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Oleaginous yeasts isolated from traditional fermented foods and beverages of Manipur and Mizoram, India, as a potent source of microbial lipids for biodiesel production

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Abstract

Purpose: Oleaginous yeasts can accumulate intracellular lipid bodies or triacylglycerides (TAGs) under nutrient limiting conditions. TAGs derived from those yeast strains are considered as an alternative to conventional plant-based oils for biodiesel production. In this study, we attempt to isolate and characterize yeast strains from selected traditional fermented foods of Manipur and Mizoram, India, and study their oleaginous attributes for biodiesel production.

Method: Fourteen potential oleaginous yeasts were isolated from fermented food samples of Manipur and Mizoram, India. The isolates were identified by 5.8S internal transcribed spacer (ITS) rRNA gene sequencing. Intracellular TAG accumulation by yeast cells were confirmed by Nile red fluorescence microscopy and spectrometry technique. The most promising isolates were evaluated for lipid accumulation having different initial carbon to nitrogen (C/N) ratios and also the full kinetic studies (depicting the glucose consumption, biomass, and lipid production) using optimum C/N ratio were estimated. Fatty acid methyl esters (FAME) profile of the transesterified lipids were analyzed by GC-MS.

Results: The identified yeast isolates belonged to seven different genera viz. *Rhodotorula*, *Pichia*, *Candida*, *Saturnispora*, *Wickerhamomyces*, *Zygoascus*, and *Saccharomyces*. Under nitrogen-limiting conditions, maximum biomass concentration of 5.66 ± 0.03 g/L and 4.713 ± 0.03 g/L was produced by *Wickerhamomyces anomalus* FK09 and *Pichia kudriavzevii* FK02, respectively. The highest lipid concentration (g lipid/L fermentation broth) of 0.58 g/L was attained by *Rhodotorula mucilaginosa* R2, followed by *Wickerhamomyces anomalus* FK09 (0.51 g/L), and *Zygoascus hellenicus* FC10 (0.41 g/L). *Rhodotorula mucilaginosa* R2 exhibited the maximum lipid content (% lipid/g dry cell weight) of (21.63 ± 0.1%) after 96 h of growth. The C/N ratio of 40 and 20 was found to be optimum for *R. mucilaginosa* R2 and *W. anomalus* FK09 with a lipid content of 22.21 ± 0.4% and 12.83 ± 0.08% respectively.

Conclusion: Newly isolated yeast strains were obtained from traditional fermented food samples of Manipur and Mizoram, India. FAME analysis of the transesterified lipid extracts suggested the potential use of yeast-derived oil as an alternative to vegetable oil for biodiesel production.

Keywords: Oleaginous yeast, Triacylglycerides, Fermented food, *Rhodotorula mucilaginosa* R2

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Introduction

Plant-based oils are the primary source for the conventional production of biodiesel and act as a substitute to the depleting fossil fuel reserves (Sitepu et al. 2014). However, huge land area requirement for crop cultivation (both edible and non-food crops) and issues over food security have led researchers to explore alternatives to sustain the biodiesel industry (Ma et al. 2018). Oleaginous yeasts store above 20% of their dry cell weight as intracellular triacylglycerols. These yeasts are considered as cell factories amenable for the commercial production of biodiesel, polyunsaturated fatty acids, and exotic fats like cocoa butter (Bellou et al. 2016, Papanikolaou and Aggelis 2019). Oleaginous yeasts predominantly belong to the genera *Rhodosporidium, Cryptococcus, Rhodotorula, Yarrowia, Lipomyces,* and *Trichosporon* (Zhu et al. 2008; Abghari and Chen 2014; Zhang et al. 2016; Deeba et al. 2017; Khot and Ghosh 2017; Blomqvist et al. 2018). Yeasts are well-suited for commercial production of microbial lipids over other oleaginous microorganisms like algae and filamentous fungi because of its ability to reach high cell densities in a short time (Sitepu et al. 2014), having similar composition of fatty acid as those of plant-based oils (Deeba et al. 2016) and capability of producing a variety of metabolic co-products including high-value carotenoids, proteins and polysaccharide rich streams of deoiled biomass, and various organic acids (Bellou et al. 2014; Kot and Kurcz 2016; Parsons et al. 2020). Furthermore, several researchers have highlighted the use of microbial lipids as a source of renewable oleochemicals like surfactants, cosmetics, lubricants, textiles, paints, plastics, and soaps (Steen et al. 2010; Zhou et al. 2016). Nutrient limitation, particularly, high C/N ratio in the growth medium has been reported to induce lipogenesis (Ratledge 2002). During such stress conditions, oleaginous yeasts accumulate high amounts of triacylglycerol in the form of intracellular lipid bodies (LBs) (Poontawee et al. 2018; Bardhan et al. 2019a). However, out of the 1600 described yeast species, only 70 are known to store lipids above 20% of their cell dry weight (Sitepu et al. 2014; Patel et al. 2019). Therefore, screening studies are crucial for the discovery of new oleaginous yeast species (Schulze et al. 2014; Lamers et al. 2016). Recent screening and molecular characterization studies have led to the identification of some novel oleaginous yeast strains which include *Cystobasidium oligophagum* JRC1 (Vyas and Chhabra 2017), *Meyerozyma guilliermondii* BI281A (Ramírez-Castrillón et al. 2017), and *Cystobasidium iriomotense* (Tanimura et al. 2018) as the potential feedstock for biodiesel production.

Oleaginous yeasts have been isolated from several fermented foods including Kefir (fermented milk product) and other dairy products like cheese, milk, and yoghurt (Jiru et al. 2016; Gientka et al. 2017). North-East Indian states have diverse ethnic backgrounds and fermented foods like soya bean, bamboo shoot, and fermented alcoholic and non-alcoholic beverages are consumed regularly by the local people (Das et al. 2016). In this study, we attempt to isolate and characterize yeast strains from some selected traditional fermented foods and beverages of Manipur and Mizoram, India, and study their ability to accumulate lipids under nitrogen-limiting conditions using glucose as a carbon source. The most promising isolates R2 and FK09 (selected on the basis of highest biomass and lipid content) were evaluated for lipid accumulation having different initial carbon to nitrogen (C/N) ratios and also the full kinetic studies (depicting the glucose consumption, biomass, and lipid production) using optimum C/N ratio were estimated. The fatty acid composition of the transesterified lipid extracts derived from the yeast strains were analyzed and compared with plant-based oils. This is the first report on the isolation of oleaginous yeasts from the fermented foods of India for lipid production and hence these strains could be used in future as a potential source of microbial lipids for biodiesel production.

Materials and methods

Chemicals and media

All the microbiological media supplements, general chemicals, and reagents like agar, malt extract, peptone, Nile red, BF_3-methanol reagent, and streptomycin were procured from HiMedia, India. Standard FAME Mix C14-22 was procured from Sigma-Aldrich, India, and analytical grade solvents like chloroform, methanol, and n-hexane were purchased from Merck Ltd., India.

