Legodi and Moganedi *Annals of Microbiology* (2023) 73:21
https://doi.org/10.1186/s13213-023-01725-8

Annals of Microbiology

ORIGINAL ARTICLE

Open Access

Selection of oleaginous yeasts isolated from sugarcane bagasse as the potential producers of single cell oils and other metabolites

Lesetja M. Legodi[1*] and Kgabo L. M. Moganedi[1]

Abstract

Purpose The microorganisms that possess diverse and improved traits for biotechnological applications provide an opportunity to address some of the current industrial challenges such as the sustainability of fuel energy and food. The aim of this study was to isolate and select the potential yeast isolates from sugarcane bagasse for single oil production.

Methods The oleaginocity of the yeasts was confirmed through a gravimetric analysis of lipids, Nile red, sulfo-phosphovanillin (SPV) and a gas chromatography method for fatty acid methyl esters (FAME). The identification of the selected yeasts was carried out through a 5.8S of the ribosomal internal transcribed spacer (ITS) and 26S ribosomal DNA (rDNA) sequences. The carbohydrate and protein contents were estimated using phenol-sulfuric acid and hot trichloroacetic acid method, respectively.

Results The yeast isolates accumulated lipids of between 28% and 67% of the dry cell weight, and 22–33% based on the SPV assay, qualifying them as oleaginous yeasts. The selected yeasts were identified as *Candida ethanolica* and *Pichia manshuriica*. The lipids contained high levels of fatty acids in the following order: oleic acid, palmitic acid, stearic acid, and linoleic acid, which made up 82% of the total lipids.

Conclusion All the six selected yeast strains have the potential to produce single cell oils and other metabolites. The fatty acids composition of the selected yeast species was found to be suitable for the applications in biofuel, nutraceutical, and food industry. The yeast strains require evaluation in lignocellulosic hydrolysate for the production of single cell oils to be cost effective.

Keywords Screening, Oleaginous yeast, *Pichia manshurica*, *Candida ethanolica*, Sinlge cel oil

Introduction

There is a global consensus about the need for sustainable and yet renewable energy to substitute and minimize the dependence on fossil fuels and the need to supply polyunsaturated fatty acids (PUFA) for food supplement. Apart from the environmental pollution caused by fossil fuels, its reserves over time will be depleted, thus making it unsustainable. The alternative fuel such as biodiesel is synthesized from oils derived from edible plants. The continuous usage of food crops for fuel has sparked public debate and concerns about future food security (El Kantar et al. 2021). These concerns resulted in the need to find alternative sources of oil. Microbial oils or single

*Correspondence:
Lesetja M. Legodi
lesetja.legodi@ul.ac.za
[1] University of Limpopo, Private Bag X 1106, Sovenga 0727, South Africa

cell oils (SCOs) can provide a sustainable supply of oil for biodiesel and other valuable products such as PUFA-rich lipids (Diwan et al. 2018; Gorte et al. 2020). The production of yeast-derived SCOs for renewable oleo-chemicals is at the preliminary stages and has attracted enormous interest for a diversified application as alternatives to edible and other non-edible oleo-chemical commodities (Bandhu et al. 2018). Single cell oils are produced by many oleaginous microorganisms, including filamentous fungi, microalgae, bacteria and yeasts (Matsakas et al. 2015). These are intracellular stored lipids comprising mainly triacylglycerols (TAGs). Microorganisms that are capable of accumulating between 20% and 80% lipid per dry biomass are called oleaginous microorganisms (Ratledge and Wynn 2002; Donot et al. 2014; Lamers et al. 2016; Ochsenreither et al. 2016; Galán et al. 2019).

High lipid content is required to decrease the processing cost per unit biomass products, just as a high growth rate is required to increase the yield (Shokrari et al. 2020). Intracellular lipid accumulation in oleaginous microorganisms is triggered by the depletion of essential nutrients, particularly nitrogen and phosphorus in the excess of carbon. Under such conditions, the cellular functions like nucleic acid and protein synthesis, eventually culminating in the cessation of cell growth. The excess carbon is metabolized into lipids through a cascade of biochemical reactions (Diwan et al. 2018). The accumulated intracellular lipids showing as lipid bodies were detected by using fluorescent Nile red dye (Younes et al. 2020). Oleaginous yeasts are a good candidate for SCOs production, mainly due to their ability to grow to high cell densities in a short time and their diverse metabolism (Lamers et al. 2016). The known oleaginous yeasts include *Cryptococcus* sp., *Yarrowia* sp., *Candida* sp., *Rhodotorula* sp., *Rhodosporidium* sp., *Trichosporon porosum* and *Lipomyces* sp. (Zhang et al. 2011; Schulze et al. 2014; Bardhan et al. 2020; Sagia et al. 2020; Caporusso et al. 2021; Chattopadhyay and Maiti 2021) and other unconventional yeasts such as *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Kazachstania unispora*, and *Zygotorulaspora florentina* (Gientka et al. 2017). The lipid content and fatty acid composition differs from one yeast species to the other.

Yeasts are attractive for SCOs and biomass production because of their ability to utilise a variety of carbon sources under limited nutrients, particularly nitrogen. These carbon sources include glucose, xylose, glycerol, starch, cellulose, and hemicellulose hydrolysate and industrial and municipal organic waste (Thevenieau and Nicaud 2013). Many of the known yeast species are meeting the requirements for the large-scale production of SCOs and are amenable to genetic manipulation. However, their drawback is the failure to achieve a yield that is close to theoretical lipid yields. Theoretical yields are influenced by the substrate used, with the yields of 0.32 g/g for glucose, 0.34 g/g for xylose (Ratledge and Wynn 2002; Donot et al. 2014; Wang et al. 2020), and 0.30 g/g for glycerol (Wang et al. 2020). This limitation encourages the continuing search for new yeasts, screening and identification of those alternative potential oleaginous yeasts with improved traits such as diverse carbon assimilation, growth in the presence of inhibitors, and tolerance to osmotic pressure (Sreeharsha and Mohan 2020).

In an effort to maximize the economic viability of SCOs production by the oleaginous microorganisms, the choice of strategy must consider the benefits of other value-added products that are co-produced in the process (Sreeharsha and Mohan 2020; Parsons et al. 2020). These products include proteins, amino acids, carbohydrates, carotenoids as well as alcohols and other oleochemicals (Parsons et al. 2020). In addition to SCOs, this study was limited to single cell protein (SCP) and carbohydrates. The use of microorganisms, mainly yeast, fungi, bacteria, and microalgae as food and feed with high SCP content is increasingly gaining research and commercial interests (Ratledge and Wynn 2002; Ritala et al. 2017; Nyyssölä et al. 2022). Yeast protein is an attractive alternative nutrient to traditional protein sources such as plants and meat; in addition, yeast protein biomass also contains trace minerals and vitamin B-group (Saed et al. 2020; Jach et al. 2022). Yeast and fungi have 50% to 55% of SCP content and contain a high protein-carbohydrate ratio, with more lysine and less methionine and cysteine (Chama 2019).

Carbohydrate in the yeast cells is stored in the form glycogen and trehalose. An accumulation of glycogen and trehalose in nutrient-limited cultures of Saccharomyces cerevisiae has a negative correlation with the specific growth rate. Thus, growth limitations by nutrients other than glucose are often resulting in a high-level accumulation of storage carbohydrates (Hazelwood et al. 2009). The variations in the content of the glycogen and trehalose within the yeast cell in response to environmental changes indicate that their metabolism is controlled by complex regulatory systems (François and Parrou 2001; Chen and Futcher 2017). The roles of glycogen and trehalose in controlling glycolytic flux, stress response, and energy stores for the building process, confer a survival and reproductive advantage to the yeast cell (François and Parrou, 2001).

Sugarcane bagasse pith is reported to be a good source of microorganisms (Martinez-Hernandez et al. 2018; Gebbie et al. 2020). To date, about 8.2% of the total number of known yeasts is made up of oleaginous yeast species, and a majority of such species are isolated from

their native habitats (Poontawee et al. 2023). In this study, yeast species were isolated from sugarcane bagasse and screened for their ability to accumulate lipids in glucose medium as a sole carbon source. The yeast species were identified as strains of *Candida* and *Pichia* species. The study further analyzed fatty acid methyl ester (FAME) and determined the carbohydrates and proteins from the de-fatted yeast biomass.

Materials and methods

Chemical and reagents

The chloroform, methanol, hexane, hydrochloric acid, sulfuric acid, Follin-Ciocalteu Phenol, and Trichloroacetic acid were obtained from Sigma-Aldrich, Germany.

Sample collection

Sugarcane bagasse (SCB) was collected in a plastic bag from a local sugar mill in Durban, KwaZulu-Natal, South Africa, and transported to the laboratory for storage at 4–8 °C.

Isolation of microorganisms

Microbial propagation

Three grams (g) of SCB were soaked in a 100-mL Scott bottle containing 100 mL of sterile distilled water for 1 h making microbial suspension. One thousand microliters of the suspension was added into 250 mL Erlenmeyer flask containing 100 mL of YPD (1% yeast extract, 2% peptone, 2% glucose) medium and Chloramphenicol (final concentration 100 µg/mL), (Ho et al. 2000) was added to inhibit bacterial growth and 0.025% (w/v) Na-propionate to suppress fungal growth (Brock and Buckel 2004; Gowda et al. 2004; Jiru et al. 2016). The culture was incubated at 30 °C, 150 rpm (Biobase Incubator, BJPX-1102C) for 24 h.

Microbial enrichment

A 10 mL of culture propagated in YPD medium contained in 15 mL conical tube was centrifuged at 2250×*g* using a bench top HERMLE centrifuge (Model Z326K, Rotor 221.19V20, Germany) for 5 min and discarded the supernatant. The pellet was re-suspended in 10 mL sterile water and used to inoculate 100 mL of nitrogen-limited medium by Ghanavati et al. (2014), with modification in a 250-mL Erlenmeyer flask. The medium composition was as follows: 40 g/L glucose, 5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L Na_2HPO_4, 0.25 g/L Na-propionate, 100 µg/mL Chloramphenicol and the pH was adjusted to 5.5. The culture was incubated at 30 °C, shaking at 150 rpm for 48 h.

Isolation of yeasts on the agar medium

The composition of the agar medium with carbon-to-nitrogen (C:N) ratio of 13 was as follows: 20 g/L glucose or xylose, 3 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L Na_2HPO_4, 0.25 g/L Na-propionate, 1 g/L yeast extract, 100 µg/mL Chloramphenicol and the pH was adjusted to 5.5. One milliliter of the enriched culture was diluted up to 10^{-6} and 100 µL of the diluted culture was spread plated onto the glucose and xylose agar. The agar plates were incubated at 30 °C, static conditions (Biobase Incubator, BJPX-1102C) for 72 h on the agar containing glucose and the 120 h agar containing xylose. Larger colonies showing different morphologies obtained from the glucose and xylose agar media were further streaked onto either the glucose agar or the xylose agar for two passages to obtain pure yeast isolates. The isolates were suspended in a sterile glycerol-enriched solution containing the following in (w/v); 2% glucose, 1% yeast extract, 1% bacto-peptone and 40% (v/v) glycerol. The yeast suspensions were stored at – 80 °C until further characterization.

