Evaluation of oleaginous yeasts isolated from lignocellulosic waste, sugarcane bagasse as a potential source for single cell oil production and other biochemical metabolites.

Lesetja Moraba Legodi (lesetja.legodi@ul.ac.za)
University of Limpopo - Turfloop Campus: University of Limpopo https://orcid.org/0000-0002-4386-2765
Kgabo L Maureen Moganedi
University of Limpopo - MEDUNSA Campus: Sefako Makgatho Health Sciences University

Research Article

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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 sustainability of fuel energy and food. The aim of this study was to evaluate the potential of yeast isolates from sugarcane bagasse for single oil production.

Methods

Oleaginocity of the yeasts was confirmed through gravimetric analysis of lipids, Nile red, sulfo-phosphovanillin (SPV) and gas chromatography method for fatty acid methyl esters (FAME). Identification of the selected yeasts was carried out through 5.8S of the ribosomal internal transcribed spacer, ITS and 26S ribosomal DNA (rDNA) sequences.

Results

The yeast isolates accumulated lipids between 28% and 67% of the dry cell weight, and 22%-33% based on SPV assay, qualifying them as oleaginous yeasts. the isolated yeasts were identified as *Candida ethanolica* and *Pichia manshuriica*.

Conclusion

The lipids contained high level of fatty acids in the following order: oleic acid, palmitic acid, stearic acid and linoleic acid, which made up 82% of the total lipids. The fatty acids composition of the selected yeast species was found to be suitable for the applications in biofuel, nutraceutical and food industry.

1. 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 supply of polyunsaturated fatty acids (PUFA) to supplement food. 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 food security. These concerns necessitated the need to find alternative sources of oil. Microbial oils, also termed single cell oils (SCO) can provide 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 SCO 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 of triacylglycerols (TAGs). Microorganisms that are capable of accumulating between 20% and 80% lipid per dry biomass are called oleaginous microorganisms (Lamers, et al., 2016; Ochsenreither, et al., 2016; Galán, et al., 2019).

Intracellular lipid accumulation in oleaginous microorganisms is triggered by the depletion of essential nutrients such as nitrogen and phosphorus in the excess of carbon. Under such conditions, the cellular functions like nucleic acid and protein synthesis and eventually cell growth ceases. The excess carbon is metabolized into lipids through a cascade of biochemical reactions (Diwan, et al., 2018). Oleaginous yeasts are thus 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).

Agro-industrial wastes have attracted much of the interest as inexpensive and abundant form of organic carbon source that can substitute refined sugars. Example of such is the sugarcane bagasse (SCB), which is a fibrous residue of cane stalk that remains after the crushing and extraction of the juice from the sugarcane. The SCB is a lignocellulosic material consisting of approximately 40–50% cellulose, 25–35% hemicellulose and 17–20% lignin and is the largest cellulosic agro-industrial waste (de Barros, et al., 2013; Motaung and Anandjiwala, 2015; Chambon, et al., 2018).In South Africa, sugarcane (*Saccharum officinarum*) is cultivated in the KwaZulu-Natal, Mpumalanga and Eastern Cape with approximately more than 20,000 registered growers. The majority of the sugarcane crop is produced in KwaZulu-Natal (Mokhena, et al., 2018). Pretreatment of SCB is necessary for efficient conversion to SCOs. The conversion process involves several stages, mainly pretreatment, hydrolysis, fermentation and lipid extraction. Pretreatment alters, in some instances removes, the structural and compositional barriers of lignocellulosic material to hydrolysis in order to improve the efficiency of hydrolysis and consequently increase the yield of simple sugars (Mosier, et al., 2005; Zhang, et al., 2018), such as glucose and xylose, which provide excess carbon source for the production of fuel energy and a variety of renewable products (Bussamra et al., 2020). Various extraction methods have been reported, and some methods rely on the use of solvents without any cell pretreatment while others

use acid, glass beads, lytic enzyme to destroy or disrupt the cell wall followed by extracting lipids using solvents. Nonetheless, extractability differs from one species to another (Suzuki et al., 1973).

Oleaginous yeasts have been studied over decades for the production TAGs, notably the challenge has been the cost of supplying the organic carbon source (Vyas and Chhabra, 2017). 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,) and unconventional yeasts such as *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Kazachstania unispora* and *Zygotorulaspora florentina* (Gientka, et al., 2017). Sugarcane bagasse pith was used previously as a solid substrate for fungal growth and a good source of microorganisms, (Martinez-Hernandez, et al., 2018). This study sought to screen and characterises oleaginous yeasts strains with diverse carbon utilization from sugarcane bagasse.

2. Materials And Methods

2.1. Chemical and reagents

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

2.2. Sample collection

Sugarcane bagasse 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.

2.3. Isolation of microorganisms

2.3.1. Microbial propagation

Three grams of sugarcane bagasse was 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) and 0.025% (w/v) Na-propionate to suppress bacterial and fungal growth. The culture was incubated at 30°C, 150 rpm (Biobase Incubator, BJPX-1102C) for 24 h.

2.3.2. Microbial enrichment

A 10 mL of culture propagated in YPD medium contained in 15 mL conical tube was centrifuged at 2250 x g using 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 Chanavati et al. 2014 with modification in 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.

2.3.3. Isolation of yeasts on agar medium

The composition of the agar medium was as follows: 20 g/L glucose/or 20 g/L 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 glucose and xylose agar. The agar plates were incubated at 30°C, static conditions (Biobase Incubator, BJPX-1102C) for 72 h on agar containing glucose and 120 h agar containing xylose. Larger colonies showing different morphologies obtained from glucose and xylose agar media were further streaked onto either glucose agar or xylose agar for two passages to obtain pure yeast isolates.

2.4. 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'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the small subunit and large subunit of the 5.8S (White, et al., 1990). Primer set NL-1 (5'- GCATATCAATAAGCGGAGGAAAAG – 3') and NL-4 (5'- GGTCCGTGTTTCAAGACGG – 3') were used to amplify the D1/D2 domain of the large subunit 26S ribosomal DNA, rDNA (Kurtzman and Robnett, 2003). 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 minutes. Subsequent denaturation was at 94°C, 30 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 1 minute. A final extension at 68°C for 10 minutes 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. 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.