Isolation of yeast strains from traditional fermented foods of Manipur and Mizoram, India

A total of ten different fermented foods were procured from the local markets of Imphal (Manipur, India) and Aizawl (Mizoram, India). These included fermented bamboo shoots (Soidon, soibum), fermented fish (Ngari), fermented fish pate (Hentek), and fermented soya beans (Hawaijar), which were collected from Manipur. The traditional fermented food from Mizoram included fermented banana flower, fermented soya beans, fermented pig fat, and fermented beverages like orange juice (hathkhora thoi) and lemon juice. Yeast extract peptone dextrose (YEPD) agar medium containing (g/L) yeast extract, 3; peptone, 5; dextrose, 10; malt extract, 3; and agar, 18 (pH 6.8) amended with 100 μg/ml of filter sterilized streptomycin was used for the selective isolation of yeast strains. The homogenate was prepared by mixing 10 g of the sample in 90 ml 0.87% (w/v) physiological saline and kept in an orbital shaker at 28 °C, 120 rpm for 30 min. One milliliter of this mixture was used for the isolation of yeast strains by spreading 100 μl of diluted sample on YEPD agar plates, which were incubated at 28 °C for 72–96 h.

Identification of the yeast strains

Fungal genomic DNA Purification Kit (HiMedia, India) was used to extract and purify the genomic DNA of all the yeast isolates according to the manufacturer's protocol. Primer set ITS-1(5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) was used to amplify the 5.8S internal transcribed spacer (ITS) region of the rRNA gene using polymerase chain reaction (PCR) (White et al. 1990). Primer set NL-1 (5′-GCATATCAAT AAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCG TGTTTCAAGACGG-3′) was used to amplify the D1/D2 domain of the 26S rRNA gene (Saikia et al. 2018). A total reaction mixture of 50 µl comprising of Dream Taq PCR 2 × master-mix, 25 µl (Thermo Scientific); nuclease free water, 19 µl; 10 µM of forward and reverse primers, 2 µl each; and template DNA (40–90 ng/µl), 2 µl, was constituted for PCR. The reaction was performed in a thermal cycler (Eppendorf) using a method described elsewhere with slight modifications (Manhar et al. 2015). The amplification parameters were as follows: initial denaturation (95 ℃, 2 min), followed by 36 cycles of denaturation (94 ℃, 1 min), annealing (54 ℃, 30 s), elongation (72 ℃, 1 min), and final elongation (72 ℃, 10 min) and then subsequently cooled at 4 ℃. PCR products were separated in 2% (w/v) agarose gel (Sigma) in 1 × Tris-acetate-EDTA buffer (pH 8.3). A 100 bp DNA ladder (Thermo Fisher Scientific) was used to ascertain the size of the obtained PCR products. Amplicons (size ranging from 355 to 790) were sequenced by the 1st BASE DNA sequencing service (Selangor, Malaysia). DNA sequences were analyzed with NCBI-BLAST (https://blast. ncbi.nlm.nih.gov). The phylogenetic tree was constructed using neighbor-joining method by CLC sequence viewer 7.6.1. Confidence levels of the clades were determined from bootstrap test (1000 replicates) as depicted on the nodes of the branches.

Growth of the yeast strains on lipid production media

Yeast strains were cultivated by introducing a loop of one colony from YEPD agar onto 50 ml YEPD broth and incubated at 28 ℃, 140 rpm. One milliliter of log phase culture (O.D was adjusted by phosphate buffer saline, pH 7, corresponding to approximately 10^7 cells/ml) was inoculated into 100 ml of lipid production medium comprising of (g/L), glucose, 30; yeast extract, 1.5; NH_4Cl, 0.5; $Na_2HPO_4.12H_2O$, 5; KH_2PO_4, 7; $CaCl_2.2H_2O$, 0.1; $MgSO_4.7H_2O$, 1.5; $FeCl_3.6H_2O$, 0.08; $ZnSO_4.7H_2O$, 0.01; $CuSO_4.5H_2O$, 0.1 and in (mg/L), $MnSO_4.5H_2O$, 0.1; $Co[NO_3]_2.6H_2O$, 0.1 and pH was adjusted to 5.5. The flasks were incubated at 28 ℃, 140 rpm for 96 h in an orbital shaker (Orbitek, Scigenics Biotech). Glucose in excess (30 g/L) was used as a carbon source and a limited concentration of 0.5 g/L NH_4Cl along with yeast extract (N total 11%) was used as the only nitrogen source (C/N molar ratio 17.81) in the culture medium. C/N ratio was calculated as the ratio of moles of carbon (glucose) to the moles of nitrogen (ammonium chloride). Different amount of ammonium chloride was added to control the C/N ratio. Further, intracellular lipid accumulation by yeast isolates growing on lipid production medium was confirmed using the Nile red staining followed by fluorescence microscopy and spectrometry.

Analysis of biomass yield, total extracted lipids, and glucose

The yeast dry weight was estimated gravimetrically; 10-ml culture broth was centrifuged at 7500 rpm for 10 min. The pellet containing the wet cell mass was washed thrice with sterile distilled water, and kept for drying at 65 ℃. Total lipids were extracted by the method of (Bligh and Dyer 1959) with slight modification. One hundred milligrams of the dried biomass was briefly treated with 1.32 ml of 4 M HCL and kept in a water bath at 75 ℃ for 1 h. To the acid hydrolyzed biomass, methanol, chloroform, and water were added sequentially in the ratio of 2:2:1.8 (v/v/v) and vortexed for 2 min after each solvent addition. The chloroform layer containing the total extracted lipids was obtained by centrifugation at 6500 rpm for 10 min and collected in a pre-weighed vial. The collected chloroform layers were kept in an oven (65 ℃) for drying until constant dry weight and the final weight of the vial was recorded. The parameters for lipid production were determined according to (Uprety et al. 2017). Residual sugar in the media was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959) using glucose as a standard.

Detection of intracellular LBs by Nile red staining and fluorescence microscopy

The intracellular lipid bodies (LBs) suggestive of lipid accumulation inside the yeast cells were detected and analyzed by the method of (Bardhan et al. 2019b). Briefly, 10 µl of Nile red solution (0.1 mg/ml in DMSO) was added to 40 µl of the culture broth. The mixture was incubated at room temperature for 5 min in dark. A fluorescence microscope (Olympus BX43) was used to observe the yeast cells under × 100 objective lens (450–490 nm excitation filter, 505 nm diachronic mirror) which allowed visualizing the stained lipid bodies (yellow-gold fluorescence). Those yeast cells having many and/or large lipid bodies were selected for quantitative analysis of neutral lipids accumulation by fluorescence spectrometry.

Quantification of the accumulated neutral lipid by fluorescence spectrometry

The fluorescence spectrometry experiment was performed as reported by (Kimura et al. 2004) with slight modifications. Two milliliters of phosphate buffer saline (pH 7.0) was added to 100 µl of the culture broth was

and mixed well. Seven hundred microliters of the cell suspension without Nile red was taken in a cuvette and the spectrum was recorded in the visual wavelength range (400–700 nm) using a fluorescence spectrometer (LS55, Perkin Elmer) with PC control equipment. The excitation was fixed at 488 nm. Ten microliters of Nile red was then added to the diluted culture broth and incubated for 5 min in dark. The cell suspension was mixed well and the spectrum was recorded again in the same wavelength range. PC control equipment was used to correct the spectra (by subtracting the emission spectra obtained before and after Nile red addition). The amount of intracellular lipids accumulated in the yeast cells corresponds to the fluorescence intensity (units) at the peak of the spectrum.