Primary screening of oleaginous yeasts for single cell oils production

The following medium composition: 30 g/L glucose or xylose, 2.5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 1 g/L Na2HPO4, 0.25 g/L, 1 g/L yeast extract and the pH adjusted to 5.5 with C:N ratio of 23 was used for the production of lipid. A total of 14 isolates were screened for their ability to accumulate lipid. A single colony from a revived culture for each isolate was inoculated into 30 mL medium contained in a 100 Erlenmeyer flask. The culture was incubated at 30 °C, shaking at 150 rpm for 72 h. After cultivation, the isolates were denoted as follows: B1, B2, C2, E1, E2, as obtained from the isolating agar medium with glucose as carbon source whereas the isolates 5-XP, 11-XP, 15-X, 6-XP, 13-X, 10-XP, 3-XP, 12-X, and 4-X xylose were the carbon source. The primary screening was effected by Nile Red dye (NR–9-diethylamino-5H-benzo [α] phenoxazin-5-one) staining, a qualitative. A Nile Red dissolved in dimethyl sulfoxide (DMSO, stock solution 0.1 mg/mL) was used as staining agent to visualize lipid bodies within the yeast. One milliliter of yeast culture harvested after 72 h was centrifuged at 7378×*g* using a bench top refrigerated microfuge SIGMA 1-14K, (Angle rotor 24 × 1.5/2 mL) for 5 min and the supernatant was discarded. The pellets of the yeasts were washed with PBS 1 X, pH 7.4 and re-suspended in the same buffer. Cell density was adjusted to OD_{600} nm ~ 1. For fluorescence microscopy, 250 µL of yeast culture was mixed with 25 µL of DMSO. A solution of NR was added to the above mixture to a final

concentration of 5 µg/mL (Priyanka et al. 2020), and incubated in the dark at room temperature for 10 min. The lipid bodies within yeast cells were visualized using a fluorescence Microscopy equipped with a Zeiss filter set 09 (Carl Zeiss, 980714, Germany), 450–490 nm excitation, 515 nm emission and 510 nm beam splitter. The presence of lipids was observed under × 100 objective lens, which enabled the visualization of stained lipid bodies. Six promising isolates were selected based on the intensity of lipid bodies for identification and further evaluation.

Molecular identification of yeast isolates

The identification of the selected yeasts was performed by determining the ribosomal internal transcribed spacer (ITS) and 26S ribosomal DNA (rDNA) sequences. Genomic DNA was extracted using the Quick-DNA™ fungal/bacterial miniprep kit (Zymo Research, catalogue no. D6005). The targeted regions were amplified using oneTaq® 2X master mix (NEB, catalogue no. M0486). The following sets of the primers, ITS-1 (5′-TCCGTA GGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCT TATTGATATGC-3′) were used to amplify the small subunit and large subunit of the 5.8S (White et al. 1990). A primer set NL-1 (5′-GCATATCAATAAGCGGAGGAA AAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACG G-3′) were used to amplify the D1/D2 domain of the large subunit 26S ribosomal DNA, rDNA (Kurtzman and Robnett 2003). The components of the PCR mix were as follows; 10 µL NEB oneTaq 2X Master mix with standard buffer, 10–30 ng/µL Genomic DNA, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM) and 7 µL nuclease free water (catalogue no E476). The following PCR conditions were used: 35 cycles including an initial denaturation step at 94 °C for 5 min. A subsequent denaturation was at 94 °C, 30 s, annealing at 50 °C for 30 s, and extension at 68 °C for 1 min. A final extension at 68 °C for 10 min was followed by holding at 4 °C.

The PCR amplicons were analyzed on a 1% agarose gel and extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, catalogue no. D4001). The extracted fragments were sequenced in the forward and reverse direction using Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000. The sequenced fragments were purified by using Zymo Research, ZR-96 DNA Sequencing clean-up Kit™ (catalogue no D4050). The purified fragments were analyzed on the ABI 3500XL Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for every sample. A CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500XL Genetic Analyzer and the results were obtained by a BLAST (BLASTN 2.11.0+) search on NCBI following procedure by Altschul et al. (1997).

Secondary screening and determination of lipid content in oleaginous yeasts

The medium composition (section 2.4) with minor adjustment for sugar (40 g/L glucose) and nitrogen (5 g/L and 2.5 g/L $(NH_4)_2SO_4$, with C:N ratio of 15 and 30, respectively) was used for screening yeasts' ability to accumulate lipid. The following isolates B2, C2, E2, 6 XP, 11 XP, and 15 X xylose were selected based on NR fluorescence results. A single colony from a revived culture for each isolate was inoculated into 100 mL medium contained in 250 Erlenmeyer flask. The culture was incubated at 30 °C, shaking at 150 rpm for 7 days. Samples (2 mL) were withdrawn every 24 h to monitor growth by the optical density, OD 600nm using spectrophotometer, (Jenway 7315 Spectrophotometry, UK). Yeast samples were harvested at the end of the cultivation period to determine biomass and the lipid content gravimetrically (Anschau et al. 2017). The lipid content was validated by the Sulfo-phosphovanillin assay.

Gravimetric determination of yeast dry biomass and lipid content

A 45-mL culture for each oleaginous yeast contained in conical tubes was centrifuged at 2250×*g*, 4 °C for 10 min using the bench top HERMLE centrifuge (Model Z326K, Rotor 221.12V20, Germany) and supernatants were discarded. The pellets were washed twice with 15 mL of BPS 1 X, pH 7.4 and dried at 60 °C using a hot air oven (Scientific, 240 L Digital incubator, Model 298, S.A) for 24 h (Pan et al. 2009; Ramirez-Castrillón et al. 2017; Bardhan et al. 2020; Younes et al. 2020). The dried yeast biomass weighed was determined by using a weighing balance (RADWAG, Model AS 220. R2, Poland). The biomass was recorded by subtracting the pre-weighed tubes from the sample tubes weight after drying.

Lipid extraction was done by applying the Acid-Heat Extraction method (Yu et al. 2015). Dried yeast biomass samples were dissolved in 3.5 mL of 4 M HCl contained in 15 mL conical tubes and incubated in a water bath with the temperature set at 95 °C for 2 h. After the incubation, the tubes were cooled to room temperature and centrifuged at 2250×*g* for 10 min. Lipid extraction was done according to Folch's method. A supernatant was decanted into a clean tube and a mixture of chloroform: methanol (2:1 v/v) was added to the solid material (pellets) to final volume of 10 mL. The tubes were shaken at 150 rpm, 25 °C for 3 h and later centrifuged using the bench top HERMLE centrifuge (Model Z326K, Rotor 221.19V20 Germany) for 15 min. The lower phase (chloroform/lipid mixture) was transferred into pre-weight

tubes and the chloroform was evaporated at 60 °C using a hot air oven. The amount of dried crude lipid was quantified gravimetrically and the lipid content was calculated. The lipid content is presented as percentage of the lipid weight relative to dry biomass weight (% w/w) (Ramirez-Castrillón et al. 2017).

Sulfo-phosphovanillin colorimetric method

Two (g) of vanillin was dissolved in warm water (60 °C) and further diluted with 85% of the Orthophosphoric acid to 500 mL (Phosphovanillin reagent). A stock solution for standard concentrations was prepared by dissolving 10 mg/100 mL of cholesterol in chloroform. A concentration range of standard solution was 50–1000 µg/mL. Both the standards and lipid samples (in triplicates) were kept at 60 °C to evaporate the solvent. After the evaporation, 0.5 mL of concentrated Sulfuric (H_2SO_4) was added to each 15 mL conical tube and closed tightly. The contents in the tubes were mixed by shaking and were later submerged in a water bath at 95 °C for 30 min and cooled at room temperature. Two hundred microliters of test samples were transferred into a clean test tube and added distilled water to make the final volume of 1 mL and mixed. A 5-mL of phosphovanillin reagent was added to the tubes for color development. The tubes were subjected to 37 °C for 10 min and the absorbance was read at 530 nm. The calibration curve was prepared with a diluted cholesterol solution and with the aid of the best fit polynominal line generated in Microsoft Excel 2010, the unknown concentrations were determined. The lipid content ($Y_{L/X}$) was calculated according to the formula below:

$$Y_{L/X=\frac{Li-Lo}{Xi-Xo}}$$

Where X_i and L_i are the dry cell weight and lipid concentration on day t_i of harvest, respectively, and X_0 and L_0 are the dry cell weight and lipid concentration on the first day (t0), respectively.

Biochemical determination of biomolecule content

Carbohydrates content

A total carbohydrate was estimated by using phenol-sulfuric acid following the method by Dubois et al. (1956). Ten milligrams of cell dry weight (CDW) for each oleaginous yeast reacted with 5 mL of concentrated H_2SO_4 and 1 mL of 5% phenol in 15 mL conical tubes. The conical tubes were submerged in a water bath with the temperature set at 90 °C for 5 min and allowed to cool prior to the reading of absorbance at A490 nm. The calibration curve was prepared with a glucose stock solution (100 mg/100 mL) and with the aid of the best fit polynominal line generated in Microsoft Excel 2010, the unknown concentrations were determined.

Protein content

The protein extraction was done by modifying the method by Slocombe et al. (2013), hot-trichloroacetic acid (TCA). Ten grams of dry cell weight for each of the oleaginous yeast was added to 10 mL of 10% of TCA in 15 mL conical tubed and vigorously vortexed for 2 min. The mixture was submerged in a water bath with the temperature set at 75 °C for 10 min and later allowed to cool at room temperature. This was followed by a centrifugation of the mixture at 2250×*g* for 10 min at 4 °C. The supernatant was discarded and the cell pellets were re-suspended in alkaline solution (2 M NaOH) and mixed by vortexing. The alkaline reaction was allowed for 10 min at room temperature. The yeast cells-alkaline mixture was centrifuged for at 2250×*g* for 10 min at 4 °C. The supernatant was retained for the protein assay. The protein assay was carried out by following the Lowry method. The reagents required for the assay are: Reagent A (2% Na_2CO_3 in 0.1 M NaOH), Reagent B (0.5% $CuSO_4.5H_2O$ in 1% $KNaC_4H_4O_6.4H_2O$) and Reagent C (2 mL of Reagent B added to Reagent A, thus making final volume of 100 mL). The assay was carried out by mixing 250 µL of the sample with 2.75 mL of reagent C, mixed and allowed reaction for 10 min at room temperature. This was followed by the addition of 300 µL of twice diluted Follin-Ciaocalteu Phenol reagent and mixed. The mixture was allowed to react for 30 min at room temperature prior to reading absorbance A600nm. Again, the calibration curve was prepared with a bovine serum albumin (BSA) stock solution (100 mg/100 mL) and with the aid of the best fit polynominal line generated in Microsoft Excel 2010, the unknown concentrations were determined.