2.5. Production of lipid under nitrogen limiting conditions

The following medium composition: 30 g/L glucose, 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 was adjusted to 5.5 was used for the production of lipid. A single colony from a revived culture for each isolate (Isolate B2, C2, E2, 6 XP, 11 XP, and 15 X) was inoculated into 100 mL medium contained in 250 Erlenmeyer flask. The culture was incubated at 30°C, shaking at 150 rpm for seven days. Samples were withdrawn every 24 h to determine weight of biomass and optical density, OD 600nm by spectrophotometer, (Jenway 7315 Spectrophotometry, UK). Dry biomass (cell dry weight, CDW) determination over growth period was done according to method described by Younes, et al. (2020). A 2 mL of each yeast culture (triplicates) was transferred to pre-weighed 2 mL microtubes and centrifuged at 7378 x g for 5 min and supernatant discarded. The pellets were washed twice with phosphate buffer saline (PBS 1 X, pH 7.4) and dried at 60°C using hot air oven (Scientific, 240 L Digital incubator, Model 298, S.A) for 24 h. Weight was determined using weighing balance (RADWAG, Model AS 220. R2, Poland).

2.6. Qualitative screening for oleaginous yeasts

A Nile Red (NR – 9-diethylamino-5H-benzo [α] phenoxazin-5-one) dissolved in dimethyl sulfoxide (DMSO, stock solution 0.1mg/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 x g using bench top refrigerated microfuge SIGMA 1-14K, (Angle rotor 24 x 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 OD600 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 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 fluorescence Microscopy equipped with 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 X objective lens, which enabled visualization of stained lipid bodies.

2.7. Qualitative and quantitative determination of lipid content inoleaginous yeasts

2.7.1. Gravimetric determination of yeast biomass

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

2.7.2. Yeast cell wall destruction and lipid extraction

Lipid extraction was done by applying Acid-Heat Extraction method (Yu, et al., 2015). A dried and wet biomass of yeasts were dissolved in 3.5 mL of 4 M HCl contained in 15 mL conical tubes and incubated in water bath with temperature set at 95°C for 2 h. After the incubation, the tubes were cooled to room temperature and centrifuged at 2250 x g for 10 min. Lipid extraction followed 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 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 hot air oven. The amount of dried crude lipid was quantified gravimetrically and lipid content was calculated. The lipid content is presented as percentage of lipid weight relative to dry biomass weight (% w/w) (Ramirez-Castrillón, et al., 2017).

2.8. Biochemical determination of biomolecule content

2.8.1. Total lipids content

For Sulfo-Phosphovanillin (SPV) method, 2 g of vanillin was dissolved in warm water (60°C) and further diluted with 85% Orthophosphoric acid to 500 mL (Phosphovanillin reagent). Stock solution for standard concentrations was prepared by dissolving 10 mg/100 mL of cholesterol in chloroform. Concentration range of standard solution was 50-1000 µg/mL. Both the standards and 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 content in the tubes were mixed by shaking and later submerged in 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 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 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 concentration expressed in terms of g/L was attained by diving the final value obtained from the SPV assay with the total volume of the chloroform-lipids prior to evaporation. The lipid content ($Y_{L/X}$) was calculated according to the formula below:

$$Y_{L/X} = \frac{Li - Lo}{Xi - Xo} \text{ Eq. (1)}$$

where, X_i and L_i are the dry call 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.

The lipid yield ($Y_{L/S}$) or biomass yield ($Y_{X/S)}$ was calculated according to the following formula:

$$Y_{L/S} = \frac{Li - Lo}{So - Si} \text{ or } Y_{X/S} = \frac{Xi - Xo}{So - Si} \text{ Eq. (2)}$$

where, S_i and S_0 are the glucose concentration on day t_i and on the first day (t_0), respectively. L_i and L_0 are the lipid concentration on day t_i and the first day (t_0), respectively.

The lipid productivity (P_{lipid}) was calculated by the formula:

$$P_{lipid}(\text{g L}^{-1}\text{ day}^{-1}) = \frac{Lmaxi}{ti} \text{ Eq. (3)}$$

where, L_{maxi} is the maximum lipid concentration on day t_i.

The biomass productivity ($P_{Biomass}$) was calculated by equation:

$$P_{Biomass}(\text{g L}^{-1}\text{ day}^{-1}) = \frac{(Xi - Xo)}{ti} \text{ Eq. (4)}$$

where, X_0 and X_i is the biomass concentration on day t_0 and t_i, respectively.

2.8.2. Carbohydrates content

A total carbohydrate was estimated by use of phenol-sulfuric acid following the method by Dubois et al., 1956 and Eldalatouy et al., 2016). Ten milligram 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 water bath with temperature set at 90°C for 5 min and allowed to cool prior to the reading of absorbance at A490nm. The calibration curve was prepared with 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.

2.8.3. Protein content

The protein extraction was done by modifying the method by Slocombe et al. (2013), hot- trichloroacetic acid (TCA). Ten milligram 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 water bath with temperature set at 75°C for 10 min and later allowed to cool at room temperature. This was followed by centrifugation of the mixture at 2250 x 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 x g for 10 min at 4°C. The supernatant was retained for protein assay. Protein assay was carried out by following the Lowry method. Reagents required for the assay are as follows; 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, 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 minutes at room temperature prior to reading absorbance A600nm. Again, the calibration curve was prepared with 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.

2.9. Lipid trans-esterification and component profile analysis

The conversion of lipid to fatty acid methyl ester (FAME) followed methods described by Yook, et al. (2019). 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 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 x g using 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 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.

3. Results

3.1. Screening of oleaginous yeasts and molecular identification

A total of 14 yeasts (B1, B2, C2, E1, E2, 5-XP, 11-XP, 15-X, 6-XP, 13-X, 10-XP, 3-XP, 12-X, 4-X) were isolated from sugarcane bagasse. The isolates were grown on carbon rich (glucose) and nitrogen limiting medium. The initial pH of the medium was 5.5 and as fermentation progresses the medium became very acidic to below pH 3 by the end of cultivation. The amount of glucose consumed was between 22.6 g/L and 28.6 g/L (Table 1).

Table 1
Glucose utilization and change in pH during cultivation of yeast isolates.

Yeast isolates	Initial glucose content (g/L)	Initial pH level of the medium	Final glucose content (g/L)	Glucose consumption rate (g/L/d)	Final pH level of the medium
B2	30	5.5	1.85 ± 0.045	4.02	2.32
C2	30	5.5	1.44 ± 0.022	4.08	2.24
E2	30	5.5	2.93 ± 0.013	3.87	2.25
6-XP	30	5.5	7.32 ± 0.041	3.24	2.29
11-XP	30	5.5	5.10 ± 0.033	3.56	2.37
15-X	30	5.5	2.73 ± 0.013	3.90	2.40

The exponential growth of yeasts began after 24 h of cultivation and maximum OD_{600} nm of 10–15.5 was attained, followed by decrease after 168 h. The yeast biomass showed increase over time of cultivation until 168 h with maximum biomass of between 2 and 3 g/L (Fig. 1a-c).