Transesterification of yeast derived lipids and GC-MS analysis

The transesterification reaction of the obtained lipids was performed according to (Morrison and Smith 1964). One milliliter of BF_3-methanolic reagent was added to 10 mg of lipid extract and the mixture was incubated at 80 °C for 30 min. A mixture of hexane and water in the ratio of 2:1 (v/v) was used to extract the methyl esters followed by centrifugation at 6500 rpm for 5 min. The upper hexane layer was analyzed by gas chromatography-mass spectrometry (GC 7890B, MS5977A) equipped with flame ionization detector (FID) and HP-5MS capillary column (Agilent, USA). One microliter of the sample was injected in split less mode (250 °C, carrier gas: helium, flow rate: 1 ml/min). Column temperature was initially set at 50 °C and raised to 180 °C at a gradient of 25 °C/min. Subsequently, it was further raised to 220 °C at a gradient of 10 °C/min and held for 1 min. Finally, the temperature was ramped at 250 °C at a gradient of 15 °C/min (Deeba et al. 2016). The retention times and mass spectra of the obtained FAMEs were identified using standard FAME mix, C14-22 (Sigma Aldrich).

Statistical analysis

The data were obtained from three independent experiments and represented as mean values with standard error using Excel 2010 Program. The statistical program SPSS version 16 was used for statistical analysis. One-way ANOVA was employed to study the differences in lipid production parameters among yeast strains. For comparison of the means, the Duncan's multiple range tests ($p < 0.05$) were used.

Results and discussion

Identification of yeast strains isolated from selected fermented foods of Manipur and Mizoram

Seventeen yeast isolates were established as pure culture from traditional fermented food and beverages of Manipur

and Mizoram, India (Fig. 1 depicts the colony morphology of some of the representative yeast isolates growing on YEPD agar medium). Amplification of 5.8S-ITS region of the rRNA gene resulted in amplicons with size ranging from 355 to 790 base pairs (Fig. 2). Amplicons corresponding to isolates FO05, FO08, and FC11 were sequenced and found to be the same organism (all showing 100% sequence similarity with *Saturnispora diversa* (KY105317.1)), so only isolate FO05 was taking into account. Similarly, isolate FK01 and R6 were sequenced and found to be 100% similar to *Rhodotorula mucilaginosa* culture CBS: 7014 (KY104857.1) and therefore only FK01 was considered in our study. The nucleotide sequences were deposited to NCBI GenBank and the accession numbers were generated as shown in Table 1. Yeast isolates belonging to seven different genera viz. *Pichia*, *Saccharomyces*, *Saturnispora*, *Candida*, *Wickerhamomyces*, *Zygoascus*, and *Rhodotorula* were identified. Figure 3 depicts the phylogenetic relationship of all the obtained yeast isolates. Based on 5.8S ITS rRNA gene analysis, 8 yeast strains were identified to belong to the phylum *Ascomycota* and 6 strains representing the *Rhodotorula* genera were placed in the phylum *Basidiomycota*. Molecular typing of yeast isolates by using ITS1 and ITS4 primers for identifying the 5.8S-ITS region of rRNA gene and NL1 and NL4 primers for the identification of D1/D2 region of 26S rRNA gene were depicted in Fig. 4. The identification of the 5.8S-ITS rDNA region is considered to exhibit the highest resolving power for discriminating closely related fungal species (Schoch et al. 2012; Filippousi et al. 2019). Oleaginous yeasts belonging to genera *Candida*, *Debaryomyces*, *Kluyveromyces*, *Kazachstania*, and *Zygotorulaspora* were also identified (based on 5.8S ITS rDNA sequence similarities) from the kefir grains (Gientka et al. 2017). In another study (Jiru et al. 2016), 18 oleaginous yeasts were identified according to gene sequences of ITS region of 5.8S rRNA gene and D1/D2 domain of the 26S rRNA gene. The diversity of yeast strains obtained in our study has been compared with the other reported studies concerning yeast diversity from food samples including several traditional fermented foods like sourdough, kefir grains, and other fermented condiments and milk products; surface and the body of red and white grapes, apples, prickly pears, damsons etc., from olive brine and vinegar were isolated and screened towards their ability to produce organic acids, polyols, and microbial lipids as shown in (Table 2).

Biomass yield and lipid content of different yeast isolates

The relative lipid production parameters (biomass concentration, lipid content, and lipid concentration) of the isolated yeast strains are shown in Table 3. Among all the tested yeast strains, significantly high biomass concentration of 4.7 ± 0.03[g] g/L and 5.6 ± 0.03[h] g/L was

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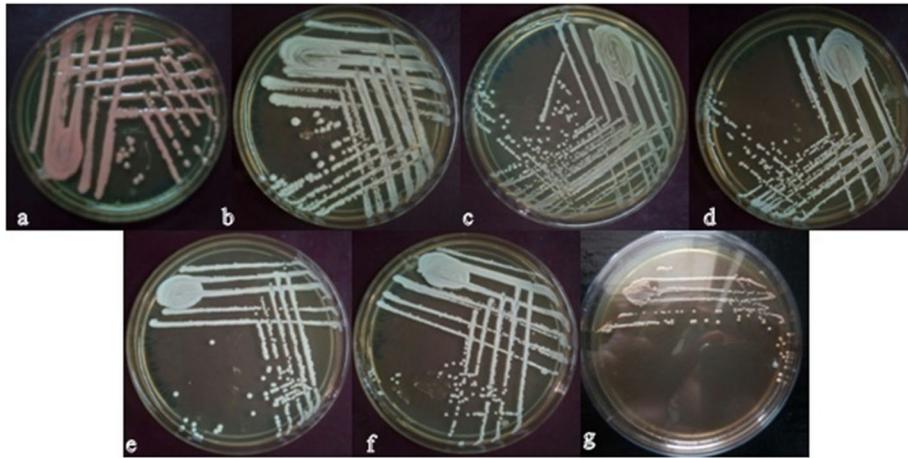
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Fig. 1 Colony morphology of yeast isolates growing on YEPD agar medium (representing one from each genera): **a** Isolate R2, **b** Isolate FK03, **c** Isolate FK04, **d** Isolate FO05, **e** Isolate FK09, **f** Isolate FC10, **g** Isolate YMA1

obtained with *Pichia kudriavzevii* FK02 and *Wickerhamomyces anomalus* FK09 and in terms of lipid concentration; 0.41[ef] g/L, 0.44[f] g/L, 0.51[g] g/L, and 0.58[h] g/L was obtained with *Zygoascus hellenicus* FC10, *Rhodotorula mucilaginosa* R4, *Wickerhamomyces anomalus* FK09, and *Rhodotorula mucilaginosa* R2, respectively. Average lipid content of yeasts has been reported to be in the range of 5 to 15% of total dry weight (TDW) (Phukan et al. 2019). However, much higher lipid content ranging from (22.1 to 68%) has been obtained with oleaginous yeasts using hydrophilic carbon sources like glucose, glycerol, and different biomass hydrolysates under various cultivation conditions (Athenaki et al. 2018). In this study, *R. mucilaginosa* R2 exhibited the maximum lipid content (21.63 ± 0.1[i]%) after 96 h of growth in glucose-based lipid production medium. The lipid content of *Rhodotorula mucilaginosa* strains, isolated from various