Lipid trans-esterification and component profile analysis

The conversion of the lipid to fatty acid methyl ester (FAME) followed the methods described by Yook et al. (2016) with some modifications. One milliliter of 2.5% H_2SO_4 in methanol (v/v) was added to the dried lipid extract (100–200 mg) and vigorously mixed. The lipid samples were incubated at 75 °C in a water bath for 45 min and cooled prior to any further processing. After cooling, 1 mL of H_2O and 2 mL of n-hexane was added and the mixture was vortexed for 5 min. This was followed by centrifugation at 2250×*g* using the bench top HERMLE centrifuge (Model Z326K, Rotor 221.19V20, Germany) for 5 min. The upper phase (n-hexane with FAMEs) was transferred into a glass vials for analysis. A mixed FAME standard (37 components) and methyl heptadeconoate were obtained from Sigma-Aldrich, USA. The FAME was determined using Gas Chromatography

equipped with a flame ionization detector (Shimadzu GC 2014-FID, Japan) and a capillary column (SP2380, Supelco Analytical) following the method by Guldhe et al. (2014). The oven temperature was programmed to start at 60 °C and kept at hold for 2 min, then increased to 160 °C at a rate of 7 °C.min^{-1} and again kept holding for 1 min. The injector and detector temperature was 250 °C and nitrogen was used as a carrier gas.

Results

Screening of oleaginous yeasts and molecular identification

Subsequent to the culturing of yeasts on either the glucose or the xylose agar medium, a total of 14 isolates were screened for their ability to grow and accumulate the lipid when cultured in limited nitrogen and high sugar media. After harvesting yeast cells at 72 h, followed the staining with NR dye denoting the presence of lipid bodies fluoresced as yellow-gold bodies (Fig. 1). The fluorescence intensity corresponds to the amount of intracellular lipids and the intensity varied between the isolates. Only six isolates showed high fluorescent intensity and the other yeast isolates fluoresced poorly (data not shown).

The exponential growth of yeasts began after 24 h of cultivation and maximum OD_{600} nm of 10–15.5 was attained at 168 h. The yeast biomass increased over time of cultivation until 168 h. The positive yeasts with regards to lipid accumulation were genetically identified. The genetic identification relied on the sequences of the ITS region of the 5.8S rDNA and D1/D2 domains of the 26S rDNA, which revealed that these isolates belonged to two genera viz. *Candida* and *Pichia* species. The six yeasts are *Candida ethanolica* B2, *C. ethanolica* C2, *C. ethanolica* E2, *C. ethanolica* 6-XP, *Pichia. manshurica* 11-XP, and *P. manshurica* 15-X, where the last letter and number denoted strain type. These yeasts were further subjected to secondary screening for lipid production in high sugar (40 g/L glucose) and limited nitrogen (i.e., C:N ratios of 15 and 30) media.

Secondary screening of oleaginous yeasts in shake flask cultivation

Microbial lipid production involves numerous phases, namely; cultivation and lipid accumulation, biomass harvesting, and lipid extraction. There exist challenges to effectively extract the lipid due to the structural complexity of the microbial cell wall, which is a barrier to solvent penetration. Therefore, pretreatment or cell disruption of the cell wall is a key step prior to solvent extraction (Zainuddin et al. 2021). The synthesis and accumulation of lipid in oleaginous microorganisms is known to be activated when the nutrients, particularly nitrogen or phosphorus becomes deficient (Pan et al. 2009). At the end of cultivation, the yeast cultures were harvested for determining the yeast biomass, lipid content. The biomass was influenced by the level of nitrogen source in the medium. This ranged from 258 mg to 339 mg of yeast biomass in low C: N ratio of 15 and from 123 mg to 186 mg of yeast biomass in high C: N ratio, nitrogen limited medium

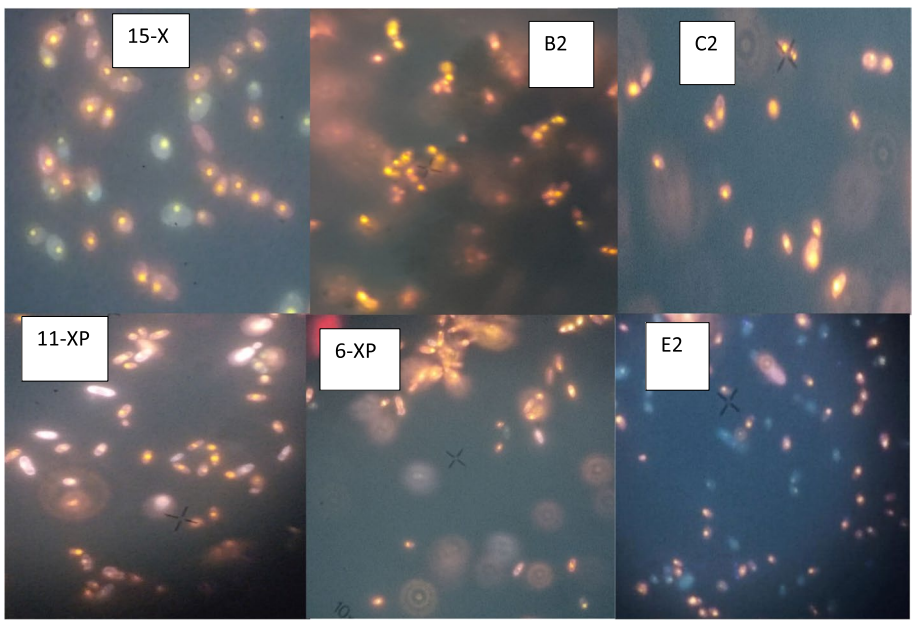

Fig. 1 Microscopic examination lipid bodies within yeast cells stained with Nile red fluorescent dye

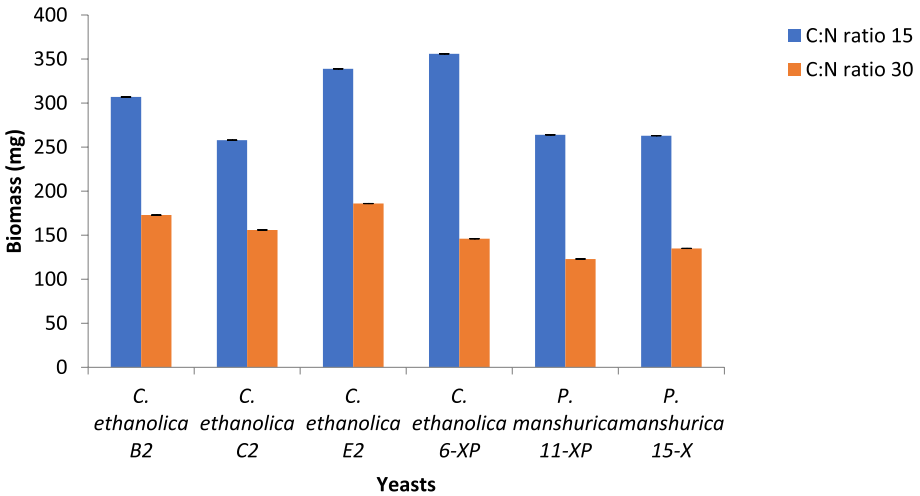

Fig. 2 Biomass of yeast species grown in limited nitrogen conditions

(Fig. 2). *Candida ethanolica* 6-XP, *C. ethanolica* B2 and *C. ethanolica* E2 attained higher biomass under both C:N ratios' conditions. These yeast strains exceed 300 mg biomass in a medium with low C:N ratio whereas the biomass of *C. ethanolica* C2, *P. manshurica* 11-XP and *P. manshurica* 15-X was slightly over 250 mg. The least yeast biomass recovered was for all the *Pichia* strains under the studied conditions.

The ability of yeast strains to accumulate lipid is expressed as lipid content. The lipid content for *C. ethanolica* strains E2 (47%) was higher than all other yeast species in low C:N ratio. When the nitrogen in the medium was reduced to 2.5 g/L, all the yeast species increased the accumulated lipids with the exception of *C. ethanolica* E2 strain (Fig. 3). *Pichia manshurica* 15-X, *P. manshurica* 11-XP, and *C. ethanolica* 6-XP accumulated higher lipids with their lipid content of 67%, followed by 50% and 46%, respectively. The lipid content for all the *Candida* strains relative to their dry biomass was greater than 20% for yeasts cultured in all nitrogen limited conditions. For the *Pichia* strains, high nitrogen (i.e., 5 g/L $(NH_4)_2SO_4$) suppressed the accumulation of lipids, which were below 20%. Conversely, the low nitrogen in the medium stimulated synthesis and accumulation of lipid in *Pichia* strains more than the *Candida* strains. The lipid content for all yeast grown under low nitrogen ranged from 28 to 67% g lipids/g dry biomass (Fig. 3).

Since the gravimetric method is prone to the over estimation of the lipid content, the SPV colorimetric assay was used to validate the oleagenocity of the yeasts. When

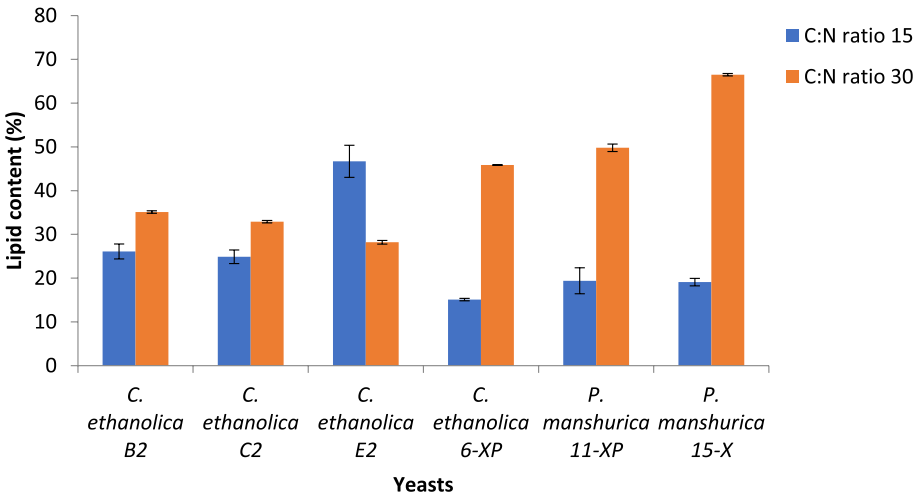

Fig. 3 Gravimetric determination of lipid content of yeast species grown under limited nitrogen conditions

the SPV reacts with lipids, it produces a pink to purple color. The color intensity is proportional to the lipid in the mixture. Based on the SPV assay, the lipid content for all yeast species grown in a medium with a low C:N ratio were less than 20%, whereas it was slightly higher in a high C:N ratio, which ranged from 22 to 33% (Fig. 4) and was lower than the lipid recorded gravimetrically.

Both the gravimetric and SPV methods confirmed the accumulation of lipids by all yeasts species when grown in a low nitrogen medium (C: N ratio of 30). Based on the SPV assay results, the de-fatted biomass harvested from nitrogen limited medium were further used for determining the carbohydrates and proteins.

Carbohydrates and proteins contents

Apart from the lipids (or SCO), yeast cells contain carbohydrates, proteins and other components that can be used in food and animal feed industries as nutritional supplements. The six de-fatted oleaginous yeasts contained carbohydrates and proteins in the range 10–35% and 21–50%, respectively. *Candida ethanolica* 6-XP contained the lowest carbohydrate level of 10%. The highest level of protein was attained by *P. manshurica* 15-X, which was 50% of the yeast biomass. The results show a trade-off between the two components, namely; carbohydrates and proteins of the oleaginous yeast cell dry weight (Fig. 5).