After 72 h, the yeast cultures were harvested under fluorescence microscopy using the nile red fluorescent dye for the detection of accumulated intracellular lipids. Six of the yeast isolates (B2, C2, E2, 6-XP, 11-XP, and 15-X) were identified as potential oleaginous yeasts based on the presence of the lipid bodies stained and fluorescing as yellow-gold bodies (Fig. 2). The fluorescence intensity often corresponds to the amount of intracellular lipids and the intensity varied between the isolates.

The six isolates were genetically identified based on the sequences of the ITS region of the 5.8S rDNA and D1/D2 domains of the 26S rDNA. The molecular identification revealed that these isolates belonged to two genera viz. *Candida* and *Pichia* (Table 2).

Table 2
Molecular identification of oleaginous yeasts based on 5.8S of the ITS region of the rDNA and the 26S of the D1/D2 domain of the rDNA.

Yeast isolate code no.	Yeast isolate taxa	Sequence length (bp)	Sequence identity (%)	GenBank Accession no
B2	*Candida ethanolica*	576	99	KY283163.1
C2	*Candida ethanolica*	582	99	KY283163.1
E2	*Candida ethanolica*	581	99	KY283163.1
6-XP	*Candida ethanolica*	580	99	KY283163.1
11-XP	*Pichia manshurica*	580	100	MK394164.1
15-X	*Pichia manshurica*	581	100	MK394164.1

3.2. Lipid production in shake flask cultivation

The yeasts, *C. ethanolica* B2, *C. ethanolica* C2, *C. ethanolica* E2, *C. ethanolica* 6-XP, *P. manshurica* 11-XP, *P. manshurica* 15-X were further investigated for lipid production in high sugar and nitrogen, 2.5-5.0 g/L medium. 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). After 7 days of cultivation, the yeast cultures were harvested and biomass, lipid content, lipid concentration and lipid productivity were determined. The biomass was determined gravimetrically whilst the lipid was determined by both the gravimetric method and the SPV assay. The 5 g/L nitrogen medium yielded a higher yeast biomass than the 2.5 g/L nitrogen medium. The lipid mass for *C. ethanolica* strains B2, C2 and E2 remained higher than *C.*

ethanolica 6-XP and other *P. manshurica* strains. However, the lipid content was higher in yeasts cultured in low nitrogen medium with the exception of *C. ethanolica* E2 strain. The biomass ranged from 0.258 to 0.339 g in high (5 g/L) nitrogen, and 0.123 to 0.186 g,in low (2.5 g/L) nitrogen medium (Table 3). The highest biomass was attained by *C. ethanolica* E2. Lipids were more extractable from yeasts with low biomass than from high yeast biomass. This could be due to biomass-solvent ratios, which therefore necessitate the need to optimize the extraction method for high biomass. The total lipid content of all the yeasts species relative to dry biomass was greater than 20% for yeasts cultured in 2.5 g/L nitrogen medium. The lipid content ranged from 28 to 67% g lipids/g dry biomass. *P. manshurica* 15-X produced higher lipid yield (g lipids/g dry biomass) of 67%.

Table 3
Biomass, and lipid content determined gravimetrically for oleaginous yeasts cultivated in high sugar and limited nitrogen conditions for lipid production after 168 h (7 days).

Yeast species	Lipid (g), C/N: 30/5	Yeast biomass (g), C/N: 30/5	Lipid content (% g Lipid/g biomass)	Lipid (g), C/N: 30/2.5	Yeast biomass (g), C/N: 30/2.5	Lipid content (% g Lipid/g biomass)
Candida ethanolica B2	0.079	0.307	25.7	0.061	0.173	35
Candida ethanolica C2	0.065	0.258	25.2	0.051	0.156	33
Candida ethanolica E2	0.158	0.339	46.6	0.053	0.186	28
Candida ethanolica 6-XP	0.053	0.356	14.9	0.067	0.146	46
Pichia manshurica 11-XP	0.051	0.264	19.3	0.062	0.123	50
Pichia manshurica 15-X	0.050	0.263	19.0	0.09	0.135	67

The SPV assay confirmed the lipogenecity of the yeasts with the lipid content ranging from 22–33% (Table 4). The lipid content discrepancies between the SPV assay and gravimetric method could be that in the later the lipid might be containing lipo-proteins and other contaminants, as well as the sensitivity of the method, which contributed to the high lipid mass.

Table 4
Biomass, and lipid content determined by Sulfo-phosphovanillin assay for oleaginous yeasts cultivated in high sugar and limited nitrogen conditions for lipid production after 168 h (7 days).

Yeast species	Yeast biomass (g/L), C/N: 30/5.0	Lipid (g/L), C/N: 30/5.0	Lipid content (% g/L Lipid/g/L biomass, C/N: 30/5.0)	Biomass productivity (g/L/d), C/N: 30/5.0, Eq. (4)	Lipid productivity (g/L/d), C/N: 30/5.0, Eq. (3)	Yeast biomass (g/L), C/N: 30/2.5	Lipid (g/L), C/N: 30/2.5	Lipid content (% g/L Lipid/g/L biomass, C/N: 30/2.5)	Biomass productivity (g/L/d), C/N: 30/2.5 Eq. (4)	Lipid productivity (g/L/d), C/N: 30/2.5 Eq. (3)
Candida ethanolica B2	7.68	1.34 ± 0.0967	17.4	1.10	0.19	4.33	0.93 ± 0.0539	22	0.62	0,13
C. ethanolica C2	6.45	0.98 ± 0.0394	15.2	0.92	0.14	3.90	1.08 ± 0.0296	28	0.56	0.15
C. ethanolica E2	8.48	1.42 ± 0.0109	17.0	1.21	0.20	4.65	1.46 ± 0.0067	31	0,66	0.21
C. ethanolica 6-XP	8.90	1.04 ± 0.0000	12.0	1.27	0.15	3.65	0.83 ± 0.0136	23	0.52	0.12
Pichia manshurica 11-XP	6.60	0.97 ± 0.0401	15.0	0.94	0.14	3.08	0.80 ± 0.0204	26	0.44	0.11
P. manshurica 15-X	6.58	0.91 ± 0.1673	14.0	0.94	0.13	3.37	1.11 ± 0.0204	33	0,48	0.16

When the SPV reacts with lipids it produces a pink to purple color. The color intensity is proportional to the lipid in the mixture. Both the gravimetric and SPV methods confirmed the presence of lipid produced by all the yeasts species. The *C. ethanolica* E2 and *Pichia manshurica* 15-X showed high lipid productivity of 0.21 g/L/d and 0.16 g/L/d, respectively. Based on the SPV assay results, the biomass with lipid content of greater than 20% (w/w) were further used for determining the carbohydrates and proteins.

