SR, Tomy Seiko, Japan). The resulting cell pellet was resuspended in 5 mL of nitrogen-limited medium (per liter: 2.0 g $(NH_4)_2SO_4$; 7.0 g KH_2PO_4; 2.0 g NaH_2PO_4; 1.50 g $MgSO_4 \bullet 6H_2O$; and 1.0 g yeast extract) either with glucose or glycerol as C source (40 g/L) and finally transferred to 45 mL of the same medium [14,23]. The flasks were incubated in a rotary shaker at 30°C for 120 h at 150 rpm agitation. 10-mL samples were then taken to determine biomass and lipid concentrations.

To measure biomass concentration, samples were centrifuged at 10,000 × *g* for 5 min. The resulting cell pellets were washed twice with distilled water and resuspended in water. Cell suspensions were dried at 105°C to constant weight.

2.4. Lipid Extraction

Samples were centrifuged for 5 min at 10,000 × *g* and washed 2× with distilled H_2O. The pellets were resuspended in 2 mL of 4 M hydrochloric acid and incubated for 2 h at 60°C with mild shaking to hydrolyze the biomass. The acid-hydrolyzed masses were transferred to 20 mL of chloroform-methanol (1:1 v/v) mixture and mildly agitated at RT for 3 h. The acid mixtures were centrifuged at 2000 × *g* for 5 min to separate the organic and aqueous layers [24]. The lower organic layers containing the lipids were recovered using a Pasteur pipette and evaporated in a low-temperature vacuum oven and dried at 70°C and 12 psi until constant weight. All solvents used for lipid extraction, including hydrochloric acid (RCI Labscan Limited, Thailand), chloroform (RCI Labscan Limited, Thailand), and methanol (J.T. Baker®, Mumbai, India) were prepared from analytical grade reagents. All experiments were carried out in triplicates. The total lipid was expressed as the amount of lipids (g/L) extracted from yeast biomass whereas lipid content ($Y_{L/X}$) was expressed as the total lipids per dry biomass. The lipid content was calculated using Equation 1 wherein X_i and L_i are the final dry cell weight and lipid concentration, respectively. X_0 and L_0 are the initial dry cell weight and lipid concentration, respectively.

$$Y_{\frac{L}{X}} = \frac{L_i - L_0}{X_i - X_0} \quad (1)$$

The one-way analysis of variance module of IBM® SPSS® Statistics 25 (IBM Corp. Armonk, New York) was used for statistical analysis. Tukey's honestly significant difference (HSD) test for *post hoc* comparison was applied to find means that are significantly different from each other (*P* o find; followed by the compact letter display (CLD) method to identify variables that have statistically different means from the ones that do not have statistically different means. Variables were labeled with letters and were only considered as distinguishable if and only if they do not share at least one letter.

2.5. Lipid Transmethylation

The oleaginous strains which exhibited maximum lipid accumulation were selected for transmethylation and fatty acid profile analysis. Fatty acids were converted to FAME according to the AOAC Official Method 969.33 [25]. Briefly, 5 mL 1.0 N methanolic sodium hydroxide and 5 mL methanol were added to 0.5 g of the extracted lipid sample. The mixture was refluxed for 10 min. 10 mL of 14% boron trifluoride in methanol (Sigma-Aldrich, Sigma-Aldrich Pte Ltd, Singapore) was added to the mixture and refluxed again for 2 min. About 5 mL of n-heptane (Mallinckrodt, Ireland) was then added, and the mixture was refluxed further for 1 min. After heating, the mixture was allowed to cool for a few minutes. 15 mL of saturated NaCl solution was added to the mixture and shaken vigorously. After standing, the upper heptane layer was collected and added with 0.36 g anhydrous sodium sulfate to remove any excess moisture. The resulting product was subjected to gas chromatography.

2.6. Lipid Characterization

Qualitative and quantitative compositions of the fatty acids were analyzed using a Shimadzu GC-14B gas chromatograph (Shimadzu Corporation, Japan) equipped with a flame ionization detector and an Ulbon HR-SS-10 capillary column (50 m × 0.25 mm) (Shinwa Chemical Industries Ltd., Japan). 1 µL sample was injected into the machine and was carried by ultra-high purity N_2 at a constant flow of 40 mL/min. Detector and injector temperatures were set at 260°C. The temperature program used for the separation of FAME is as: 160–220°C ramped at 1.5°C/min and held for 10 min once 220°C was reached. The retention times and peak areas of the samples were compared with FAME standards (Supelco 37 FAME Mix, Sigma-Aldrich Pte Ltd, Singapore). The FTIR spectra between the microbial oils before and after transmethylation were recorded on a Shimadzu IR Prestige-21 (Japan) using the standard attenuated total reflection method.