The conversion of fatty acids to fatty acid methyl esters

The conversion of the extracted yeast lipids to FAME was carried out using acid-catalyzed trans-esterification reaction to determine fatty acid composition by Gas Chromatography. The main FAME in the lipids from all yeast species, with the exception of *C. ethanolica* B2 and

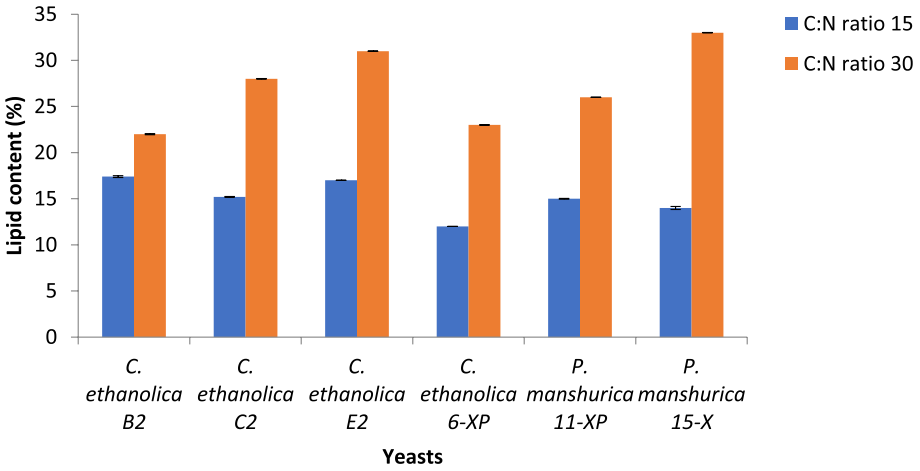

Fig. 4 Sulfo-phosphovanillin determination of lipid content in yeast species grown under limited nitrogen conditions

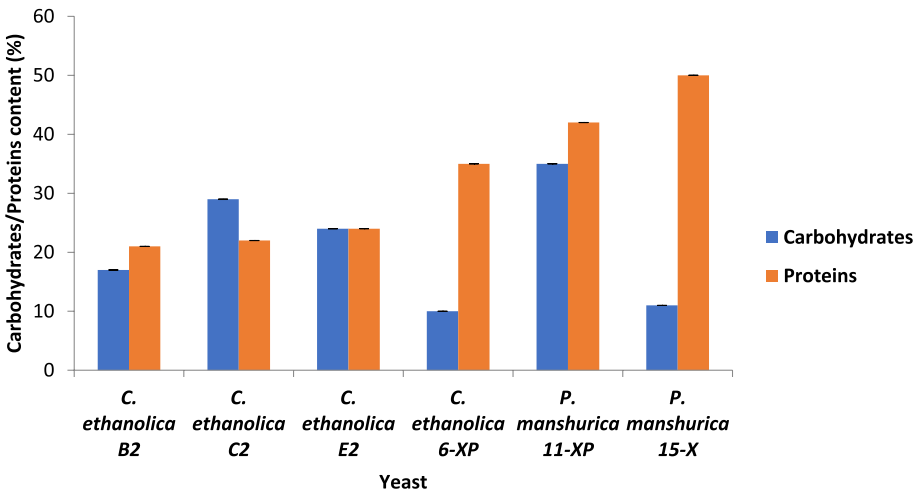

Fig. 5 Carbohydrates and proteins content in oleaginous yeasts after the production of lipids

C. ethanolica 6-XP, were found to be palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c), and linoleic (C18:2n6c) acids. Oleic acid dominated the fatty acids in *C. ethanolica* C2, *C. ethanolica* E2 and *P. manshurica* 15-X. The dominating FAME for *C. ethanolica* B2 and *C. ethanolica* 6-XP, were only saturated fatty acids (SFA), palmitic and stearic acids. *Candida ethanolica* B2 was the only strain showing the presence of margaric acid (C17:0). Eicosadienoic acid of over 4% was only present in *C. ethanolica* B2 and *C. ethanolica* 6-XP. Arachidic acid and other fatty acids were neither detected nor present in the smallest amount for all the yeast strains (Table 1).

The myristic (C14:0) and palmitoleic (16:1) acids were in moderate amounts in all the yeast species. *Pichia manshurica* 15 X lacked all the C20 fatty acids and only had lower levels of uricic (C22:1) and lignoceric (C24:0) acids. *Candida ethanolica* C2, *C. ethanolica* E2 and *P. manshurica* 11-XP exhibited the presence of most of the fatty acids with reference to the FAME 37 component standard. All *Candida* and *Pichia* strains' SCOs were dominated by SFA ranging from 45.3 to 72.2%. *Candida ethanolica* B2 and *C. ethanolica* 6-XP SCOs contained a greater amount of SFA, more than 70% content. The monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were ranged from 16.3 to 38.7% and 10.7–21%, respectively. The presence of PUFA, which is dominated by omega-6-fatty acids a linoleic (C18:2n6c), can be vital in food supplements.

Table 1 Composition of fatty acids methyl esters of lipids produced by the oleaginous yeasts

		Fatty acid methyl esters composition (%)						
Fatty acid name	**Carbon number**	**FAME 37 Standard mix**	***C. ethanolica* B2**	***C. ethanolica* C2**	***C. ethanolica* E2**	***C. ethanolica* 6-XP**	***P. manshurica* 11-XP**	***P. manshurica* 15-X**
Myristic acid	C14:0	4.98 (8.9%)	0.66 (2.8%)	3.02 (7.0%)	3.10 (7.6%)	0.59 (2.6%)	3.5 (8.4%)	2.85 (7.6%)
Palmitic acid	C16:0	7.60 (13.5%)	7.43 (31.4%)	8.05 (18.8%)	7.72 (18.8%)	8.11 (36.1%)	9.5 (23%)	7.38 (19.6%)
Palmitoleic acid	C16:1	2.22 (4.0%)	0.69 (2.9%)	2.88 (6.7%)	2.68 (6.5%)	0.45 (2.0%)	2.16 (5.2%)	2.01 (5.3%)
Margaric acid	C17:0	2.35 (4.2%)	0.30 (1.3%)	*ND -	*ND -	*ND -	*ND -	*ND -
Stearic acid	C18:0	5.33 (9.50%)	7.13 (30.2%)	6.76 (15.8%)	6.90 (16.8%)	7.17 (32%)	8.39 (20.1%)	8.06 (21.4%)
Linolenic acid	C18:3	2.97 (5.3%)	*ND -	*ND -	1.80 (4.4%)	0.37 (1.6%)	1.34 (3.2%)	1.01 (2.7%)
Elaidic acid	C18:1n9t	2.28 (4.1%)	1.21 (5.1%)	0.35 (0.8%)	0.30 (0.7%)	1.17 (5.2%)	0.32 (0.8%)	*ND -
Oleic acid	C18:1n9c	4.67 (8.3%)	2.32 (9.8%)	10.53 (24.6%)	10.13(24.7%)	2.03 (9.0%)	8.15 (19.5%)	9.78 (26.0%)
Linolelaidic acid	C18:2n6t	1.43 (2.5%)	0.29 (1.2%)	0.18 (0.4%)	*ND -	0.19 (0.8%)	0.18 (0.4%)	*ND -
Linoleic acid	C18:2n6c	1.29 (2.3%)	1.16 (4.9%)	6.29 (14.7%)	5.97 (14.6%)	1.04 (4.6%)	6.08 (14.6%)	5.67 (15.0%)
Arachidic acid	C20:0	5.62 (10.0%)	*ND -	0.55 (1.3%)	0.50 (1.2%)	*ND -	0.39 (0.90%) 0.46 (1.1%)	*ND -
Gondoic acid	C20:1	0.27 (0.5%)	0.19 (0.8%)	0.18 (0.4%) 1.89 (4.4%)	*ND -	*ND -	*ND -	*ND -
Eicosadienoic acid	C20:2	1.13 (2.0%)	1.07 (4.5%)	0.37 (0.9%)	*ND -	0.97 (4.3%)	*ND -	*ND -
Behenic acid	C22:0	5.50 (9.8%)	0.44 (1.9%)	0.21 (0.5%)	0.45 (1.10%)	0.36 (1.6%)	*ND -	*ND
Erucic acid	C22:1	3.32 (5.9%)	*ND -	0.38 (0.9%)	0.60 (1.5%)	*ND -	0.68 (1.6%)	0.56 (1.5%)
Docosanoic acid	C22:2	4.95 (8.8%)	*ND -	0.38 (0.9%)	0.60 (1.5%)	*ND -	0.29 (0.7%)	*ND -
Lignoceric acid	C24:0	0.18 (0.3%)	0.75 (3.2%)	0.82(1.9%)	0.29 (0.7%)	*ND -	0.30 (0.7%)	0.36 (0.9%)
Total fatty acids (FA)		**56.09 (100%)**	**23.65 (100%)**	**42.84 (100%)**	**41.04 (100%)**	**22.45 (100%)**	**41.74 (100%)**	**37.68 (100%)**
Total saturated FA		31.56 (56.3%)	16.71 (70.7%)	19.41 (45.3%)	18.96 (46.2%)	16.23 (72.2%)	22.54 (54%)	18.65 (49.5%)
Total monounsaturated FA		12.75 (22.7%)	4.41 (18.6%)	16.59 (38.7%)	13.71 (33.4%)	3.65 (16.3%)	11.31 (27.1%)	12.35(32.8)
Total polyunsaturated FA		11.77 (21.0%)	2.52 (10.7%)	7.22 (16.9%)	8.37 (20.4%)	2.58 (11.5%)	7.89 (18.9%)	6.68 (17.7%)

* *ND* refers to non-detected

Discussion

Screening of oleaginous yeasts and molecular identification

After the processing of sugarcane, the bagasse is left piled up in an open and uncontrolled environment. The sugarcane bagasse (SCB) becomes inhabited and colonized by microorganisms which proliferate as they assimilate carbon and other nutrients. Sugarcane bagasse is a fibrous lignocellulosic residue. The microorganisms include bacterial, yeast, fungi, etc., which in concerted efforts break down the bagasse to make carbon accessible. The microbiotas on the SCB are a potential resource for the discovery of useful, novel microorganisms and enzymes for industrial applications (Gebbie et al. 2020).

Candida ethanolica and *P. manshurica* are normally associated with alcoholic, vinegar fermentation and cocoa bean fermentation and fermentation (or rotting) of plant materials (Maura et al. 2016; Tolieng et al. 2018; Xing et al. 2018). These yeasts are found associated with the SCB and have not been studied extensively for their ability to accumulate lipids. The most studied oleaginous yeasts species belong to the genera of *Rhodotorula*, *Rhodosporidium*, *Yarrowia*, *Candida*, *Cryptococcus*, *Rhizpus*, *Trichosporon*, and *Lipomyces* (Pan et al. 2009; Zhang et al. 2011; Thevenieeau and Nicaud, 2013; Schulze et al. 2014; Bardhan et al. 2020, Caporusso et al. 2021). These oleaginous microorganisms store neutral lipids in the form of TAGs and accounting between 20% and 80% of their cell weight (Ratledge 2004; Lamers et al. 2016; Ochsenreither et al. 2016). The synthesis and accumulation of lipid in oleaginous microorganisms is known to be activated when the nutrients, particularly nitrogen or phosphorus becomes deficient (Pan et al. 2009). Under these conditions, oleaginous yeast strains produce lipids as a protective mechanism (Shi et al. 2013). Among the heterotrophic microorganisms, oleaginous yeasts are regarded as an alternative source for lipid production because of the relatively high biomass yield, fast lipid accumulation, lipid yield, and productivity (Vasconcelos et al. 2018).