3.3. Carbohydrates and proteins contents

Apart from lipids (SCO), yeast cells contain carbohydrates, proteins and other components that can be used in biofuels, food and animal feed industries as nutritional supplements. The six 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, viz carbohydrates and proteins of the oleaginous yeast cell dry weight (Fig. 3).

The maximum yields (Eqs. 1 and 2) expressed in g product formed per gram glucose consumed for the biomass, lipids, carbohydrates and proteins for the six oleaginous yeasts are shown in Fig. 4. Based on the stoichiometric analysis of every gram of carbon (glucose) consumed, only between 10–20% of the carbon was used for achieving biomass. The biomass yield ranged between 0.124 g (12.4%) and 0.172 g (17.2%) biomass per gram of glucose consumed.

The highest biomass was attained by *C. ethanolica* E2 (17.2%), whilst the *Pichia* strains produced the lowest biomass (12.4%). More of the carbon (glucose) metabolized was either lost as carbon dioxide or use in the formation of other products. Figure 5 summarises the carbon utilization for selected yeast strains, *C. ethanolica* E2. The substrate consumption, nitrogen consumption, carbon dioxide production, and biomass production are illustrated. The effect of biomass on biochemical metabolites is examined, which highlights the importance of achieving high biomass. The results suggest that more carbon could have been used for cell maintenance other than increasing biomass or carbon redirected to formation of other products and some liberated as carbon dioxide (CO_2).

3.4. The conversation of fatty acids to fatty acid methyl esters

The conversion of the extracted yeast lipids to FAME was carried out using acid-catalyzed transesterification reaction to determine fatty acid composition by Gas Chromatography. The main FAME in the lipids from all yeast species, with exception of *C. ethanolica* B2 and *C. ethanolica* 6-XP, were found to be palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c) and linoleic (C18:2n6c) acids. Their percentage ranged between 74% and 82% of the FAME analysed and appeared higher than the reference standard. The highest percentage (82%) was achieved by *P. manshurica* 15-X (Table 5). 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, palmitic and stearic acids making 61.6% and 68% for *C. ethanolica* B2 and *C. ethanolica* 6-XP, respectively. *Candida ethanolica* B2 was the only strain showing the presence of margaric acid (C17:0).

The moderate FAME were myristic (C14:0) and palmitol (16:1) acids, making up approximately 14% of the total lipids 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. *C*andida *ethanolica* strains and *P. manshurica* strains produced saturated fatty acids (SFA) ranging from 45–54% of their total lipids (fatty acids methyl esters), whereas strains of *C. ethanolica* B2 and 6-XP accumulated saturated fatty acids between 68% and 72% of their total lipids analyzed.

The monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids were in between 16% – 33% and 11% – 20%, respectively. The presence of PUFA, which is dominated by omega-6-fatty acids a linoleic (C18:2n6c) can be vital in food supplements.

Table 5
The 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	0.66	3.02	3.10	0.59	3.5	2.85
Palmitic acid	C16:0	7.60	7.43	8.05	7.72	8.11	9.5	7.38
Palmitoleic acid	C16:1	2.22	0.69	2.88	2.68	0.45	2.16	2.01
Margaric acid	C17:0	2.35	0.30	*ND	*ND	*ND	*ND	*ND
Stearic acid	C18:0	5.33	7.13	6.76	6.90	7.17	8.39	8.06
Linolenic acid	C18:3	2.97	*ND	*ND	1.80	0.37	1.34	1.01
Elaidic acid	C18:1n9t	2.28	1.21	0.35	0.30	1.17	0.32	ND
Oleic acid	C18:1n9c	4.67	2.32	10.53	10.13	2.03	8.15	9.78
Linolelaidic acid	C18:2n6t	1.43	0.29	0.18	*ND	0.19	0.18	ND
Linoleic acid	C18:2n6c	1.29	1.16	6.29	5.97	1.04	6.08	5.67
Arachidic acid	C20:0	5.62	*ND	0.55	0.50	*ND	0.39 0.46	*ND
Gondoic acid	C20:1	0.27	0.19	0.18 1.89	*ND	*ND	*ND	*ND
Eicosadienoic acid	C20:2	1.13	1.07	0.37	*ND	0.97	*ND	*ND
Behenic acid	C22:0	5.50	0.44	0.21	0.45	0.36	*ND	*ND
Erucic acid	C22:1	3.32	*ND	0.38	0.60	*ND	0.68	0.56
Docosanoic acid	C22:2	4.95	*ND	0.38	0.60	*ND	0.29	*ND
Lignoceric acid	C24:0	0.18	0.75	0.82	0.29	*ND	0.30	0.36
Total fatty acids (FA)		**53.73**	**23.65**	**42.84**	**41.04**	**22.46**	**41.74**	**37.68**
Total saturated FA		29.20	16.71	19.41	18.96	16.23	22.54	18.65
Total monosaturated FA		12.75	4.41	14.32	13.71	3.65	11.31	12.35
Total polyunsaturated FA		11.77	2.52	7.22	8.37	2.58	7.89	6.68
*ND refers to non-detected.								

4. Discussion

Screening of oleaginous yeasts and molecular identification

Amongst the heterotrophic microorganisms, oleaginous yeasts are regarded as alternative source for lipid production because of the relatively high biomass yield, fast lipid accumulation, lipid yield and productivity (Vasconcelos, et al., 2018). The most studied oleaginous yeasts species belong to the genera of *Rhodotorula*, *Rhodosporidium*, *Yarrowia*, *Candida*, *Cryptococcus* and *Lipomyces* (Pan et al., 2009). 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). *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; Xing, et al., 2018; Tolieng, et al., 2018). These yeast species have not been studied extensively for their ability to accumulate lipids.

This study demonstrated the lipid producing capabilities of *Candida ethanolica* and *Pichia manshurica* isolates. Vincent et al. (2018) isolated and identified oleaginous yeasts from different sources, fruit surfaces, sugarcane juice, sago effluent and agricultural soil. Six species were identified, *P. manshurica* (1/21), *Candida krusei* (8/21), *C. parapsilosis* (1/21), *Pichia guilliermondii* (2/21), *Clavispora lusitaniae* (1/21) and *K. marxianus* (4/21). The limitation of the study was that the growth kinetics, lipid production and lipid profiles of these species were not assessed. The oleaginocity of the yeasts was based on fluorescent microscopy using Sudan IV staining dye under light microscope (Vincent, et al., 2018).