2.7. Morphological and Phylogenetic Characterization of Oleaginous Yeast Strain

The morphological attributes of the selected yeast strain were observed. Cell shape, dimension, and reproductive features of the strain grown in a liquid YM medium were also observed microscopically (Supplementary Method).

Yeast strains were identified by sequencing the 5.8S-ITS region [26]. Isolates cultured on YEPD medium for 24 h at 30 °C were subjected to DNA extraction using Quick DNA™ Fungal/Bacterial DNA Extraction Kit following the manufacturer's specifications (Zymo Research, California). The fungus-specific universal primers ITS-1 (5′→3': TCCGTAGGTGAACCT GCGG) and ITS-4 (5'→3': TCCTCCGCTTATTGATATGC) were used to amplify the 5.8S-ITS region by polymerase chain reaction. The PCR mixture contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1.0 µM each of ITS-1 and ITS-4, 1.25 U Taq pol (Invitrogen), 200 ng DNA template, and to 50 µL, nuclease-free water. The PCR steps were as follows: (1) 95°C for 3 min; (2) 94°C for 1 min; (3) 60°C for 1 min; (4) 72°C for 2 min; (5) 72°C for 5 min; and (6) 4°C. Steps 2–4 were repeated for 30 cycles [27]. The amplicons were sent for sequencing to Macrogen (South Korea). The basic local alignment search tool of the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for related sequences and to identify the yeast isolates. A phylogram was generated using the QIAGEN CLC Sequence Viewer ver. 8.0 (https://digitalinsights.

qiagen.com/). The ITS-containing region of BUB8 was deposited to NCBI with accession number OP808033.1.

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Oleaginous Yeasts

A total of 258 colonies that appeared morphologically to be yeasts were isolated from the epiphytic ferns, moss, roots, orchids, bark, and leaves of several forest trees in the MMFR. Twenty-two colonies that were blue after staining with Sudan Black B were selected for further studies. The presence of lipids is usually demonstrated using fat stains such as Sudan Black B, Sudan III, or Nile red staining [28,29]. This staining technique, in combination with replica plating, serves as a qualitative screening method to select potential oleaginous yeasts isolated from different sources such as sludge, soil, and other environmental samples [30]. The putative oleaginous yeast isolates from this study were obtained from Narra, Bagtikan, and Malaruhat Pula trees. Oleaginous yeasts have been obtained from various natural habitats such as soils, plant and flower surfaces, mangroves, bark-beetles, tree exudates, and water, among others [31,32].

3.2. Biomass and Lipid Production of Selected Yeast Isolates

Biomass production, lipid accumulation, and lipid contents of the isolates were evaluated in a nitrogen-limited medium supplemented with glucose [Figure 2]. A nitrogen-limited medium was used since lipid accumulation is induced by nitrogen exhaustion. To be considered oleaginous, lipid content must be more than 20% of cell biomass [33]. BTB8, MPB8, MPB7, NFR2, and NF15 are not oleaginous yeasts due to their low lipid content (10.83 ± 0.52, 11.56 ± 0.70, 17.07 ± 6.32, 17.46 ± 2.16, and 19.52 ± 3.94%, respectively, all of which are statistically indistinguishable since they share the same CLD *a*). NFR9 was the best lipid-yielding strain (55.10 ± 1.70%) however, its biomass accumulation was low (1.68 ± 0.07 g/L). Ideally, oleaginous yeasts should be able to grow at high densities since lipids are extracted from the cells [34]. Among the oleaginous yeast isolates, NFR6 and BUB8 accumulated the most biomass (10.09 ± 0.12 and 8.87 ± 0.17 g/l, respectively) and had good lipid content. The two strains were thus selected for the succeeding study based on Tukey's test and CLD.