Nile red dye used for primary screening is regarded as an effective and quickest way for the detection of accumulated lipids inside oleaginous microorganisms (Bajwa et al. 2018). This fluorimetric assay is considered a suitable, accurate, and reliable method to estimate intracellular lipid production among yeasts of distinct genera and species (Miranda et al. 2020). The yeasts differed in cell sizes, which upon staining the cells, revealed different degrees of fluorescence intensity. The yeast that appeared larger fluoresced vividly, showing yellow-golden bodies inside the yeast. This observation was in congruent with the results reported by Elfeky et al. (2019). However, due to the complex and relatively resistant cell wall of yeasts, which limit the efficient permeability of fluorogenic dye or impede full recovery of the intracellular lipid, the process requires a pretreatment step (Zainuddin et al. 2021). The resistant cell wall causes inconsistencies of Nile red staining, which makes it challenging to optimize and present a robust protocol for all species or new strains (Hicks et al. 2019). Although a yeast cell wall differs in thickness, the addition of DMSO improves the dye penetration, which makes it possible to detect lipid bodies (Sitepu et al. 2012). The yeasts that revealed poor fluorescence were excluded them in the subsequent analyses. A low fluorescent intensity of the yeast strains suggested non-oleaginocity, thus poor lipid accumulation (Ramirez-Castrillón et al. 2021). The higher the fluorescence intensity, the higher intracellular the lipid content in yeast strains (Miranda et al. 2020). There is a need for using the fluorescence emission quantum yield (ϕ_{fl}) when standard fluorescence methods based on Nile red are employed to estimate yeast lipids. This ϕ_{fl} enabled a closest correlation between Nile red and gravimetric method for the determination of lipid content (Morales-Palomo et al. 2022).

The C:N ratio represents the amount of carbon and nitrogen available in the medium and they are essential for microbial growth and functioning. Nitrogen facilitates the synthesis of amino acids, proteins, and nucleic acids, whereas carbon serves as a structural unit and energy source (Khanal et al. 2019). It is one of the important parameters that has a greater influence on the production of SCOs (Braunwald et al. 2013). A high C:N ratio in the medium favours an increase in lipid yield and productivity, and also has an adverse effect on the yeast biomass and growth rate (Robles-Iglesias et al. 2023). This observation is congruent with the results in Fig. 2, which showed a lower biomass when a C: N ratio was 30 compared to a C:N ratio of 15. A strong dependence between nitrogen concentration in the medium and resulting biomass was evident in the current study. An increase in C:N ratio from 2 to 100 affected the *Rhodotorula kratochvilovae* biomass by 4-fold reduction (Robles-Iglesias et al. 2023). After cultivation, the SCOs determination requires cell wall disruption or pretreatment method prior to lipid extraction using solvents (Zainuddin et al. 2021). The choice of a microbial cell wall pretreatment technique depends on the type of microorganisms studied because of their cell wall composition, which limit the extractability of lipids (Patel et al. 2019). After primary screening, yeast cells that displayed a high fluorescence intensity were further screened following the gravimetric and SPV methods before the selection of the oleaginous yeasts. This was done to avoid false or pseudo-oleaginous yeasts being selected. The Gravimetric method, which determines the microbial lipid content by weight of the extracted lipids relative to the weight of

the yeast biomass, was used as a reference method to validate other methods (Chen et al. 2018; Patel et al. 2019).

The C:N ration of less than 20 results in poor lipid accumulation, whereas the C:N ration of 30 or more enhances the synthesis and lipid accumulation. The optimum C:N ratio for lipid production is affected by the substrate used. For instance, *R. toruloides* exhibited varying C:N ratios that yielded maximum lipid in xylose, acetic acid, glycerol, and hydrolysate (Lopes et al. 2020), and was also influenced by the microorganisms employed (Beopoulos et al. 2009). The theoretical lipid yield is 0.32 g/g carbon consumed from glucose and 0.34 g/g carbon consumed from xylose (Ratledge and Wynn 2002; Donot et al. 2014; Wang et al. 2020), and 0.3 g/g carbon consumed from glycerol (Wang et al. 2020). However, it is reported that a C:N ratio less than 20 results in poor lipid accumulation, whereas C: N ratios between 30 and 80 enhances the synthesis and accumulation of lipid (Beopoulos et al. 2009).

In this study, a hot-acid pretreatment method was applied, followed by Folch's method to extract lipids. All the yeast species, viz. *C. ethanolica* strains and *P. manshurica* accumulated the lipid content of over minimum 20% (w/w) under cultivation of a high C:N ratio of 30 medium. *Candida ethanolica* strains showed a high capacity to accumulate lipid under both C: N ratio of 15 and C: N ratio of 30 of nitrogen cultivation conditions as shown in (Fig. 3), thus making them oleaginous yeasts (Patel et al. 2020). However, the lipid content of *P. manshurica* strains was between 50 and 67% higher than for *C. ethanolica* strains (28–46% w/w) in a high C: N ratio of 30, thus nitrogen-limited conditions. Consistent with these results, to demonstrate efficiency of acid-pretreatment of yeast cells, Bonturi et al. (2015) reported lipid content of 42% (w/w) and 40% (w/w) for *Rhodotorula toruloides* and *Lipomyces starkeyi*, respectively. Acid-treatment of *L. starkeyi* improved a higher lipid recovery yield, irrespective of the solvent used (Bonturi et al. 2015; Kruger et al. 2018). *Rhodotorula glutinis* var. rubescens LOCKR13 has accumulated lipid content of 34.15% (w/w) (Gientka et al. 2022). For *Rhodosporidium toruloides*-1588, the optimum C:N ratio of 70 produced 43% (w/w) of lipid in undetoxified wood hydrolysate medium. In addition, the strain consumed 70% of xylose (Saini et al. 2021). Acid treatment combined with a heating process seems to facilitate the lipid extraction by disintegrating the cell walls.

The ability of *Candida ethanolica* and *P. manshurica* to accumulate lipids was previously reported; however, the lipid content was below 20% (w/w). For instance, the lipid content from *C. ethanolica* AM320 was 15% (w/w) (Wang et al. 2018) and for *C. ethanolica* M1 and *P. manshurica* were 12% (w/w) and 15% (w/w), respectively (Arous et al. 2017). These results arguably show that the accumulation of lipid inside the microorganisms could be dependent on the species type or strain, and cultivation conditions (e.g., substrate, temperature, pH, aeration). This effect was demonstrated in this study where a nitrogen concentration in the medium influenced how much of lipid synthesis and accumulation resulted. To the best of our knowledge, we are the first to report the oleaginocity (i.e., lipid content over 20%) of the *C. ethanolica*. The growth stage at which cells are harvested also has an effect on the accumulated lipid (Liu et al. 2010). The lipids content for the *P. manshurica* CHC34 was 11.43% (w/w) and 9.52% (w/w) at the early and late stage of cultivation, respectively (Areesirisuk et al. 2015). The maximum lipid content is also dependent on the type of nitrogen source. Using ammonium sulphate and ammonium nitrate as a nitrogen sources at a C:N ratio of 200, *R. toruloides* RZL2 accumulated 59% (w/w) and 63% (w/w), respectively (Ye et al. 2021).

Only a few studies, Polburee et al. (2015) and Planonth and Chantarasiri (2022) reported higher lipid content in *P. manshurica*. The *P. manshurica* strain DMKU-UbC9(2) accumulated lipid content of 64.8% (w/w) in glycerol medium (Polburee et al. 2015), which was comparable to the lipid content of *P. manshurica* 15-X. Recently, *P. manshurica* strain Y2 that was isolated from *Lansium domesticum* fruit had lipid content of 43.03% (w/w), (Planonth and Chantarasiri 2022). These observations are congruent with our results that oleaginous *P. manshurica* strains exist, which also reveals that numerous known yeast species or strains and even new microorganisms have not been extensively studied for their ability to produce lipids intracellular.

The oleaginocity of *C. ethanolica* and *P. manshurica* strains under the current study were verified by the SPV method. This method also requires cell disruption, followed by lipid extraction and was used to quantify the lipid content of *Candida* and *Pichia* species. It has advantages over both the Folch extraction and gravimetric methods for lipid analysis since the latter in its estimation of lipids may erroneously include lipophilic proteins and pigments, and therefore record high lipid values. The results of the SPV assay confirmed the oleaginocity of the yeast species. However, the lipid content discrepancies between the SPV assay and gravimetric method could be attributed to lipo-proteins and other contaminants, which contributed to the high lipid mass. The acid-thermal reaction (treatment) of the SPV assay degraded the lipophilic proteins and other products thereby reducing contaminants of lipids (Mishra et al. 2014; Patel et al. 2019). Therefore, the SPV method could be employed as a reliable assay for the quantification of lipids to facilitate an efficient screening of oleaginous microorganisms (Byreddy et al. 2016). The results showed the percent lipid

content of the *C. ethanolica* and *P. manshurica* reduced to between 22 and 33% (w/w), less than gravimetric lipid content. The lipid content of *P. manshurica* strains 15-X remained higher when determined by both gravimetric and SPV methods. It is pivotal to note that obtaining high lipid content is crucial to off-set the processing costs per unit biomass (Shokrari et al. 2020).

Carbohydrate and proteins

Yeast responds to stress by accumulating either carbohydrates or lipids. The microbial response in nutrient limitations, particularly nitrogen or phosphorus requires robust metabolism to attain maximum cell growth (biomass). In nitrogen limitation, cellular carbohydrates such as glycogen and trehalose increase and further contribute to the increase of the lipid content while protein and RNA content are reduced (Yu et al. 2020). The availability of carbohydrates and proteins as co-metabolites with lipids is desirable as it would make the microbial oil production process cost-effective. Not all studied yeasts contained sufficient carbohydrates and proteins. Most of the carbohydrates were obtained from *Pichia* and *Candida* strains with the exception of the *C. ethanolica* 6XP and *P. manshurica* strains 11-XP, which had less carbohydrate and accumulated a higher protein content of 42–50% when grown in nitrogen limited and high glucose medium.

Different types of substrates influence the amount of carbohydrates and proteins (Kumar et al. (2017). *Pichia guilliermondii* contained 35%. 8–44.2% of carbohydrates and 12.4–24.6% of proteins (Kumar et al. (2017). Tchakouteu et al. (2015) studied the interaction between the synthesis of intracellular total carbohydrates and cellular lipids in *Cryptococcus curvatus* under nitrogen-limited and nitrogen-excess condition with lactose and sucrose as carbon source. The strain accumulated a high quantity of intracellular total sugars (up to 68% w/w) at the early stage of fermentation when nitrogen availability was sufficient and at the end of fermentation when nitrogen was depleted, the intracellular total sugar decreased to 20%. In excess nitrogen, the intracellular total sugar were determined from the yeast culture in the late stage of cultivation (168 h), which according to the above observation by Tchakouteu et al. (2015), may attribute the low carbohydrate content (i.e., less than 50%) to that effect. There seems to be a trade-off between accumulated lipids and carbohydrates. Besides the fact that both macromolecules compete for the available carbon flux from sugar and that the excess lipid accumulation occurs at the late stage of cultivation, carbohydrates accumulation is favoured in the early stage under nitrogen limited conditions. Growing under a nitrogen limited medium, a short and transitory intracellular carbohydrates accumulation in *Rhodotorula glutinis* CECT 1137 occurred prior to lipid accumulation (Cescut et al. 2014). From our results, it also clear that the levels of these macromolecules is dependent on the strain and species type. Another important factor is the cultivation conditions, thus either aerobic or anaerobic.