Qualitative detection of lipids

The *C. ethanolica* and *P. manshurica* strains were isolated from sugarcane bagasse and selected based on their ability to grow in a high sugar and limited nitrogen medium. This was because the lipid accumulation capacity of oleaginous microorganisms requires limited or depleti nitrogen to redirect excess carbon to lipid synthesis (Pan, et al., 2009). Nutrition is a major factor; the number of lipid bodies inside the cells is reduced at low C/N ratio (Garay et al., 2014). The cells size differences also influence the fluorescence intensity. The larger cells accumulate more lipid bodies, fluoresce becomes intense and lipid bodies appear more golden (Elfeky, et al., 2019). Yeast cells showing high fluorescence intensity are selected for quantitative analysis of neutral lipids by other methods.

Oleaginous yeast regulates their mechanisms to varying degrees depending on the strain in for adaptation under limited nitrogen conditions in order to alter the lipid metabolism and enhance lipid production. The adaptation introduces the modification in the expression of genes or the enzyme machinery involved in the lipid biosynthesis pathway (Patel, et al., 2020). Gravimetric method determines the microbial lipid content by weight of the extracted lipids relative to the weight of the yeast biomass. It is frequently used as reference standard to validate other methods (Chen, et al., 2018; Patel, et al., 2019). Several laboratory methods for extracting lipids are established, however, there is no single method that ensures 100% recovery of intracellular lipids. The recovery efficiency of the lipids from the oleaginous microbial biomass is hindered by the cell wall, which differs between organisms. In order to disrupt the cell wall, mechanical and chemical methods or these methods in combinations are utilized. Such methods including hot-acid hydrolysis, microwave irradiation, sonication, high pressure homogenization, bead beating and swelling by osmotic pressure have been applied to disintegrate cell wall to make the lipids extractable (Geciova, et al., 2002; Patel, et al., 2018). In this study, the hot-acid method was applied to disintegrate the cell wall of the yeasts in order to increase permeability of the extracting solvent, Folch's method. All the yeast species, viz. *C. ethanolica* strains and *P. manshurica* isolated accumulated lipid content of over minimum 20% (w/w), thus making them oleaginous yeasts. The lipid content of *P. manshurica* strains was between 50%-67% higher than for *C. ethanolica* strains (28%-46% w/w).

Few studies as indicated below have reported the production of lipids by *C. ethanolica* and *P. manshurica* and the reported lipids content are usually less than 20% (w/w) of dry cell weight. The accumulated lipid is found to be dependent on the species type, strain, substrate and cultivation conditions. For instance, cultivation of *C. ethanolica* AM320 in inulin hydrolysate (fructose and glucose) produced 15% (w/w) of lipid relative to its cell dry weight (Wang, et al., 2018). Arous, et al. (2017) also reported low lipid content by *C. ethanolica* M1 and *P. manshurica* with lipid of 12% (w/w) and 15% (w/w), respectively. These lipids levels are lower than the lipid content obtained in this study. A lipid content of 64.8% (w/w) by *P. manshurica* DMKU-UbC9(2) strain (Polburee, et al., 2015) growing in glycerol medium was comparable to the lipid content of *P. manshurica* 15-X obtained in this study. The harvesting time at any stage of cultivation also influences the accumulation of lipids. Harvesting at a late cultivation stage correlates with high lipid content (Liu, et al., 2010). Although there is limited literature on the lipid content of *C. ethanolica* and *P. manshurica*, other related yeast strains are reported. *Pichia etchellsii* BM1 accumulated 25% (w/w) of lipid making it oleaginous yeast (Arous, et al., 2017). *Pichia manshurica* CHC34, *Candida parapsilosis* CH08 and *Pseudozyma parantarctica* CHC28 accumulated high lipids in day 1 of cultivation compared to day 5 of cultivation in glucose medium. The lipids content for the yeasts above, following the same order of names in day 1 and 5 were 11.43%, 24.21%, 43.54% and 9.52%, 9.72%, 39.89%, respectively (Areesirisuk, et al., 2015).

Other candida species, *C. tropicalis* L2 and *C. quercitrasa* L3 have accumulated very little lipid in glucose medium, 2.29% and 2.17% (w/w), respectively. An exception was for *C. tropicalis* S1, which has lipid content of 20.95%. However, cultivation of *Trichosporon mycotoxinivorans* S2 in glucose + xylose medium resulted in lipid content of 44.86% (w/w), (Sagia, et al., 2020). Similar lipid content (44% w/w) is attained by *Rhodosporidium* TJUWZ4 cultured in glucose medium (Wang, et al., 2017). *Trichosporon* sp. (RW) growing in glucose medium yielded 35.98% (w/w), (Brar et al., 2017). In cases of low lipid content, the extraction efficiency of the lipid can be increased by optimized microbial cell wall pretreatment. The choice of microbial cell wall pretreatment technique differs with the type of microorganisms studied because of their cell wall

composition, which limit extractability of lipids (Patel, et al., 2019). The extraction of lipids from acid-treated cells with hexane showed higher lipid content of 42% (w/w) and 40% (w/w) for *Rhodotorula toruloides* and *Lipomyces starkeyi*, respectively. Similar results were obtained using Folch's method (Bonturi, et al., 2015). Acid-treatment of *L. starkeyi* led to higher lipid recovery yield (Kruger, et al., 2018).

The SPV requires cell disruption and lipid extraction. This assay has advantages over Folch extraction and gravimetric quantification. The gravimetric estimation of lipids may erroneously include lipophilic proteins and pigments. Therefore, during the acid-thermal reaction (treatment) of the SPV assay the lipophilic proteins and other products are degraded (Patel, et al., 2019). *Candida* strains grew better in both media than the *Pichia* strains. *Candida ethanolica* E2 and *C. ethanolica* 6-XP attained the highest biomass of 8.48 g/L and 8.9 g/L, respectively. Biomass and lipids are known to be depended on the type of the medium and conditions used. Guo et al. (2019) cultivated wild type *Rhodosporidium toruloides* RC 2.1389 and *R. toruloides* R-ZL2 and R-ZY13 UV mutants were cultured in FM medium. The maximum amount of lipid produced by the wild type yeast RC was 1.56 g/L after 96 h of cultivation. The mutants R-ZL2 and R-ZY13 produced 2.24 and 2.15 g/L. The lipids productivity was low for wild type yeast, 16.29 ± 1.6 mg/L/h. The mutants exhibited high productivity of 23.32 ± 1.8 and 22.40 ± 2.5 mg/L/h for R-ZL2 and R-ZY13 mutants, respectively (Guo et al., 2019). In shake flask cultivation, wild type *Lipomyces starkeyi* (WT) and UV mutants *L. starkeyi* A1 and A3 were cultivated in a medium containing 30% glucose and 70% xylose for 168 h. The mutants A1 showed slight improvement in biomass (13.74 g/L) and lipid content (39.60% ± 1.3), whilst mutant A3 showed improvement in lipid content (38.11% ± 1.2) when compared to biomass 12.32 g/L and lipid content 35.02 ± 1.59 for wild type yeast (WT), (Tapia et al., 2012). In fed-batch both the biomass and lipid content increased in all the *L. starkeyi* yeasts. After 144 h of cultivation, mutant A1 showed higher biomass 88.7 g/L and lipid content of 55% compared to *L. starkeyi* (WT) with biomass of 76 g/L and lipid content of 43.8%. The lipid productivity for mutant A1 was 0.34 g/L/h higher than 0.26 g/L/h for wild-type (Tapia, et al., 2012).