The biomass and lipid production of NFR6 and BUB8 were evaluated in the same medium but with glycerol as substrate [Figure 3]. BUB8 had significantly higher biomass (8.08 ± 0.19 g/l) and lipid (2.91 ± 0.12 g/l) production, with a lipid content of 36.03 ± 0.75%. The cultivation studies demonstrated that BUB8 is capable of both *de novo* and *ex novo* lipid accumulation. This is a remarkable attribute since it suggests that the isolated strain can utilize various materials including wastes of agricultural and industrial origins. Other microbes capable of utilizing both pathways include *Yarrowia*, *Cryptococcus*, *Rhodospiridium*, *Geotrichum*, and *Trichosporon* [35,36]. The utilization of glycerol as substrate will encourage circular bioeconomy for biodiesel processing. Crude glycerol is the major waste product of biodiesel processing using plant oils and biomass-based substrates with at least 10% for every batch of biodiesel [37]. This by-product has been used as an alternative feedstock for single-cell oil production by the oleaginous yeasts, *Yarrowia lipolytica*, *Cryptococcus curvatus*, *Trichosporonoides spathulate*, and *Candida viswanathii* [38-41]. Crude glycerol mixed with hemicellulosic hydrolysate was also evaluated for lipid production by *R. toruloides* [42].

Most oleaginous yeasts can utilize various sugar carbon sources via the *de novo* lipid biosynthesis pathway but only a few strains can accumulate lipids through the *ex novo* route [8]. There are major differences between the two pathways in terms of biochemical mechanisms. In the *de novo* pathway, nitrogen depletion in the culture medium causes cellular AMP to rapidly decrease. This leads to the accumulation of citric acid, which is then cleaved by ATP-citrate lyase to acetyl-COA. Acetyl-CoA is further converted to TAGs. It should be noted that ATP-citrate lyase is unique to oleaginous yeasts [43,44]. The *de novo* pathway is a non-growth-associated process. The *ex novo* pathway, on the other hand, is growth-associated and is entirely independent of nitrogen levels [9,14]. Hydrophobic substrates such as fatty acids, oils, and TAGs from the culture medium would result to the unmodified form of the lipids [44].

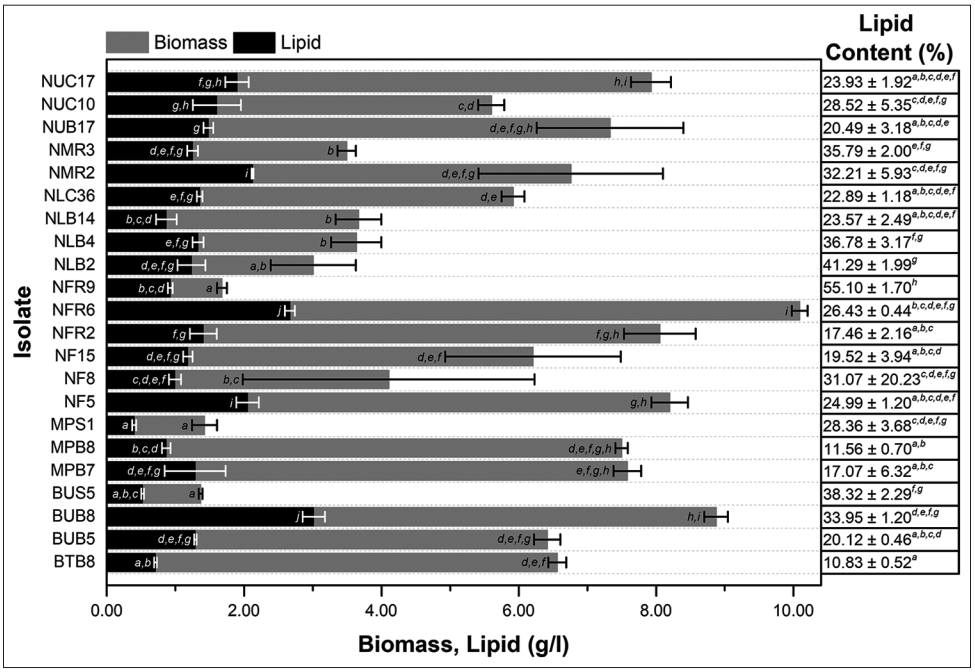

Figure 2: Biomass and lipid production and lipid content of the isolates. Values that share the same italicized letters are not statistically different.