Yeast is the widely accepted microorganism for the production of single cell protein (SCP) because of its high nutritional quality and consumer acceptance (Lapeña et al. 2020). Microbial (including algal) proteins are referred to as SCP, if the microorganism contains 30% or more of proteins (Glencross et al. 2020; Lapeña et al. 2020). Our results show that the protein contents are higher than the carbohydrates, suggesting the existence of continuous synthesis and accumulation of protein in those yeasts at the late stage of cultivation (168 h) in limited or depleted nitrogen medium. The protein content differed remarkably between yeast species, but remained the same for the *C. ethanolica* strains with exception of *C. ethanolica* strain 6-XP. The biomass of *P. manshurica* strains contained the most protein (42–50%). Juszcyk et al. (2013) reported protein content that ranges from 35.1 to 49%, with the highest protein content attained from *Y. lipolytica* strain S21 biomass.

The biomass of *Y. lipolytica* clade species (YADE and YAGA) contained protein content ranging from 13.2 to 31.9%. This protein content (31.9%) is comparable to the reported protein content of *C. ethanolica* strain 6-XP in this study. However, it is known that upon the depletion of nitrogen in the growth medium, the synthesis of intracellular proteins and nucleotides ceases as excess carbon is redirected into lipid synthesis (Zhang et al. 2016). This occurs to activate autophage, which is a mechanism that allows yeast cells to maintain viability. This process makes the proteins or amino acids resulting from cell death of other yeasts available for a continuous synthesis of proteins required for survival (Reggiori and Klionsky 2013). For instance, *Lipomyces starkeyi* AS2.1560 in response to limited-nitrogen condition after 96 h of cultivation activated protein degradation process and amino acid biosynthesis to salvage and redistribute nitrogen sources for suboptimal cell growth (Liu et al. 2010). The requirement for autophagy during starvation may be due to the need to recycle biological polymers (proteins, nucleic acids, carbohydrates, lipid bilayers, etc.) into building blocks for reuse under conditions where they may not be available outside the cell (Abeliovich and Klionsky 2001).

In de-fatted yeast biomass, the protein and lipid content of approximately 30% protein and 12% lipid would be sufficient for use in animal feed production (Rakicka-Pustulka et al. 2021). The analysis of yeast proteins, particularly *Yarrowia* reveal high lysine, leucine, threonine and valine as well as small quantities of

cysteine and methionine (Juszcyk et al. 2013). The composition of proteins varies between yeast and strains. Rakicka-Pustulka et al. (2021) reported protein rich in aspartic and glutamic acid, alanine, and lysine. The protein was poor in serine, methionine and cysteine.

Both SCO and SCP have biotechnological applications in food and animal feed diets as sources of amino acids, omega-3-lipids and bioactive molecules (Glencross et al. 2020). Co-production of SCO together with either carbohydrates or proteins will make the process of lipid production cost-effective and sustainable, particularly if a waste carbon substrate is used.

The conversation of fatty acids to fatty acid methyl esters

Fatty acids composition is usually influenced by the yeast strain type and the carbon source utilized during growth (Gientka et al. 2017). The FA produced by all the *C. ethanolica* strains and *P. manshurica* strains are mainly SFA, followed by MUFA and PUFA. Then again, *P. manshirica* 15-X produced abundance amount of palmitic, stearic, oleic, and linoleic acids making up 82% of the total lipids. This composition was similar to SFA in a lipid produced by *R. toruloides*-1588, namely palmitic, stearic, oleic, and linoleic acids when grown in undetoxified wood hydrolysate (Osorio-González et al. 2023) and fatty acids produced by *Cryptococcus curvatus* DSM 70022 (Annamalai et al. 2018). These fatty acids are reported to be comparable to those of plant or vegetable oils (Annamalai et al. 2018). Other oleaginous yeasts that showed similar fatty acids profile include *Trichosporon* sp. (RW), *Thodotorula glutins*, *Rhodotorula babjevae*, *Lipomyces starkeyi*, and *Lipomyces lipofer* species (Brar et al. 2017; Shapaval et al. 2019). These fatty acids profiles can also differ between species and strains. For instance, *Trichosporon mycotoxinivorans* S2's major fatty acids were palmitic, oleic and stearic (Sagia et al. 2020). The fatty acids for *Rhodosporidium* TJUWZ4 were oleic, palmitic and linoleic acids (Wang et al. 2017). This confirms that specific yeast strains will produce the lipids of different fatty acids compared to the others. Polyunsaturated fatty acids for all yeast strains were dominated by linoleic acid (C18:2n6c), constituted up to 20% of the total fatty acids. To improve this content, Kolouchova et al. (2016) found that under limited nitrogen using $(NH4)_2SO_4$ as a nitrogen source, the unsaturated fatty acids increases with cultivation time. The level of linoleic acid for *Candida* sp., *Rhodotorula glutinis*, *Yarrowia lipolytica*, and *Trichosporum cutancum* was between 15.6% and 53.4%. Therefore, cultivation conditions and species or strain type greatly influence the fatty acid profile.

Conclusion

All the *Candida* and *Pichia* screened demonstrated the potential to produce single cell oils. *Pichia manshurica* 15-X accumulated higher rate of lipid and protein than the other yeasts. FAME analysis of lipid from *P. manshurica* 15-X revealed the suitability of the lipids based on the fatty acids composition for application in the biofuel and food industry. In addition, the co-products of lipids such as carbohydrates and proteins make the lipid production process cost-effective with potential application in biofuel and animal feed. It is imperative that all the selected yeasts be evaluated further under optimized conditions using high C: N ratios in lignocellulosic hydrolysate for lipid production.

Acknowledgements
Not applicable.

Authors' contributions
LML conceptualized the ideas, designed, planned, and executed the experimental research work. LML took a lead in analyzing the results, and structuring and writing of the manuscript. KLMM critically reviewed and edited the entire manuscript. The author also assisted with analysis and interpretation of the results. All authors read and approved final manuscript.

Authors' information
LML holds Ph.D in microbiology and is currently a postdoctoral fellow at the University of Limpopo.
KLM holds Ph.D in microbiology and is currently an Associate Professor at the University of Limpopo.

Funding
No funding available.

Availability of data and materials
The data sets generated during and or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 11 January 2023 Accepted: 9 June 2023
Published online: 20 June 2023

References

Abeliovich H, Klionsky DJ (2001) Autophage in yeast: mechanistic insights and physiological function. Microbiol Mol Biol Rev 65(3):463–479. https://doi.org/10.1128/MMBR.65.3.463-479.2001

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17):3389–3402

Annamalai N, Sivakumar N, Oleskowics-Popiel P (2018) Enhanced production of microbial lipids from waste office paper by the oleaginous yeast *Cryptococcus curvatus*. Fuel 217:420–426. https://doi.org/10.1016/j.fuel.2017.12.108

Anschau A, Caruso SS, Kuhn RC, Franco TT (2017) Validation of sulfo-phosphoanillin (SPV) method for the determination of lipid content in oleaginous microorganisms. Braz J Chem Eng 34(01):19–27. https://doi.org/10.1590/0104-6632.20170341s20140222

Areesirisuk A, Chiu CH, Yen TB, Liu CH, Guo JH (2015) A novel oleagionous yeast strain with high lipid productivity and its application to alternative biodiesel production. Appl Biochem Microbiol 51(4):411–418. https://doi.org/10.1134/S0003683815030035

Arous F, Azabou S, Triantaphyllidou I-E, Aggelis G, Jaouani A, Nasri M, Mechichi T (2017) Newly isolated yeasts from Tunisian microhabitats: Lipid accumulation and fatty acid composition. Eng Life Sci 17:226–236. https://doi.org/10.1002/elsc.201500156

Bajwa K, Bishnoi NR, Toor M et al (2018) Isolation, screening, characterization of indigenous oleaginous bacteria: evaluation of various carbon and nitrogen sources as substrated for single celled oil producing bacteria. Asian J Biotechnol Bioresour Technol 3(1):1–12. https://doi.org/10.9734/AJB2T/2018/39260

Bandhu S, Knot MB, Sharma T, Sharma OP, Dasgupta D, Mohapatra S, Hazra S, Khatri OP, Ghosh D (2018) Single cell oil from oleaginous yeast grown on sugarcane bagasse derived xylose: an approach toward novel biolubricant for low friction and wear. ACS Sustain Chem Eng 6:275–283. https://doi.org/10.1021/acssuschemeng.7b02425

Bardhan P, Gupta K, Kishor S, Chattopadhyay P, Chaliha C, Kalita E, Goud VV, Mandal M (2020) Oleaginous yeasts isolated from traditional fermented foods and beverages of Manipur and Mizoram, India, as a potent source of microbial lipids for biodiesel production. Ann Microbiol 70:20. https://doi.org/10.1186/s13213-020-01562-z

Beopoulos A, Cescut J, Haddouche R, Uribelarrea J-L, Molina-Jouve C, Nicaud J-M (2009) *Yarrowia lipolytica* as a model for bio-oil production. Prog Lipid Res 48:375–387. https://doi.org/10.1016/j.plipres.2009.08.005

Bonturi N, Matsakas L, Nilsson R, Christakopoulos P, Miranda EA, Berglund KA, Rova U (2015) Single cell oil producing yeasts *Lipomyces starkeyi* and *Rhodosporidium toruloides*: selection of extraction strategies and biodiesel property prediction. Energies 8:5040–5052. https://doi.org/10.3390/en8065040

Brar KK, Ak Sarma, Aslam M, Polikarpov I, Chadha BS (2017) Potential of oleaginous yeast *Trichosporon* sp., for conversion of sugarcane bagasse hydrolysate into biodiesel. Bioresour Technol 242:161–168. https://doi.org/10.1016/j.biortech.2017.03.155

Braunwald T, Schwemmlein L, Graeff-Hönninger S, French WT, Hernandez R, Holmes WE, Claupein W (2013) Effect of different C/N ratios on carotenoid and lipid production by *Rhodotorula glutinis*. Appl Microbiol Biotechnol 97:6581–6588. https://doi.org/10.1007/s00253-013-5005-8

Breddy AR, Gupta A, Barrow CJ, Puri M (2016) A quick colorimetric method for total lipid quantification in microalgae. J Microbiol Methods 125:28–32. https://doi.org/10.1016/j.mimet.2016.04.002

Brock M, Buckel W (2004) On the mechanism of action of the antifungal agent propionate propionyl-CoA inhibits glucose metabolism in *Aspergillus nidulans*. Eur J Biochem 271:3227–3241. https://doi.org/10.1111/j.1432-1033.2004.04255x