samples including dairy products, varied in the range of 19.67 ± 0.28 to 38.61 ± 0.39% (Jiru et al. 2016). The biomass and lipid content of some of the yeast strains obtained in this study has been compared with other reported oleaginous yeasts as shown in (Table 4). *Saturnispora diversa* FO05 and *Zygoascus hellenicus* FC10 were isolated from traditional fermented beverage, hathkhora thoi of Mizoram. *S. diversa* FO05 was found to be slow growing yeast with the least biomass production (2.09 ± 0.05[a] g/L) among all the isolated yeast strains. However, the lipid concentration in *S. diversa* FO05 was found to be 0.39[de] g/L with a lipid output of 18.57 ± 0.6[h]%. *S. diversa* belongs to the phylum *Ascomycota*, which was recently transferred from *Candida diversa* (Kurtzman 2015). To the best of our knowledge, there are no reports on microbial lipid production by *S. diversa* and *Z. hellenicus*. However, *S. diversa* and *Z.*
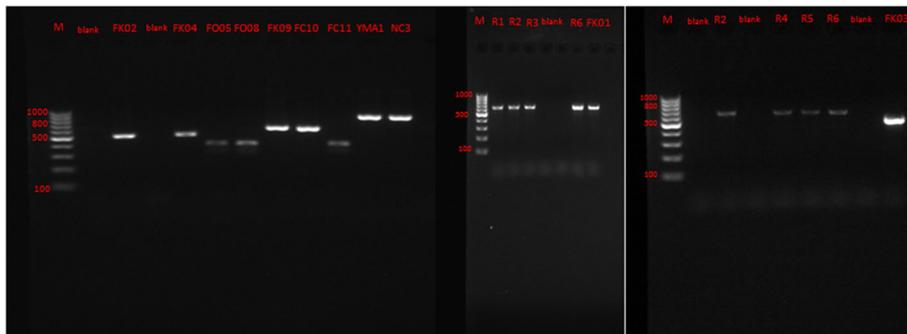

Fig. 2 Electrophoretic separation of PCR amplification products on 2% agarose gel (M-100 bp DNA ladder, FK02-*Pichia kudriavzevii* isolate FK02, FK04-*Candida parapsilosis* isolate FK04, FO05-*Saturnispora diversa* isolate FO05, FO08-*Saturnispora diversa* isolate FO08, FK09-*Wickerhamomyces anomalus* isolate FK09, FC10-*Zygoascus hellenicus* isolate FC10, FC11-*Saturnispora diversa* isolate FC11, YMA1-*Saccharomyces cerevisiae* isolate YMA1, NC3-*Saccharomyces cerevisiae* isolate NC3, R1-*Rhodotorula mucilaginosa* isolate R1, R2-*R. mucilaginosa* isolate R2, R3-*R. mucilaginosa* isolate R3, R4-*R. mucilaginosa* isolate R4, R5-*R. mucilaginosa* isolate R5, R6-*R. mucilaginosa* isolate R6, FK01-*R. mucilaginosa* isolate FK01, FK03-*Pichia kudriavzevii* isolate FK03)
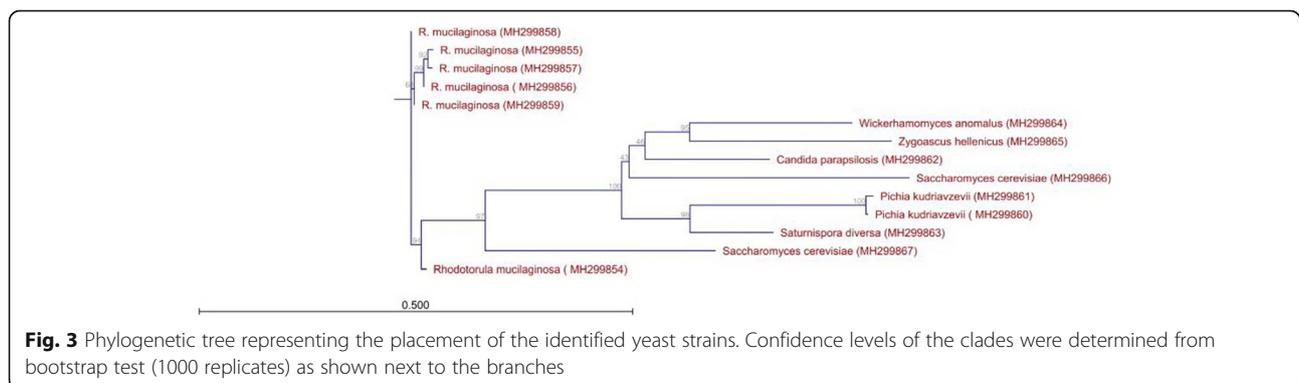
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Table 1 Sequence analysis of 5.8S-ITS region of rRNA gene of the isolates

SL no.	Isolate no.	5.8S-ITS region of rRNA gene		Closest-related species	GeneBank accession no.
		Size of PCR product (bp)	Homology (%)		
1	FK02	481	99	*Pichia kudriavzevii* strain BSFL-3 (KY457575.1)	MH299860
2	FK04	477	100	*Candida parapsilosis* strain h56b (KP674744.1)	MH299862
3	FO05	355	99	*Saturnispora diversa* (KY105317.1)	MH299863
4	FK09	572	100	*Wickerhamomyces anomalus* (KT175180.1)	MH299864
5	FC10	563	99	*Zygoascus hellenicus* (AY447032.1)	MH299865
6	YMA1	790	100	*Saccharomyces cerevisiae* strain TU9 (KU535590.1)	MH299866
7	NC3	787	100	*Saccharomyces cerevisiae* YJM1592 (CP006433.1)	MH299867
8	R1	568	100	*Rhodotorula mucilaginosa* isolate2 (MG020687.1)	MH299854
9	R2	527	100	*R. mucilaginosa* strain S05 (KX866274.1)	MH299855
10	R3	518	100	*R. mucilaginosa* strain RH2 (MG969795.1)	MH299856
11	R4	571	100	*R. mucilaginosa* strain SBM-IAUF-2 (MG711888.1)	MH299857
12	R5	572	100	*R. mucilaginosa* strain AUMC 11221 (KY611824.1)	MH299858
13	FK01	569	99	*R. mucilaginosa* culture CBS:7014 (KY104857.1)	MH299859
14	FK03	459	100	*Pichia kudriavzevii* strain YB-25 (MF062195.1)	MH299861

hellenicus have been reported to produce ethanol from various monosaccharides (Kodama et al. 2013; Jaiboon et al. 2016). Similarly, *S. cerevisiae* strains YMA1 and NC3 should produce ethanol on glucose-based medium despite aerobic conditions that were imposed ("Crabtree"-positive yeasts) and therefore accumulate lower quantities of reserve lipids (De Deken, 1966; Sitepu et al. 2014).

The C/N molar ratio initially used for screening of yeast strains for lipid accumulation (Table 4) was 17.81. Two strains *W. anomalus* FK09 and *R. mucilaginosa* R2 (selected on the basis of highest biomass and lipid content respectively) were tested for lipid accumulation using glucose-based medium having different C/N molar ratios (20, 40, 60, 80, and 100) and also a full kinetic studies (depicting the glucose consumption, total dry weight, and lipid concentration) using optimum C/N ratio were estimated (Fig. 5). The C/N ratio of 40 and 20 was found to be optimum for *R. mucilaginosa* R2 and *W. anomalus* FK09 with a lipid content of 20.67% and