3.3. Characterization of Microbial Oil

The lipid content and fatty acid profiles differ among species; however, the main fatty acids made by oleaginous yeast are acknowledged to be similar to those of vegetable oils [33,45-47]. They consist of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and α-linolenic acid (C18:3). Notably, this is the oil composition (and the lipid classes of C16 to C18) that has been prescribed for biodiesel production [48,49]. In particular, the typical relative composition of lipids in oleaginous yeasts consists of oleic acid > palmitic acid > linoleic acid = stearic acid [50]. The fatty acid profile of strain BUB8 exhibited oleic acid as the most abundant fatty acid present, followed by palmitic acid, linoleic acid, and stearic acid [Table 1]. This profile closely resembles the TAGs produced from glycerol by *R. toruloides* [51].

Table 1: Fatty acid methyl ester profile of transmethylated lipid extracted from BUB8.

Number of carbon atoms	Common name	Composition (% w/w)
C12	Lauric acid	0.07±0.01
C14	Myristic acid	1.23±0.01
C15	Pentadecylic acid	0.06±0.00
C16:0	Palmitic acid	23.54±0.90
C16:1	Palmitoleic acid	1.29±0.04
C17:0	Margaric acid	0.14±0.01
C17:1	Heptadecenoic acid	0.39±0.02
C18:0	Stearic acid	4.68±0.10
C18:1	Oleic acid	52.59±0.37
C18:2	Linoleic acid	12.33±0.06
C20:0	Arachidic acid	1.04±0.01
C20:1	Paullinic acid	0.23±0.01
C20:2	Eicosadienoic acid	0.23±0.01
C20:3	Mead acid	0.39±0.01
C20:4	Arachidonic acid	0.56±0.01
C22	Behenic acid	0.46±0.01
C24	Lignoceric acid	0.83±0.01

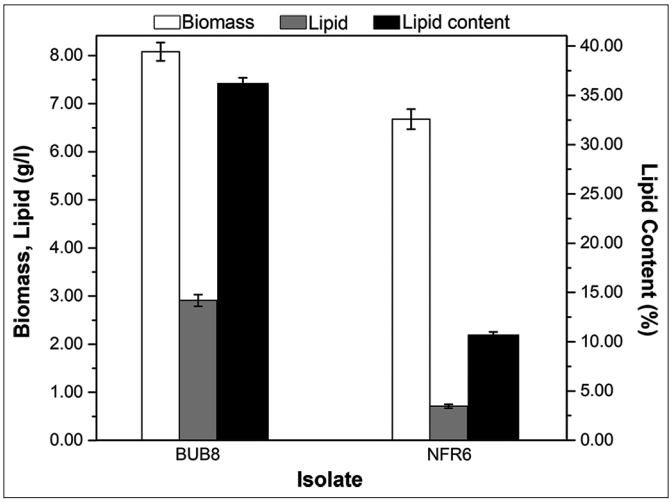

Figure 3: Biomass and lipid production and lipid content with glycerol as sole carbon source.

The transmethylation of the fatty acids was further confirmed by ATR-FTIR. The FTIR spectra (Appendix B) of pure and transmethylated microbial oil extracted from BUB8 showed the following peaks at 2850 and 2920 cm^{-1} corresponding to the symmetric and asymmetric stretching around the –CH of methylene groups; 1740 cm^{-1} (stretching of the ester carbonyl group); 1160 cm^{-1} (ester stretching (–CO)); and 720 cm^{-1} (unsaturated alkene) [52,53]. A band is present at 1430 cm^{-1} in the FAME spectra, which are attributed to asymmetric $–CH_3$ bending vibration. The stretching of $O=C–CH_3$ bonds, represented by peaks at around 1190 cm^{-1}, typical in FAME but not in microbial oils, was also observed [53]. Only subtle differences can be observed between the spectra since the FAME sample is chemically similar to its precursor.

The fatty acid composition is a major determinant of the quality of biodiesel [54,55]. Fuel properties such as cetane number, heat of combustion, kinematic viscosity, melting point, and oxidative stability are assessed through fatty acid compositional analysis [56-58]. The presence of high levels of monounsaturated fatty acids is desirable for a biodiesel substrate because of the favorable balance they conferred on these parameters [5,59]. In assessing biodiesel feedstocks, a mixture of high amounts of monounsaturated fatty acids and a balanced level of saturated and polyunsaturated fatty acids is preferable for biodiesel production [60]. Studies have demonstrated that biodiesel production from oleic acid has the property ideal to be a diesel substitute and thus BUB8 would be a good contender for biodiesel production [61,62].