Carbohydrates and proteins

Yeast responds to stress by accumulating either carbohydrates or lipids. Oleaginous yeast strains produce lipids as a protective mechanism under the stressed conditions, e.g. depleted nitrogen (Shi, et al., 2013). The microbial response in nutrient limitations, particularly nitrogen or phosphorus requires robust metabolism in order to attain maximum cell growth (biomass). In nitrogen limitation, cellular carbohydrates (glycogen and trehalose) increases as well as increasing lipid content while protein and RNA content are reduced (Yu, et al., 2020). Yu et al. (2020) showed that a reduction in mRNA prompted the cells to increase gene-specific translation efficiency of genes (~ 74%) and this could be actively regulated or arising naturally as a result of low ribosomes in the cells.

Our results show that *P. manshurica* strains 11-XP and 15-X accumulated higher protein content of 42–50%, which corresponded with high lipids (w/w) of the strains and less carbohydrates. Both the *P. manshurica* 11-XP and *C. ethanolica* C2 strains revealed higher carbohydrates content of 35% and 29%, respectively. Most microorganisms studied under nutrient stress, such as limited nitrogen the accumulation of lipids was directly proportional to the accumulation of carbohydrates. The increase in lipids was accompanied by increase in carbohydrates contents. Kumar et al. (2017) studied oleaginous yeast *Pichia guillierrmondii* that was cultivate in different carbon sources (20 g/L); molasses, crude glycerol, distillery waste water and corn steep liquor. The accumulated carbohydrates (35%.8–44.2%) were higher than the protein content (12.4% – 24.6%) in *P. guilliermondii*. It was found that the type of carbon substrate influenced the levels of the accumulated carbohydrates and proteins (Kumar, et al., 2017). In Cyanobacteria, *Arthrospira* sp. PCC 8005 limited-nitrogen increased the carbohydrates content (from 14%-74%) in the biomass and decreased protein content from 37% – 10%. Transcriptomic and proteomic analysis indicated that de novo protein synthesis was down-regulated in nitrogen limited culture. Instead, the degraded proteins were partially converted into carbohydrates through gluconeogenesis (Depraetere, et al., 2015).

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 recycled proteins or amino acids resulting from cell death of other yeasts, also called autophage was necessary for maintaining cell viability. 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). Tchakouteu et al. (2014) studied 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 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 is depleted the intracellular total sugar decreased to 20%. In excess nitrogen, the intracellular total sugar remains high (Tchakouteu, et al., 2014). Microbial (including algal) proteins are referred to as single cell proteins (SCP) if the microorganism contains 30% or more of proteins (Glencross, et al., 2020; Lapeña, et al., 2020). Yeast is widely accepted for the production of SCP because of the protein nutritional quality and acceptability among the consumers (Lapeña, et al., 2020). 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 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 (Gientka, et al., 2017). The fatty acids produced by all the *C. ethanolica* strains and *P. manshurica* strains are dominated by SFA, followed by MUFA and PUFA. Then again, *P. manshirica* 15-X produced abundant amount of palmitic, stearic, oleic and linoleic acids making up 82% of the total lipids. This composition was similar to fatty acids produced by *Cryptococcus curvatus* DSM 70022 and the fatty acids are comparable to those of plant or vegetable oils (Annamalai, et al., 2018). Other oleaginous yeasts such as *Trichosporon* sp. (RW), *Thodotorula glutins* strains, *Rhodotorula babjevae* strains, *Lipomyces starkeyi* strains and *Lipomyces lipofer* strains had their lipids dominated by plamitic, stearic, oleic and linoleic acids (Shapaval, et al., 2020; Brar, et al., 2017). *Trichosporon mycotoxinivorans* S2's major fatty acids were palmitic, oleic and stearic (Sagia et al., 2010). The fatty acids for *Rhodosporidium* TJUWZ4 were oleic, palmitic and linoleic acids (Wang, et al., 2017). This confirms that specific yeast strains will produce 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. In order to improve this content, Kolouchova, et al. (2016) found that under limited nitrogen using (NH4)2SO4 as 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%.

5. Conclusion

Oleaginous yeasts were isolated from sugarcane bagasse and the yeast belonged to the genera *Candida* and *Pichia*. *Pichia manshurica* 15-X accumulated 67% (w/w) of lipid. FAME analysis had revealed the suitability of the lipids based on the fatty acids composition for application in the biofuel and food industry. The co-products of lipids such as carbohydrates and proteins will also have application in animal feed.

Declarations

Ethical statement:

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data sets generated during and or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

LML conceptualized the ideas, designed, planned and executed the experimental research work. LML took a lead in analysing the results, structuring and writing of the manuscript. KLMM critically reviewed the entire manuscript and assisted with analysis and interpretation of the results. All authors read and approved final manuscript.

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Author's information (optional)

LML holds Ph.D in microbiology and is currently a postdoctoral fellow at University of Limpopo.

KLM holds Ph.D in microbiology and is currently Associate Professor at the University of Limpopo.

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Figures

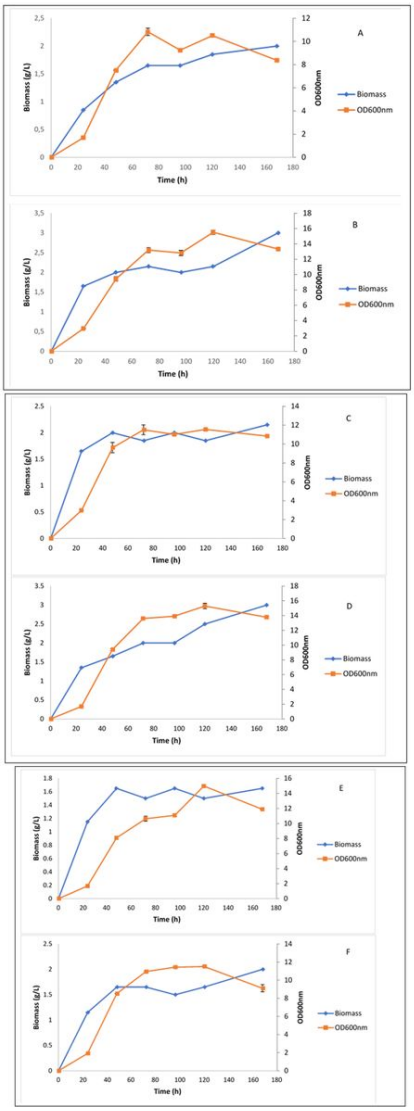

Figure 1

1a Time-course of yeast growth in synthetic medium containing glucose as carbon source for 168 h: (A) Isolate B2 and (B) Isolate C2.