Caporusso A, Capece A, De Bari I (2021) Oleaginous yeasts as cell factories for the sustainable production of microbial lipids by the valorization of agri-food wastes. Fermentation 7:50. https://doi.org/10.3390/fermentation7020050

Cescut J, Fillaudeau L, Molina-Jouve C, Uribelarrea J-L (2014) Carbon accumulation in *Rhodotorula glutinis* induced by nitrogen limitation. Biotechnol Biofuels 7:164. https://doi.org/10.1186/s13068-014-0164-0

Chama NT (2019) Production of single-cell protein from different substrates. Aust J Sci Technol 3(4):148–153

Chattopadhyay A, Maiti MK (2021) Lipid production by oleaginous yeasts. Adv Appl Microbiol 116:1–98. https://doi.org/10.1016/bs.aambs.2021.03003

Chen Z, Wang L, Qiu S, Ge S (2018) Determination of microalgal lipid content and fatty acid for biofuel production. BioMed Res Int 2018:1503126. https://doi.org/10.1155/2018/1503126

Chen Y, Futcher B (2017) Assaying glycogen and trehalose in yeast. Bio-Protoc 7(13):1–9. https://doi.org/10.21769/BioProtoc.2371

Diwan B, Parkhey P, Gupta P (2018) From agro-industrial wastes to single cell oils: a step towards prospective biorefinery. Folia Microbiol 63(5):547–568. https://doi.org/10.1007/s12223-018-0602-7

Donot F, Fontana A, Baccou JC, Strub C, Schorr-Galindo S (2014) Single cell oils (SCOs) from oleaginous yeasts and molds: production and genetics. Biomass Bioenergy 68:135–150. https://doi.org/10.1016/j.biombioe.2014.06.016

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Fred S (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28(3):350–356. https://doi.org/10.1021/ac60111a017

Elfeky N, Elmahmoudy M, Guo Zhang Y, JL, Bao Y, (2019) Lipid and carotenoid production by *Rhodotorula glutinis* with a combined mode of nitrogen, sulfur and aluminium stress. Appl Sci 9:2444. https://doi.org/10.3390/app9122444

El Kantar S, Khelfa A, Vorobiev E, Koubaa M (2021) Strategies for increasing lipid accumulation and recovery from *Y. lipolytica*: a review. OCL 28:51. https://doi.org/10.1051/ocl/2021038

François J, Parrou JL (2001) Reserve carbohydrate metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev 25:125–145. https://doi.org/10.1111/j.1574-6976.2001.tb00574.x

Galán B, Santos-Merino M, Nogales J, de la Cruz F, García JL (2019) Microbial oils as nutraceuticals and animal feeds. In: H. Goldfine (ed). Health consequences of microbial interactions with hydrocarbons, oils, and lipids. Handbook of Hydrocarbon and Lipid Microbiology. *Springer Nature,* Switzerland. https://doi.org/10.1007/978-3-319-72473-7_34-1

Gebbie L, Dam TT, Ainscough R, Pafreyman R, Cao L, Harrison M, ÓHara I, Speigut R (2020) A snapshot of microbial diversity and function in an undisturbed sugarcane bagasse pile. BMC Biotechnol 20:12. https://doi.org/10.1186/s12896-020-00609y

Ghanavati H, Nahvi I, Roghanian R (2014) Monitoring growth and lipid production of new isolated oleaginous yeast *Cryptococcus aerius* UIMC 65 on glucose and xylose cultures. Biotechnol Bioprocess Eng 19:468–477. https://doi.org/10.1007/s12257-014-0007-7

Gientka I, Kieliszek M, Jermacz K, Błażejak S (2017) Identification and characterization of oleaginous yeast isolated from Kefir and its ability to accumulate intracellular fats in deproteinated potato wastewater with different carbon sources. BioMed Res Int 2017:6061042. https://doi.org/10.1155/2017/6061042

Gientka I, Wirkowska-Wojdyla M, Ostrowska-Ligeza E et al (2022) Enhancing red yeast biomass yield and lipid biosynthesis by using waste nitrogen source by glucose fed-batch at low temperature. Microorganisms 10:1253. https://doi.org/10.3390/microorganisms10061253

Glencross BD, Huyben D, Schrama JW (2020) The application of single-cell ingredients in aquaculture feeds – a review. Fishes 5:22. https://doi.org/10.3390/fishes5030022

Gorte O, Hollenbach R, Papachristou I, Steinweg C, Selve A, Frey W, Syldatk C, Ochsenreither K (2020) Evaluation of downstream processing, extraction, and quantification strategies for single cell oil produced by the oleaginous yeasts *Saitozyma podzolica* DSM 27192 and *Apiotrichum porosum* DSM 27194. Fronts Bioeng Biotechnol 8:355. https://doi.org/10.3389/fbioe.2020.00355

Gowda NKS, Malathi V, Suganthi RU (2004) Effect of some chemical and herbal compounds on growth of *Aspergillus parasiticus* and aflatoxin production. Anim Feed Sci Technol 116:281–291. https://doi.org/10.1016/j.anifeedsci.2004.02.008

Guldhe A, Singh B, Rawat I, Ramluckan K, Bux F (2014) Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production. Fuel 128:46–52. https://doi.org/10.1016/j.fuel.2014.02.059

Hazelwood LA, Walsh MC, Luttik MAH, Daran-Lapujade P, Pronk JT, Daran J-M (2009) Identity of the growth-limiting nutrient strongly affects storage carbohydrate accumulation in anaerobic chemostat cultures of Saccharomyces cerevisiae. Appl Environ Microbiol 75(21):6876-6885. https://doi.org/10.1128/AEM.01464-09

Hicks RH, Chuck CJ, Scott RJ, Leak DJ, Henk DA (2019) Comparison of Nile red and cell size analysis for high-throughput lipid estimation within oleaginous yeast. Eur J Lipid Sci Technol 121(11):1800355

Ho S-P, Hsu T-Y, Chen M-H, Wang W-S (2000) Antibacterial effect of chloramphenicol, thiamphenicol and florfenicol against aquatic animal bacteria. The J V Med Sci 62(5):479–485

Jach ME, Serefko A, Ziaja M, Kieliszek M (2022) Yeast protein as an easily accessible food source. Metabolites 12:63. https://doi.org/10.3390/metabol2010063

Jiru TM, Abate D, Kiggundu N, Pohl C, Groenewald M (2016) Oleaginous yeasts from Ethopia. AMB Express 6:78. https://doi.org/10.1186/s13568-016-0242-8

Juszcyk P, Tomaszewska L, Kita A, Rymowicz W (2013) Biomass production by novel strains of *Yarrowia lipolytica* using raw glycerol, derived from biodiesel production. Bioresour Technol 137:124–131. https://doi.org/10.1016/j.biortech.2013.03.010

Khanal SK, Nindhia TG, Nitayavardhana S (2019) Biogas from wastes: processes and applications. In Sustainable Resource Recovery and Zero Waste Approaches, Taherzadah M.J. et al (eds), Elsevier B.V. 165-174. https://doi.org/10.1016/B978-0-444-64200-4.00011-6

Kolouchova I, Maťátkova O, Sigler K, Masák J, Řezanka T (2016) Production of palmitoleic and linoleic acid in oleaginous and non-oleaginous yeast biomass. Intl J Anal Chem 2016:7583684

Kruger JS, Cleveland NS, Yeap RY, Dong T, Ramirez KJ, Nagle NJ, Lowell AC, Beckham GT, McMillan JD, Biddy MJ (2018) Recovery of fuel-precursor lipids from oleaginous yeast. ACS Sustain Chem Eng 6(3):2921–2931. https://doi.org/10.1021/acsuschemeng.7b01874

Kumar RR, Dhanarajan G, Bhaumik M, Chopra J, Sen R (2017) Performance evaluation of a yeast biorefinery as a sustainable model for co-production of biomass, bioemulsifier, lipid, biodiesel and animal-feed components using inexpensive raw materials. Sustain Energy Fuels 1:923–931. https://doi.org/10.1039/c7se00010c

Kurtzman CP, Robnett CJ (2003) Phylogenetic relationships among yeasts of the "*Saccharomyces* complex" determined from multigene sequence analyses. FEMS Yeast Res 3(4):417–432

Lamers D, van Biezen N, Martens D, Peters D, van de Zilver E, Jacobs-van Dreumel N, Wijffels RH, Lokman C (2016) Selection of oleaginous yeast for fatty acid production. BMC Biotechnol 16:45. https://doi.org/10.1186/s12896-016-0276-7

Lapeña D, Kosa G, Hansen LD, Mydland LT, Passoth V, Horn SJ, Eijsink VGH (2020) Production and characterization of yeasts grown on media composed of spruce-derived sugars and protein hydrolysates from chicken by-products. Microb Cell Factories 19:19. https://doi.org/10.1186/s12934-020-1287-6

Liu H, Zhao X, Wang F, Jiang X, Zhang S, Ye M, Zhao ZK, Zou H (2010) The proteome analysis of oleaginous yeast *Lipomyces starkeyi*. FEMS Yeast Res 11:42–51. https://doi.org/10.1111/j.1567-1364.2010.00687.x

Lopes HJS, Bonturi N, Kerkhove EJ, Miranda EA, Lahtvee PJ (2020) C/N ratio and carbon source-dependent lipid production profiling in *Rhodotorula toruloides*. Appl Microbiol Biotechnol 104:2639–2649. https://doi.org/10.1007/s00253-020-10386-5

Martinez-Hernandez E, Amezcua-Allieri MA, Sadhukhan J, Anell JA (2018) Sugarcane bagasse valorization strategies for bioethanol and energy production. In: de Oliveira, A.B. (ed). Sugarcane Technology and Research. IntechOpen; 71-83.