3.4. Characterization and Molecular Identification of BUB8

Due to its significant lipid productivities both in glucose and glycerol and FAME and FTIR profiles, BUB8 was considered for further identification and characterization. The colonies of BUB8 are pink in color, circular, smooth, shiny, opaque, convex with an entire margin, and measuring 1.0–2.0 mm in diameter. SEM imaging showed that BUB8 cells appear to be ellipsoidal to subcylindrical [Figure 4]. The cells are arranged in singles, pairs, and clusters, exhibiting unipolar budding. Furthermore, the cells measure 3.0–5.0 × 2.0–3.0 µm (length × width) [Figure 4].

ITS-PCR is widely used for the rapid identification and taxonomic study of yeasts from various sources [27,63-65]. To identify strain BUB8, the 5.8S-ITS rDNA was amplified, sequenced, and compared against available sequences. Phylogenetic analysis showed that BUB8 shares 99% identity with *Rhodotorula paludigena* [Figure 5]. The evolutionary tree showed the relationship of *Rhodotorula* spp. with related genera such as *Sporidiobolus* and *Sporobolomyces*, all of which belong to the Order Sporidiobolales, subphylum Pucciniomycotina. These genera are referred to as the "red yeasts," producing pigmented colonies [66,67]. *Rhodosporidiobolus* is more closely related to *Rhodotorula* than to *Sporobolomyces* as shown

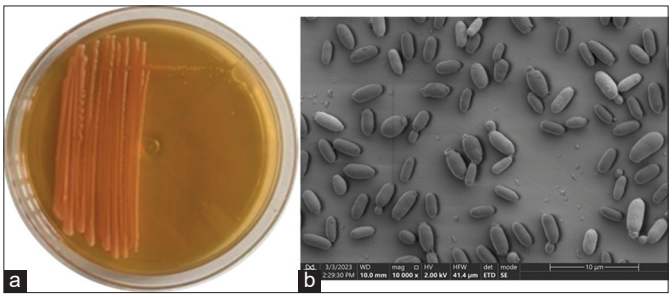

Figure 4: Appearance of BUB8 cells in petri plate (a) and under SEM (b).