12.82% respectively. In case of *R. mucilaginosa* R2, the lipid content increased significantly from 7.08 ± 0.1^a to 20.67^d% when the C/N molar ratio was increased from 20 to 40. Further increasing the C/N ratio (from 40 to 100) lowered the lipid yield. This could be attributed to the fact that high glucose concentration results in a high osmotic pressure and also the sharp decrease in pH due to excessive glucose consumption as confirmed by recording the pH of the culture medium after fermentation (pH 3.51) (Zhu et al. 2008). Lower the biomass yield, higher is the lipid content in the cells (Kolouchova et al. 2016). At C/N ratio of 100, the lipid content significantly decreased with increased biomass yield. Similar results were obtained by Zhu et al. (2008) but at a much higher C/N ratio above 140. Filippousi et al. (2019) reported non-negligible production of cellular lipids (16.9 to 29.9% of TDW) by *Yarrowia lipolytica* and *Debaromyces* strains under nitrogen-limiting condition using glycerol-based carbon source and also a
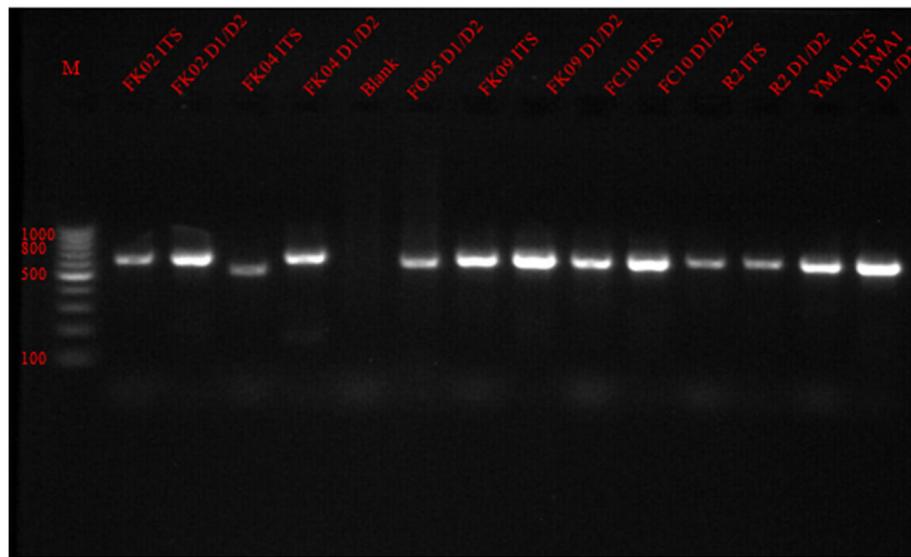

Fig. 3 Phylogenetic tree representing the placement of the identified yeast strains. Confidence levels of the clades were determined from bootstrap test (1000 replicates) as shown next to the branches

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Fig. 4 Molecular typing of yeast isolates by using 5.8 S ITS (internal transcribed spacer)-primer and 26S rRNA gene D1/D2 region-primer, M-molecular marker (100 bp DNA ladder), FK02-*Pichia kudriavzevii*, FK04-*Candida parapsilosis*, FO05-*Saturnispora diversa*, FK09-*Wickerhamomyces anomalus*, FC10-*Zygoascus hellenicus*, R2-*Rhodotorula mucilaginosa*, YMA1-*Saccharomyces cerevisiae*

decrease in lipid content as the fermentation time increased from 24 to 120 h. The low lipid output at later stages of growth despite nitrogen limitation was attributed to the metabolic shift towards synthesis of endopolysaccharides.

For both strains FK09 and R2, the growth of yeast strains were accompanied by the production of organic acids that led to significant lowering of pH at all stages of growth. The synthesis of microbial lipids and/or polysaccharides with secretion of low-molecular weight metabolites like polyols (mannitol) or citric acid by most of the genera including *Cryptococcus*, *Yarrowia*, and *Rhodotorula* under nitrogen-limiting conditions using glucose or glycerol as a sole carbon source has been reported by Athenaki et al. (2018) and Filippousi et al. (2019). The glucose consumption in case of both the strains R2 and FK09 was faster during the early stages of growth until 96 h after which it became stabilized. This was followed by lipid accumulation phase in case of *Rhodotorula mucilaginosa* R2. Similar results were obtained by Vyas and Chhabra (2019) using oleaginous yeast *Cystobasidium oligophagum* JRC1. The glucose assimilation capacity of *R. mucilaginosa* R2 was low (9.18 g/L) as compared to *W. anomalus* FK09 (23.29 g/L) possibly due to low pH of the culture media that limited the utilization of glucose from the media by *Rhodotorula mucilaginosa* R2, whereas *Wickerhamomyces anomalus* FK09 continued to assimilate glucose at lower pH. In *R. mucilaginosa* R2, the lipid accumulation phase started after 72 h with maximum production (lipid content of 21.89%) at 120 h of incubation.

Subsequently, the lipid accumulation in five of the selected yeast strains (on the basis of high biomass concentration and/or high lipid content in lipid production medium) was evaluated by Nile red staining, fluorescence microscopy, and spectrometry techniques.

Detection of LBs by Nile red staining and fluorescence microscopy

Nile red staining was performed in selected yeast isolates representing five different genera. Yeast strains belonging to the genera *Rhodotorula*, *Wickerhamomyces*, *Zygoascus*, *Pichia*, and *Saturnispora* contained lipid bodies (present as yellow-gold emissions). The shape, number, and localization of lipid bodies were found to vary among the yeast isolates belonging to different genera. Cells of *R.mucilaginosa* R2, *W. anomalus* FK09, *Z. hellenicus* FC10, *S. diversa* FO05, and *P. Krudivazevi* FK03 produced around 2–3 lipid bodies within the cytoplasm. In contrast, cells of *Saccharomyces cerevisiae* YMA1 did not contain any neutral lipid bodies (indicated by an absence of yellow-gold emission). As shown in Fig. 6, fluorescence emission observed was comparable to several oleaginous yeasts and fungi as reported by other studies (Viñarta et al. 2016; Bardhan et al. 2019b. Hydrophobicity of the lipid affects its fluorescence emission (Poontawee et al. 2017). Neutral lipids fluoresce yellow-gold whereas a red emission is observed with polar lipids in the presence of Nile red (Kimura et al. 2004).

Table 2 Comparison of diversity of yeast strains isolated from food samples with other reported studies