1b Time-course of yeast growth in synthetic medium containing glucose as carbon source for 168 h: (C) Isolate E2 and (D) Isolate 6-XP.

1c Time-course of yeast growth in synthetic medium containing glucose as carbon source for 168 h: (E) Isolate 11-XP and (F) Isolate 15-X

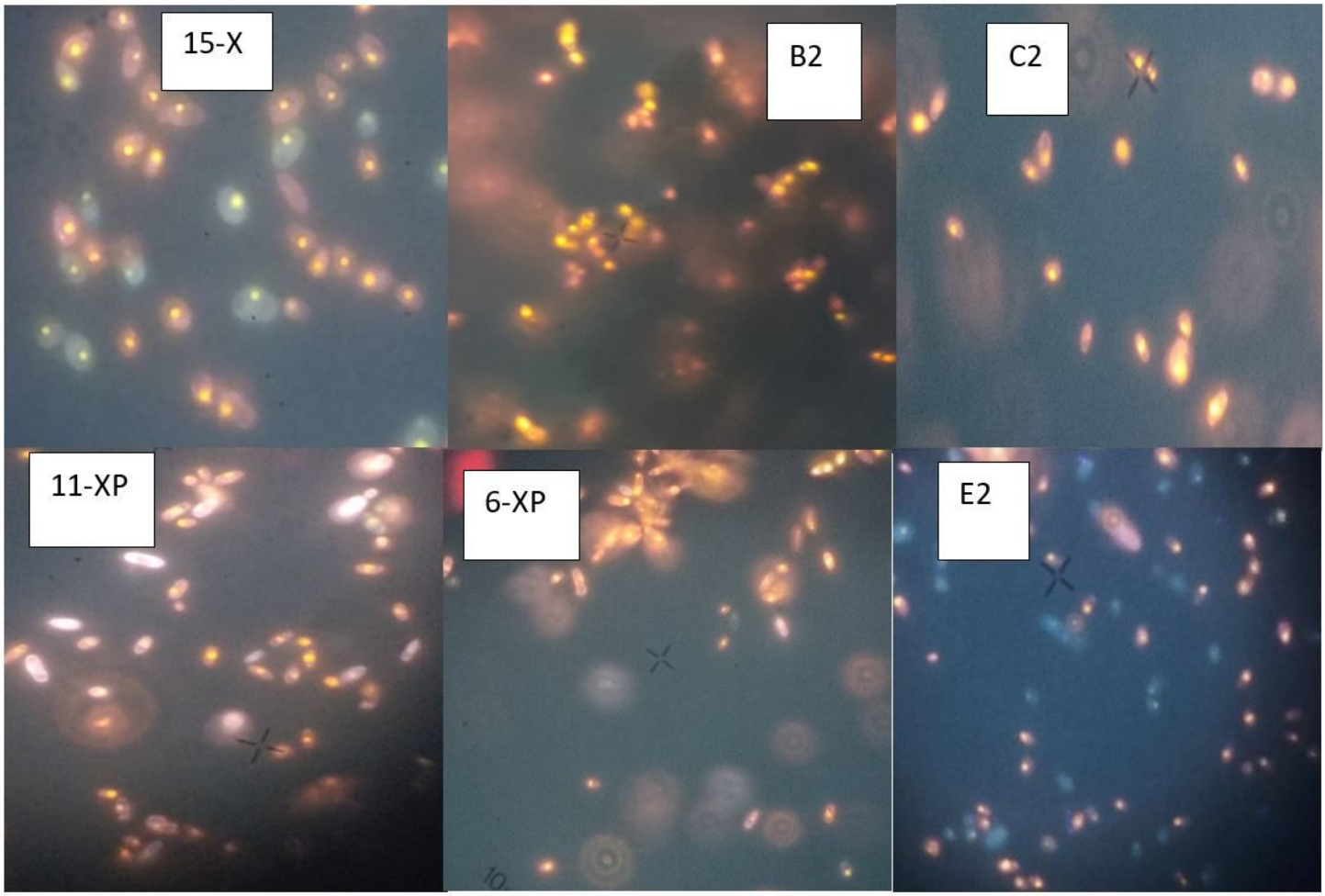

Figure 2

Microscopic examination of yeast cells stained with nile red fluorescent dye. The cells were grown in high sugar (30% glucose) and limited nitrogen medium and harvested after 72 h.