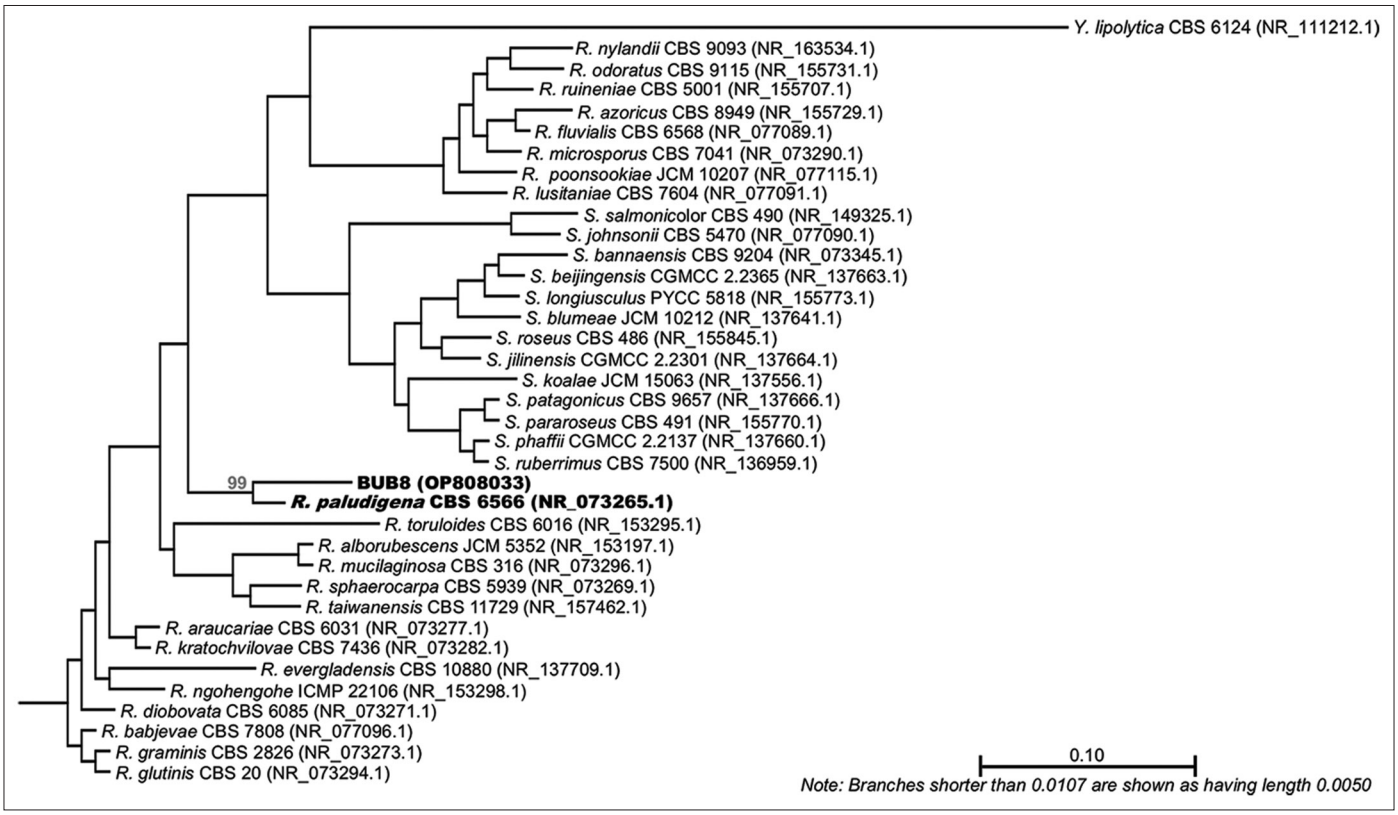

Figure 5: Phylogeny of BUB8. The phylogram was constructed using the neighbor-joining method. GenBank accession numbers for the ITS regions are shown in brackets. The evolutionary distances were computed using the Kimura 2-parameter method. Bar, 0.110 substitutions per nucleotide position. Distance calculations were done based on 1000 replicates.

in the tree [68]. *Rhodotorula* species are lipid-accumulating yeasts that have been considered to produce microbial oils as biodiesel feedstock [69-72].

R. paludigena was only characterized as oleaginous in recent years, and thus, few publications on its lipid productivity were available [58]. In a recent study, a novel deep-sea strain, *R. paludigena* P4R5, can produce high levels of intracellular lipids (16.9 g/L) and extracellular mixture (48.5 g/L) of mannitol esters of 3-OH C14, C16 and C18 fatty acids simultaneously [73]. The intracellular lipids were composed mostly of C16 and C18 fatty acids, which could serve as substrates for biodiesel production. Likewise, the lipid production potential of another *R. paludigena* strain (CM33) was also evaluated using molasses and crude glycerol found to be suitable substrates for the microorganism [74]. When grown in molasses, it produced remarkable biomass (16.5 g/L) and lipid (6.1 g/L) concentrations with 37% lipid content. These results, together with the information provided by this study, highlight the potential of *R. paludigena* of contributing to a high-value carbon chain through the transformation of various substrates into lipids for biodiesel production.

4. CONCLUSION

Microbial oils are explored as substitute feedstock for the synthesis of biodiesel because they are comparable to vegetable oils in terms of fatty acid profiles. Oleaginous yeasts are promising microorganisms characterized by their significant intracellular accumulation of fatty acid in the form of triglycerides. The selection of potential oleaginous yeasts isolated from the MMFR was based on primary screening using Sudan Black stain, biomass, and lipid productivities. This study has identified a promising oleaginous strain isolated from the upper bark of a Bagtikan tree. It has presented both *de novo* and *ex novo* lipid biosynthesis, as it exhibited high lipid production using glucose and glycerol, respectively as substrates. FAME and FT-IR analyses have shown the presence of fatty acids, the most abundant of which is oleic acid, that are applicable for biodiesel production. Molecular identification of BUB8 using ITS-rDNA rDNA sequencing revealed 99% sequence homology with *R. paludigena.* This oleaginous yeast could be used for further optimization to harness its potential for biodiesel production and make it a better alternative to petroleum-based diesel. Likewise, this strain could kickstart developments of local microbial-based technology for sustainable biodiesel production in the Philippines to augment the production of coco-methyl ester biodiesel.

5. ACKNOWLEDGMENT

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6. AUTHOR CONTRIBUTIONS

IG Pajares and PJ Requiso performed microbiology, fermentation, and molecular biology experiments whereas LM Fabro conducted the chemical analyses. IG Pajares and KM Ramos prepared the

manuscript. AK Raymundo is the first author's dissertation adviser. All authors have seen and approved the manuscript and all its contents.

7. FUNDING

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data obtained in the study are represented as table or figures available in the main text and appendices.

11. PUBLISHER'S NOTE

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