Food samples	Diversity of isolated yeast strains	Application	References
Various types of sourdoughs	*Yarrowia lipolytica*	Lipids and organic acids	(Papanikolaou et al. 2009)
Sourdough	*Torulaspora delbrueckii, Candida humilis, Saccharomyces cerevisiae, Kazachstania exigua, Wickerhamomyces anomalus,* and *Pichia kudriavzevii*	Food (ethanol and higher alcohols, organic acids, probiotic)	(De Vuyst et al. 2016)
Fermented condiments (Soumbala, Maarri, Bikalga), fermented milk (traditional yogurt) of Burkina Faso	*Clavispora lusitaniae, Kluyveromyces marxianus, Saccharomyces cerevisiae, Candida tropicalis, Rhodotorula mucilaginosa, Pichia kudriavzevii,* and *Cyberlindnera fabianii*	Probiotics	(Cissé et al. 2019)
Brazilian savannah fruits	*Candida glabrata, Candida parapsilosis, Meyerozyma guilliermondii*	Biofuel and food	(Camargo et al. 2018)
Coconut Inflorescence sap	*Lachancea fermentati, Candida tropicalis, Shizosaccharomyces pombe, Pichia kudriavzevii, Saccharomyces cerevisiae, Saccharomycodes ludwigii, Wickerhamomyces anomalus, Zygosaccharomyces rouxii, Hanseniaspora guilliermondii,* and *Pichia manshurica*	Bioethanol	(Udomsaksakul et al. 2018)
Dairy products, oilseeds (soybean, sesame, and sunflower seeds), deli meats, date palm juice, flowers, and rotten fruits	*Cyberlindnera fabianii, Rhodotorula mucilaginosa, Rhodotorula babjevae, Yarrowia lipolytica, Candida viswanathii, Candida tropicalis, Trichosporon asahii,* and *Naganishia albida*	SCO for biodiesel, cellulase, lipase	(Ayadi et al. 2018)
Red and white grapes, apples, prickly pears, pears, damsons, plums, melons, pomegranates and figs	*Metschnikowia pulcherrima*	SCO for biodiesel	(Maina et al. 2017)
Olive brine, vinegar	*Candida boidinii, Candida ethanolica, Candida metapsilosis, Candida parapsilosis, Candida pararugosa, Pichia manshurica, Pichia membranifaciens, Pichia etchellsii, Pichia anomala, Kluyveromyces marxianus*	SCO for biodiesel, extracellular lipases	(Arous et al. 2017a)
Samples including dairy products (cheese, milk, and yoghurt)	*Cutaneotrichosporon curvatus, Rhodotorula kratochvilovae, Rhodotorula dairenensis*	SCO for biodiesel	(Jiru et al. 2016)
Kefir grains	*Debaryomyces hansenii, Candida inconspicua, Kluyveromyces marxianus, Zygotorulaspora florentina,* and *Kazachstania unispora*	SCO for biodiesel	(Gientka et al. 2017)
Fermented bamboo shoots (Soidon, soibum), fish (Ngari), fish pate (Hentek), soya beans (Hawaijar), banana flower, pig fat, orange juice (hathkhora thoi), and lemon juice	*Pichia kudriavzevii, Saccharomyces cerevisiae, Saturnispora diversa, Candida parapsilosis, Wickerhamomyces anomalus, Zygoascus hellenicus,* and *Rhodotorula mucilaginosa*	SCO for biodiesel	(This study)

Table 3 Biomass concentrations (g/L), lipid concentration (g/L), and lipid content (%) after 96 h of growth; strains were grown in shake flasks containing 100 ml of lipid production medium at 28 ℃

Isolate	Total dry weight (g/L)	Lipid concentration (g/L)	Lipid content (% w/w)
S. diversa FO05	2.09 ± 0.05[a]	0.39 ± 0.006[de]	18.6 ± 0.65[h]
R. mucilaginosa R3	2.40 ± 0.003[b]	0.34 ± 0.003[c]	14.36 ± 0.10[ef]
P. kudriavzevii FK03	2.50 ± 0.004[c]	0.27 ± 0.003[b]	11.02 ± 0.12[c]
Z. hellenicus FC10	2.57 ± 0.043[c]	0.41 ± 0.005[ef]	16.32 ± 0.10[g]
R. mucilaginosa R2	2.70 ± 0.006[d]	0.58 ± 0.003[h]	21.63 ± 0.16[i]
R. mucilaginosa R1	2.78 ± 0.042[de]	0.37 ± 0.037[cd]	13.53 ± 1.35[de]
S. cerevisiae NC3	2.85 ± 0.028[e]	0.37 ± 0.004[cd]	12.98 ± 0.07[d]
S. cerevisiae YMA1	2.86 ± 0.026[e]	0.27 ± 0.003[b]	9.48 ± 0.04[b]
R.mucilaginosa R4	2.87 ± 0.042[e]	0.44 ± 0.004[f]	15.60 ± 0.07[fg]
R. mucilaginosa R5	3.09 ± 0.044[f]	0.20 ± 0.02[0a]	6.63 ± 0.69[a]
R. mucilaginosa FK01	3.11 ± 0.049[f]	0.17 ± 0.004[a]	5.737 ± 0.06[a]
C. parapsilosis FK04	3.11 ± 0.005[f]	0.35 ± 0.003[cd]	11.34 ± 0.09[c]
P. kudriavzevii FK02	4.71 ± 0.038[g]	0.27 ± 0.003[b]	5.84 ± 0.05[a]
W. anomalus FK09	5.66 ± 0.033[h]	0.51 ± 0.007[g]	9.13 ± 0.07[b]

Strains are arranged in increasing order of biomass concentrations. The data are given as averages of three replicates with standard error. Values followed by the different letters are significantly different at *p* < 0.05

Detection of the accumulated intracellular lipid by fluorescence spectrometry

Intracellular lipids were detected by Nile red fluorescence spectrometry with excitation at 488 nm. Excitation wavelength range of 480–490 nm is commonly used for the fluorescence measurements of intracellular neutral lipids (Kimura et al. 2004). Emission spectra with different fluorescence intensities were obtained with all the yeast isolates in either 565–585 nm or 600–605 nm range (Fig. 7). The peak of fluorescence intensity corresponds to the amount of accumulated intracellular lipids. The fluorescence intensity varied with different genera of yeast strains as reported by (Ramírez-Castrillón et al. 2017). *Wickerhamomyces anomalus* FK09 was found

to accumulate the maximum intracellular neutral lipids. Several studies have reported the accumulation of intracellular lipid bodies by oleaginous yeast *W. Anomalus* (Arous et al. 2017b; Souza et al. 2017).

Fatty acid composition

The primary FAME constituents were evaluated by GC-MS and identified from NIST11.L database. Fatty acid (FA) analysis in yeast is important both form biodiesel standpoint as well as to differentiate closely related yeast strains at generic and specific level (Phukan et al. 2019). FA profile of all the yeast isolates predominantly contained palmitic acid (C16:0) 19.24–30.76%, stearic acid (C18:0) 3.13–12.69%, oleic acid (C18:1) 40.45–62.7%,

Table 4 Comparison of lipid production parameters with other reported oleaginous yeasts