Matsakas L, Bonturi N, Miranda E, Rova U, Christakopoulos P (2015) High concentrations of dried sorghum stalks as a biomass feedstock for single cell oil production by *Rhodosporidium toruloides*. Biotechnol Biofuels 8(1):6

Maura YF, Balzarini T, Borges PC, Evrard P, Vuyst LD, Daniel H-M (2016) The environmental and intrinsic yeast diversity of Cuban cocoa bean heap fermentations. Int J Food Microbiol 233:34–43. https://doi.org/10.1016/j.ijfoodmicro.2016.06.012

Miranda C, Bettencourt S, Pozdniakova T et al (2020) Modified high-throughput Nile red fluorescence assay for the rapid screening of oleaginous yeasts using acetic acid as carbon source. BMC Microbiol 20:60

Mishra SK, Suh WI, Farooq W et al (2014) Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. Bioresour Technol 155:330–333. https://doi.org/10.1016/j.biortech.2013.12.077

Morales-Palomo S, Liras M, González-Fernández C, Tomás-Dejó E (2022) Key role of fluorescence quantum yield in Nile red staining method for determining intracellular lipids in yeast strains. Biotechnol Biofuels Bioprod 15:37. https://doi.org/10.1186/s13068-022-02135-9

Nyyssölä A, Suhonen A, Ritala A, Oksman-Caldentey K-M (2022) The role of single cell protein in cellular agriculture. Curr Opin Biotechnol 75:102686

Ochsenreither K, Glück C, Stressler T, Fischer L, Christoph S (2016) Production strategies and applications of microbial single cell oils. Front Microbiol 7:1539. https://doi.org/10.3389/fmicb.2016.01539

Osorio-González CS, Saini R, Hegde K, Brar SK, Lefebvre A, Ramirez AV (2023) Carbon/Nitrogen ratio as a tool to enhance the lipid production in Rhodosporidium toruloides-1588 using C5 and C6 wood hydrolysates. J Clean Prod 384:135687

Pan L-X, Yang D-F, Shao L, Li W, Chen G-G, Liang Z-Q (2009) Isolation of oleaginous yeast from the soil and studies of their lipid producing capacities. Food Technol Biotechnol 47(2):215–220

Parsons S, Allen MJ, Chuck CJ (2020) Coproducts of algae and yeast-derived single cell oils: a critical reviews of their role in improving biorefinery sustainability. Bioresour Technol 303:122862

Patel A, Antonopoulou I, Enman J, Rova U, Christakopoulos P, Matsakas L (2019) Lipids detection and quantification in oleaginous microorganisms: an overview of the current state of the art. BMC Chem Eng 1:13. https://doi.org/10.1186/s42480-019-0013-9

Patel A, Karageorgou D, Rova E et al (2020) An overview of potential oleaginous microorganisms and their role in biodiesel and omega-3-fatty acid-based industries. Microorganisms 8(3):434. https://doi.org/10.3390/microorganisms8030434

Planonth S, Chantarasiri A (2022) The oleaginous yeast *Pichia manshurica* isolated from *Lansium domesticum* fruit in Thailand and its fatty acid composition of single cell oil. Biodivers 23(2):801–809

Polburee P, Yongmanitchai W, Lertwattanasakul N, Ohashi T, Fujiyama K, Limtong S (2015) Characterizatin of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-deived crude glycerol. Fungal Biol 119(12):1194–1204

Poontawee R, Lorliam W, Polburee P, Limtong S (2023) Oleaginous yeasts: Biodiversity and cultivation. Fungal Biol Rev 44:100295

Priyanka P, Kinsella GK, Henehan GT, Ryan B (2020) Nile red assay development for the estimation of neutral lipids in *Chlorella emersonii* and *Pseudokirchneriella subcapitata*. The Eur Biotech J 4(4):216–222. https://doi.org/10.2478/ebtj-2020-0025

Rakicka-Pustulka M, Miedzianka J, Jama D et al (2021) High-value-added products derived from crude glycerol via microbial fermentation using *Yarrowia* clade yeast. Microb Cell Factory 20:195. https://doi.org/10.1186/s12934-021-01686-0

Ramirez-Castrillón M, Jaramillo-Garcia VP, Rosa PD et al (2017) The oleaginous yeast *Meyerozyma guilliermondii* BJ281A as a new potential biodiesel feedstock: Selection and lipid production optimization. Front Microbiol 8:1776. https://doi.org/10.3389/fmicb.2017.01776

Ramirez-Castrillón M, Jaramillo-Garcia VP, Barros HL et al (2021) Nile red incubation time before reading fluorescence greatly influences the yeast neutral lipids quantification. Front Microbiol 12:619313

Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochemie 86:807–815. https://doi.org/10.1016/j.biochi.2004.09.017

Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–51. https://doi.org/10.1016/s0065-2164(02)51000-5

Reggiori F, Klionsky DJ (2013) Autophagic process in yeast: mechanism, machinery and regulation. Genetics 194:341–361. https://doi.org/10.1534/genetics.112.149013

Ritala A, Häkkinen ST, Toivari M, Wiebe MG (2017) Single cell protein-state-of-the-art, industrial landscape and patents 2001–2016. Front Microbiol 8:2009. https://doi.org/10.3389/fmicb.2017.02009

Robles-Iglesias R, Naveira-Pazos C, Fernández-Blanco C, Veiga MC, Kennes C (2023) Factors affecting the optimization and scale-up of lipid accumulation in oleaginous yeasts for sustainable biofuels production. Renew Sustain Energy Rev 171:113043

Saeed MS, Saeed A, Adnan M (2020) Production and utilization of single cell proteins – an overview. Curr Res Agri Far 1(4):9–12

Sagia S, Sharma A, Singh S, Chaturvedi S, Nain PKS, Nain L (2020) Single cell oil production by novel yeast *Trichosporon mycotoxinivorans* for complete and ecofriendly valorization of paddy straw. Electron J Biotechnol 44:60–68. https://doi.org/10.1016/j.ejbt.2020.01.009

Saini R, Osorio-Gonzales CS, Hegde K, Brar SK, Vezina P (2021) Effect of creating a fed-batch like condition using carbon to nitrogen ratios on lipid accumulation in *Rhodosporidium toruloides*. Bioresour Technol 337:125354

Schulze I, Hansen S, Großhans S, Rudszuck T, Ochsenreither K, Syldatk C, Neumann A (2014) Characterization of newly isolated oleaginous yeasts – *Cryptococcus podzolicus*, *Tricosporon porosum* and *Pichia segobiensis*. AMB Express 4:24

Shapaval V, Brandenburg J, Blomqvist J, Tafintseva V, Passoth V, Sandgren M, Kohler A (2019) Biochemical profiling, prediction of total lipid content

and fatty acid profile in oleaginous yeast by FTIR spectroscopy. Biotechnol Biofuels 12:140. https://doi.org/10.1186/s13068-019-1481-0
Shi J, Feng H, Lee J, Chen WN (2013) Comparative proteomics profile of lipid-cumulating oleaginous yeast: An iTRAQ – coupled 2-D LC – MS/MS analysis. PLOS ONE 8(12):e85532
Shokravi Z, Shokravi H, Chyuan OH et al (2020) Improving lipid productivity in microalgae by bilateral enhancement of biomass and lipid contents: a review. Sustainability 12:9083. https://doi.org/10.3390/su12219083
Sitepu IR, Ignatia L, Franz AK et al (2012) An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. J Microbiol Methods 9:321–328. https://doi.org/10.1016/j.mimet.2012.09.001
Slocombe SP, Ross M, Thomas N, McNeill S, Stanley MS (2013) A rapid and general method for measurement of protein in microalgal biomass. Bioresour Technol 129:51–57. https://doi.org/10.1016/j.biortech.2012.10.163
Sreeharsha RV, Mohan SV (2020) Obscure yet promising oleaginous yeasts for fuel and chemical production. Trends Biotechnol 38(8):873–887. https://doi.org/10.1016/j.tibtech.2020.02.004
Tchakouteu SS, Chatzifragkou A, Kalantzi O, Koutinas AA, Aggelis G, Papanikolaou S (2015) Oleaginous *Cryptococcus curvatus* exhibits interplay between biosynthesis of intracellular sugars and lipids. Eur J Lipid Sci Technol 115:657–672. https://doi.org/10.1002/ejlt.201400347
Thevenieau F, Nicaud J-M (2013) Microorganisms as sources of oils. OCL 20(6):D603. https://doi.org/10.1051/ocl/2013034
Tolieng V, Kunthiphun S, Savarajara A, Tanasupawat S (2018) Diversity of yeasts and their ethanol production at high temperature. J Appl PharmSci 8(02):136–142. https://doi.org/10.7324/JAPS.2018.8221
Vasconcelos B, Teixeira JC, Dragone G, Teixeira JA (2018) Optimization of lipid extraction from the oleaginous yeast *Rhodotorula glutinis* and *Lipomyces kononenkoae*. AMB Express 8:126. https://doi.org/10.1186/s13568-018-0658-4
Wang G, Liu L, Liang W (2018) Single cell oil production from hydrolysates of inulin by a newly isolated yeast *Papiliotrema laurentii* AM113 for biodiesel making. Appl Biochem Biotechnol 184:168–181. https://doi.org/10.1007/s12010-017-2538-9
Wang J, Ledesma-Amaro R, Wei Y, Ji B, Ji X-J (2020) Metabolic engineering for increased lipid accumulation in *Yarrowia lipolytica – a review*. Bioresour Technol 313:123707. https://doi.org/10.1016/j.biortech.2020.123707
Wang Q, Cui Y, Sen B, Ma W, Zheng RL, Liu X, Wang G (2017) Characterization and robust nature of newly isolated oleaginous marine yeast *Rhodosporidium* spp. From coastal water of Northern China. AMB Express 7:30. https://doi.org/10.1186/s13568-017-0329-x
White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal DNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J and T.J. White (Eds). PCR Protocols: a guide to methods and Applications. Academic Press, Inc, New York. 315-322.
Xing X, Wang Y, Huo N, Wang R (2018) *Candida ethanolica* strain Y18 enhances aroma of Shanxi aged-vinegar. Food Sci Technol Res 24(6):1069–1081. https://doi.org/10.3136/fstr.24.1069
Ye Z, Sun T, Hao H, He Y, Liu X, Guo M, Chen G (2021) Optimising nutrients in the culture medium of Rhodosporidium toruloides enhances lipids production. AMB Express 11:149. https://doi.org/10.1186/s13568-021-01313-6
Yook SD, Sanchez RS, Ha JH, Park JM (2016) Effects of the ratio of carbon to nitrogen concentration on lipid production by bacterial consortium of sewage sludge using food wastewater as a carbon source. Korean J Chem Eng 33(6):1805–1812. https://doi.org/10.1007/s11814-016-0058-x
Younes S, Bracharz F, Awad D, Qoura F, Mehlmer N, Brueck T (2020) Microbial lipid production by oleaginous yeast grown on *Scenedesmus obtusiusculus* microalgae biomass hydrolysate. Bioprocess Biosyst Eng 43:1629–1638. https://doi.org/10.1007/s00449-020-02354-0
Yu X, Dong T, Zheng Y, Miao C, Chen S (2015) Investigations on cell disruption of oleaginous microorganisms: hydrochloric acid digestion is an effective method for lipid extraction. Eur J Lipid Sci Technol 117:730–737. https://doi.org/10.1002/ejlt.201400195
Yu R, Campbell K, Pereira R, Björkeroth J, Qi Q, Vorontsov E, Sihlbom C, Nielsen J (2020) Nitrogen limitation reveals large reserves in metabolic and translational capacities of yeast. Nat Commun 11:1881. https://doi.org/10.1038/s41467-020-15749-0
Zainuddin MF, Fai CK, Ariff AB, Rios-Solis L, Halim M (2021) Current pretreatment/cell disruption and extraction methods used to improve intracellular lipid recovery from oleaginous yeasts. Microorganisms 9:251. https://doi.org/10.3390/microorganisms9020251
Zhang J, Fang X, Zhu X-L, Li Y, Xu H-P, Zhao B-F, Chen L, Zhang X-D (2011) Microbial lipid production by the oleaginous yeast *Cryptococcus curvatus* O3 grown in fed-batch culture. Biomass Bioenergy 35:1906–1911. https://doi.org/10.1016/j.biombioe.2011.01.024
Zhang H, Wu C, Wu Q, Dai J, Song Y (2016) Metabolic flux analysis of lipid biosynthesis in the yeast *Yarrowia lipolytica* using 3C-labled glucose and gas chromatography mass spectrometry. PLOS ONE 11(7):e0159187. https://doi.org/10.1371/Journal.pone.0159187

Publisher's Note