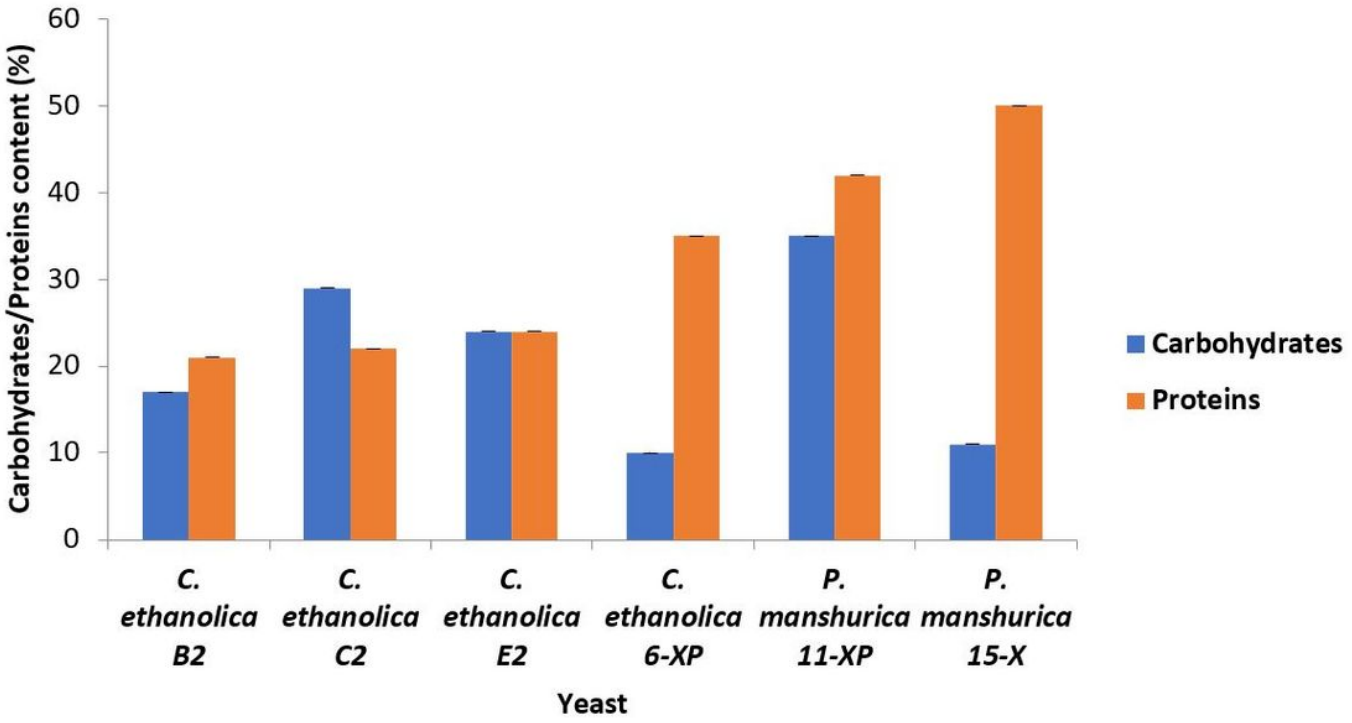

Figure 3

The content of carbohydrates and proteins in oleaginous yeasts during the production of lipids.

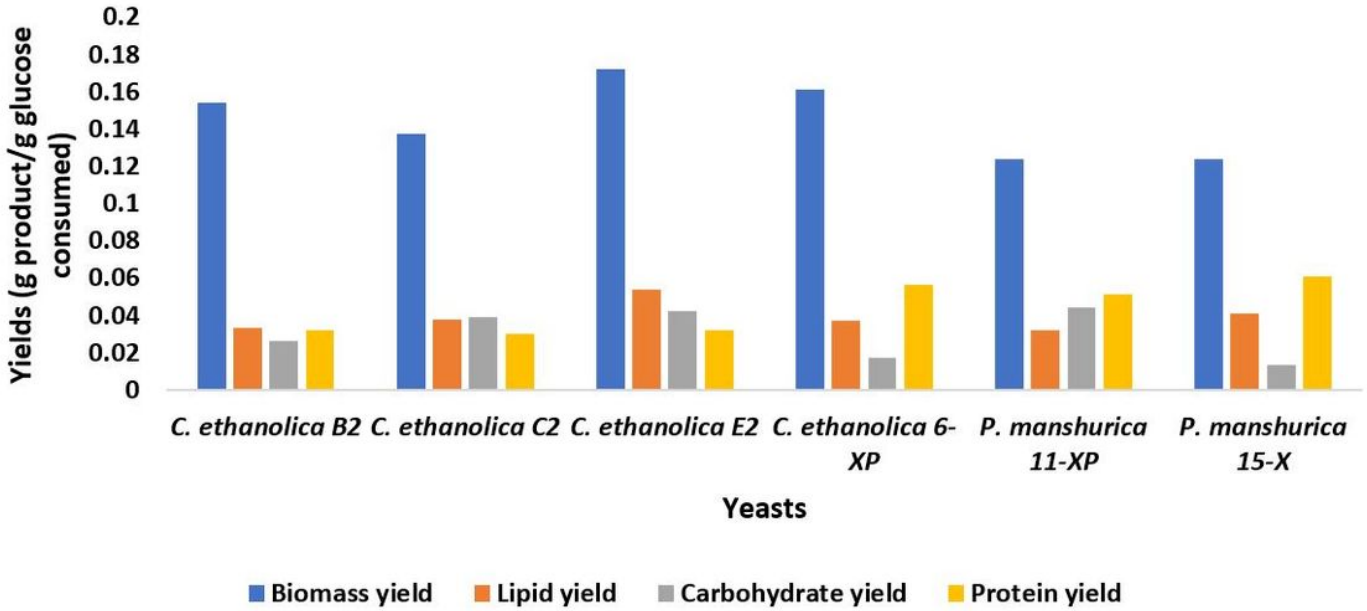

Figure 4

The yields of biomass, lipids, carbohydrates and proteins for oleaginous yeast cultivated in synthetic medium using glucose as carbon source.

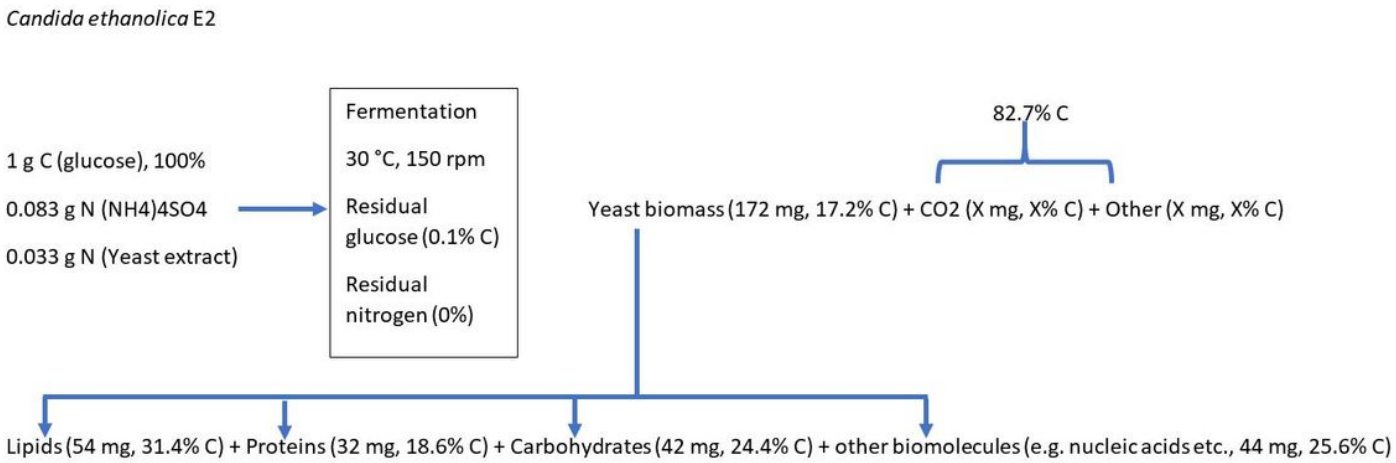

Figure 5

Illustration of the carbon utilization by C. *ethanolica* E2 during lipid production.