| Yeast strain	Feedstock	Incubation time (h)	Stress (nutrient limitation)	Lipid conc. (g/L), lipid content (%)	Reference
Rhodotorula mucilaginosa IIPL32	Sugarcane bagasse (xylose)	56	Nitrogen	0.17 g/g, 15.6 g/L (biomass conc.)	(Khot and Ghosh 2017)
Wickerhamomyces anomalus strain EC28	Deproteinized cheese whey	96	Nitrogen	0.65 ± 0.01, 24.00 ± 0.24	(Arous et al. 2017b)
Debaryomyces etchellsii	Deproteinized cheese whey	96	Nitrogen	0.4 ± 0.05,15.9 ± 0.93	(Arous et al. 2016)
Meyerozyma guilliermondii BI281A	Pure glycerol	120	–	108 mg/L, 34.97	(Ramírez-Castrillón et al. 2017)
Cystobasidium oligophagum JRC1	Untreated cheese whey (100%)	168	–	4.57 ± 0.13, 21.79 ± 1.00	(Vyas and Chhabra 2019)
Rhodotorula mucilaginosa R2	Glucose	96	Nitrogen	0.58 ± 0.009, 21.63 ± 1.2	(This study)
Wickerhamomyces anomalus FK09	Glucose	96	Nitrogen	0.51 ± 0.007, 9.13 ± 0.2	(This study)
Zygoascus hellenicus FC10	Glucose	96	Nitrogen	0.41 ± 0.004,16.3 ± 0.4	(This study)
Saturnispora diversa FO05	Glucose	96	Nitrogen	0.39 ± 0.008, 18.57 ± 0.7	(This study)
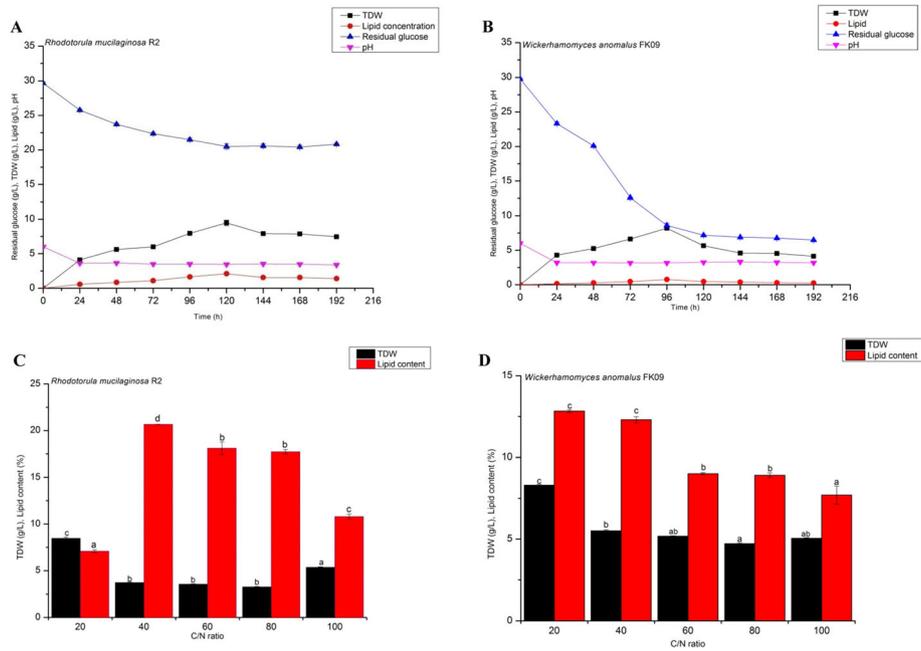
Fig. 5 Full growth kinetics depicting the glucose consumption (g/L), total dry weight (g/L), and lipid concentration (g/L) for **a** *Rhodotorula mucilaginosa* R2 and **b** *Wickerhamomyces anomalus* FK09. Optimization of C/N ratio for **c** *Rhodotorula mucilaginosa* R2 and **d** *Wickerhamomyces anomalus* FK09
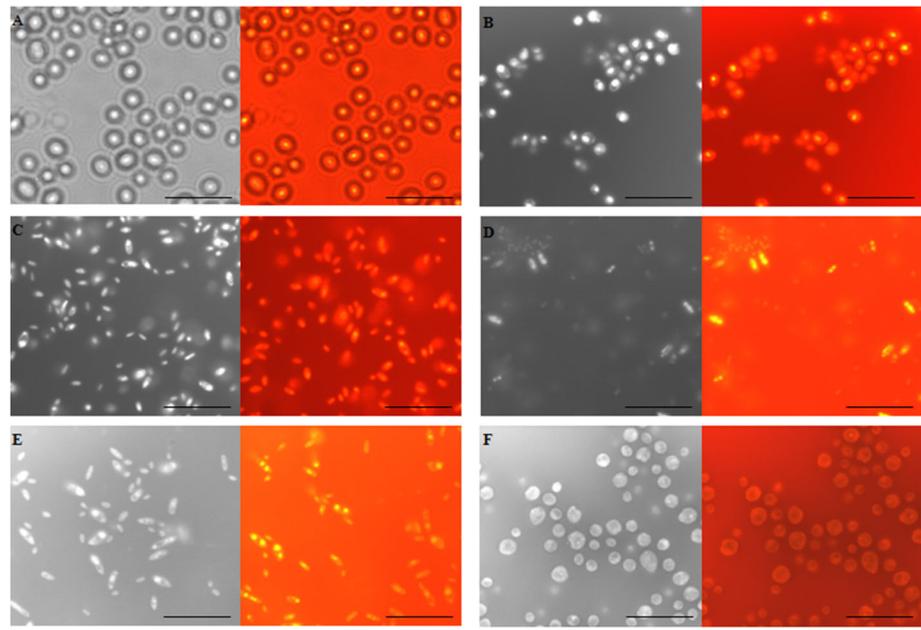

Fig. 6 Fluorescence micrographs of Nile red stained lipid bodies from yeasts: **a** *R. mucilaginosa* isolate R2, **b** *Wickerhamomyces anomalus* isolate FK09, **c** *Saturnispora diversa* isolate FO05, **b** *Pichia krudiavzevii* isolate FK03, **e** *Zygoascus hellenicus* isolate FC10, and **f** *Saccharomyces cerevisiae* isolate YMA1 after 96 h of culture in lipid production medium. Photomicrographs were taken under × 100 oil immersion objective, bar indicates 2 μM
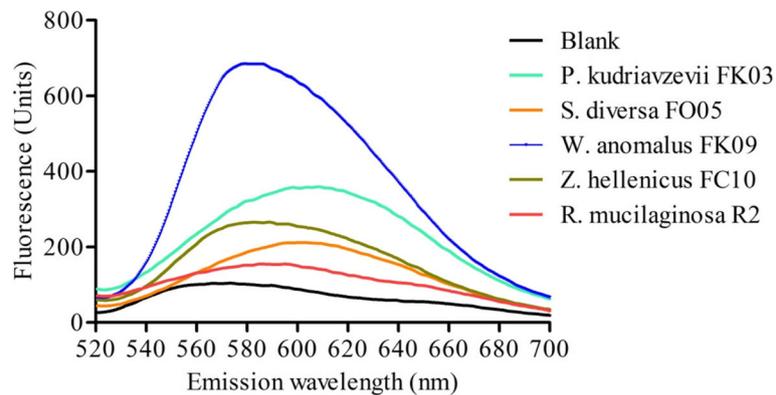
Fig. 7 Emission spectra of different yeast cell suspension with Nile red (0.1 mg/ml in DMSO)

and linoleic acid (C18:2) 11.45–31.09%. Whereas medium chain (e.g., C12 and C14) or long-chain (e.g., C18:3, C20 and C22) FAs were produced in negligible quantities (≤ 1% w/w of total FAs). As predicted, in yeast oil, the presence of intracellular stored lipids (stearic and oleic acids) is higher as compared to membrane lipids (linoleic acid) (Thiru et al. 2011). Figure 8 depicts the GC-MS chromatogram of the standard FAME mix and FAME derived from representative yeast strain *R.*

mucilaginosa R2. The relative abundance of FA in the yeast isolates, representing each of the seven different genera, is shown in (Table 5). Eighteen-carbon FA was the most abundant (56.72–78.63%) in all the yeast isolates. Our results are comparable to FA composition of lipids produced by *Cryptococcus podzolicus*, *Trichosporon porosum*, *Pichia segobiensis*, *Cryptococcus vishniaccii*, and *Rhodosporidium fluvial*, as reported by other authors (Schulze et al. 2014; Deeba et al. 2016;
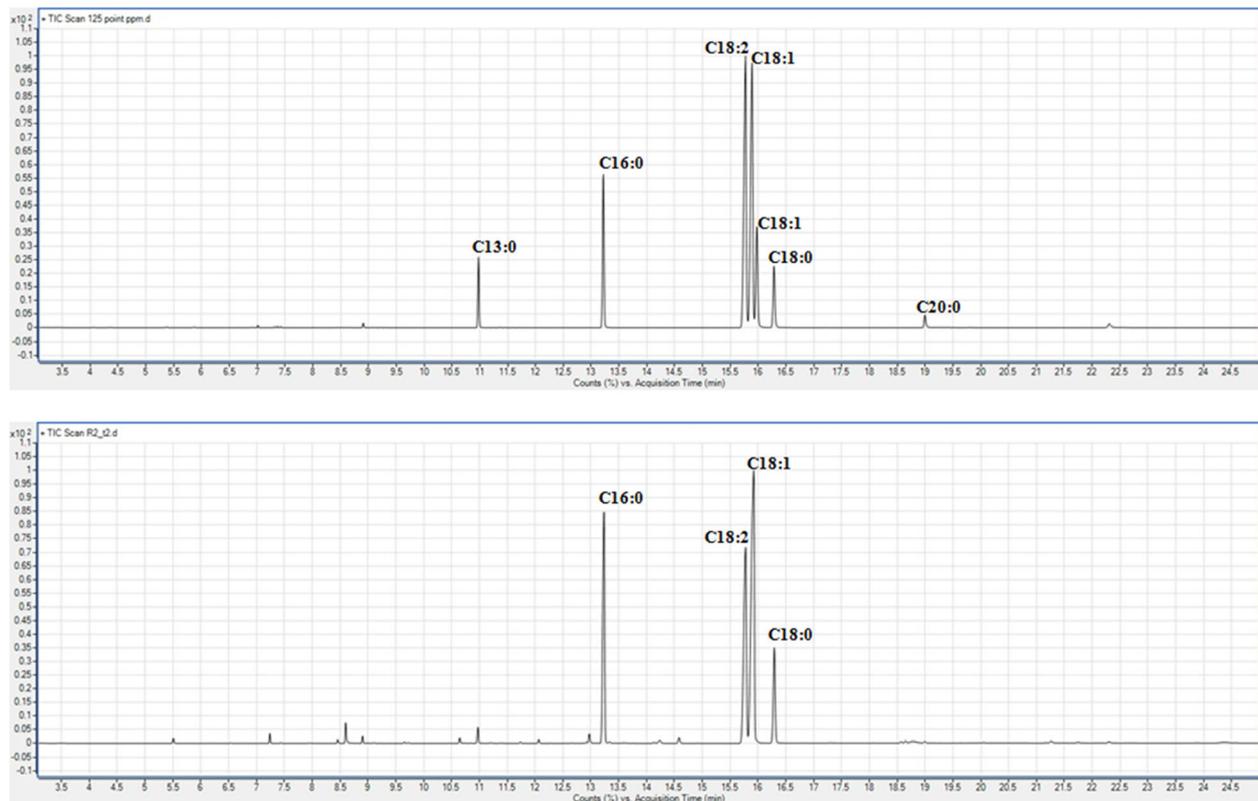

Fig. 8 GC-MS chromatogram of standard FAME mix and FAME derived from representative yeast strain *Rhodotorula mucilaginosa* R2

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Table 5 The total percentage of fatty acid methyl esters (FAMEs) produced by different yeast strains when grown on lipid production medium at 28 °C, 140 rpm for 96 h and its comparison with plant-based oils

Yeast Strain	Relative abundance of FA (w/w %)			
	C16:0 (palmitic acid)	C18:0 (stearic acid)	C18:1 (oleic acid)	C18:2 (linoleic acid)
*Rhodotorula mucilaginosa is*olate R2	20.89 ± 1.05	9.9 ± 1.37	40.45 ± 3.48	28.75 ± 3.17
Pichia kudriavzevii isolate FK02	19.86 ± 0.6	3.13 ± 0.065	49.42 ± 0.63	27.58 ± 0.07
Candida parapsilosis isolate FK04	20.13 ± 0.8	5.7 ± 0.14	62.7 ± 0.36	11.45 ± 0.6
Saturnispora diversa isolate FO05	19.24 ± 1.1	6 ± 0.18	56.58 ± 1.01	18.16 ± 1.93
Wickerhamomyces anomalus isolate FK09	19.59 ± 0.3	3.41 ± 0.14	45.88 ± 0.17	31.09 ± 0.69
Zygoascus hellenicus isolate FC10	19.51 ± 1.3	9.3 ± 0.84	51.23 ± 5.4	19.89 ± 1.8
Saccharomyces cerevisiae YMA1	30.57	12.69	56.72	Not detected
Plant-based oils				
Palm oil	32–46.3	4-6.3	37–53	6–12
Soya bean oil	11	4	23.4	53.2
Jatropha oil	14.66	6.86	39.08	32.48

Poontawee et al. 2016). The FA compositional profiles of the yeast strains were found to be comparable with vegetable oil such as rapeseed oil, palm oil, soya bean oil, and Jatropha oil which are conventionally used for biodiesel production (Vyas and Chhabra 2017). In our study, the concentration of mono-unsaturated FA (C18:1) was found to be in the range of (40.45–62.7%) for all the tested yeast strains and proportionately higher (i.e., ≥ 50% w/w of total FAs) in case of *Candida parapsilosis* FK04, *Saturnispora diversa* FO05, *Zygoascus hellenicus* FC10, *and Saccharomyces cerevisiae* YMA1 representing similarity in FA composition to rapeseed oil which is considered as the model oil for biodiesel synthesis (Papanikolaou et al. 2017). Lipids having FA composition with high amount of saturated FA (≥ 30% w/w of total FAs) were found in case of *Rhodotorula mucilaginosa* R2, *Zygoascus hellenicus* FC10, and *Saccharomyces cerevisiae* YMA1 representing similarity with animal fats and palm oil (Maina et al. 2018). Moreover, it was observed for all strains tested presented elevated quantities of linoleic acid (≥ 15% w/w of total FAs) with the highest content of polyunsaturated FA (C18:2) in case of *W. anomalus* FK09 (31.09 ± 0.69%), whereas *C. parapsilosis* FK04 has the lowest concentration of C18:2 (11.45%) and in *S. cerevisiae* YMA1, this acid was negligible. Similar findings were obtained with *C. pararugosa* strain BM24, *C. metapsilosis* strain EL2, and *C. parapsilosis* strain LV2, having high percentage of oleic acid and the low percentage of linoleic acid (Arous et al. 2017a).

Conclusion

A total of 14 yeast isolates belonging to seven different genera were established as pure culture from the traditional fermented food and beverages of Manipur and Mizoram, India. Molecular identification of all the yeast isolates was performed by PCR amplification of 5.8S-ITS and D1/D2 domain of the 26S rRNA gene of the isolates. The total lipid content of all the obtained yeast isolates varied between 5.7 ± 0.1 and 21.63 ± 1.2%. *Rhodotorula mucilaginosa* R2 gave the highest lipid content (21.63 ± 1.2^i%). The lipid content in two of the most promising strains *R. mucilaginosa* R2 and *W. anomalus* FK09 (selected on the basis of highest lipid content and biomass) increased to 22.21 ± 0.4% and 12.83 ± 0.08% using C/N 40 and 20 respectively. The ability of the some of the yeast strains to grow well and accumulate non-negligible quantities of intracellular lipid droplets having similar fatty acid composition to that of vegetable oil suggests their suitability for further studies. Particularly, the pink yeast *R. mucilaginosa* R2 may provide an alternative platform for SCO production and therefore this organism should be studied in detail. The production of metabolic co-products like carotenoids, endopolysaccharides, polyols, and organic acids by most yeast isolates are valuable renewable resources as commodity chemicals.

Ethics approval and consent to participate
N/A.

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Competing interests

The authors declare that they have no conflict of interest.

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