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Final Report

Project: Production of Yeast Cell Oil from Sugar cane Leaves for Biodiesel Production

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Phosphate Supplementation of Sugarcane Leaves Hydrolysate Enhanced Oil Accumulation in *Yarrowia* NG17

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บทคัดย่อ *Cryptococcus humicola* NG2, *Cyberlindnera subsufficiens* NG8.2, *Rhodotorula mucilaginosa* MG11-2.3 และ *Yarrowia* NG17 เป็นยีสต์น้ำมันซึ่งใช้น้ำตาลไซโลสได้ ที่แยกได้ในงานวิจัยนี้ ในอาหารที่มีอัตราส่วนของคาร์บอนต่อไนโตรเจนต่ำ *Yarrowia* NG17 สามารถสะสมน้ำมันภายในเซลล์ได้สูงที่สุดเท่ากับ 27.9% (w/w dry weight; DW) โดยน้ำมันที่สะสมมีกรดไขมันคือ oleic acid และ palmitic acid เป็นองค์ประกอบหลักเท่ากับ 57.6% และ 25.4% ตามลำดับ ระหว่าง sugarcane leaves hydrolysate (SLH) และ detoxified SLH พบว่า *Yarrowia* NG17 ผลิตน้ำมันใน SLH ได้มากกว่าใน detoxified SLH แต่ปริมาณ oleic acid และ palmitic acid ซึ่งเป็นกรดไขมันที่เป็นองค์ประกอบหลักของน้ำมันลดลงเหลือเพียง 48.55% และ 22.1% ตามลำดับ ค่า pH ที่เหมาะสมที่สุดสำหรับการผลิตน้ำมันของ *Yarrowia* NG17 ใน SLH คือ 6.5 การเติม 0.1% (w/v) KH_2PO_4 ลงไปใน SLH (pH 6.5) ซึ่งนำมาเลี้ยง *Yarrowia* NG17 ทำให้ปริมาณน้ำมันที่ *Yarrowia* NG17 ผลิต เพิ่มขึ้นจาก 5.07 กรัม/ลิตร เป็น 6.67 กรัม/ลิตร

คำสำคัญ: แคลเซียมไฮดรอกไซด์ การลดความเป็นพิษ สารยับยั้ง ไบโอดีเซล ไซโลส

Abstract The xylose utilizing oleaginous yeasts *Cryptococcus humicola* NG2, *Cyberlindnera subsufficiens* NG8.2, *Rhodotorula mucilaginosa* MG11-2.3 and *Yarrowia* NG17 were newly isolated. *Yarrowia* NG17 had the highest accumulated oil content at 27.9% (w/w dry weight; DW) with a major fatty acid composition of oleic (57.6%) and palmitic (25.4%) acids when grown in a high carbon/nitrogen ratio (C/N) medium. *Yarrowia* NG17 gave a higher oil yield in sugarcane leaves hydrolysate (SLH) than in detoxified SLH, but this was lower compared to in the high C/N medium, while the oleic and palmitic acid compositions were reduced to 48.5% and 22.1%, respectively, in SLH compared to in the high C/N medium. Optimal pH for oil accumulation in SLH was 6.5. Supplementation of SLH (pH 6.5) with 0.1% (w/v) KH_2PO_4 increased the oil yield of *Yarrowia* NG17 from 5.07 to 6.67 g/L.

Key words: Ca(OH)2; detoxification; inhibitor; biodiesel; xylose

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INTRODUCTION

Biodiesel has similar features to petroleum diesel but with lower emissions of nitrogen dioxide, sulfur dioxide and dusts than petroleum diesel upon combustion (Innocent *et al.* 2013). Oleaginous yeasts are defined as yeasts that accumulate intracellular lipids as oil droplets to more than 20% (w/w dry weight; DW), while yeasts in some genera, such as *Lipomyces* sp., *Rhodosporidium* sp. and *Rhodotorula* sp., can accumulate oil up to 70% (w/w DW) (Kitcha and Cheirslip 2011). The oil is mainly in the form of triacylglycerols (Sitepu *et al.* 2014) with a fatty acid composition that is similar to vegetable oil (Steen *et al*. 2010). Therefore, yeast oil can be used as a raw material for biodiesel production. Differences in the oil accumulation level and its fatty acid composition within yeasts has been reported to be strain-specific (Sitepu *et al*. 2013), which highlights the importance of screening for novel oleaginous yeast strains. Oil production from yeast has the advantages over plants that it does not require a large plantation area, has no limitation on topography and seasonality and gives a 100-fold higher oil productivity. The yeasts can utilize various kinds of simple sugars, including those derived from lignocellulose, which is a complex component found in plant cell walls (Atabani *et al.* 2012). Lignocellulose consists of the three kinds of polymer of (i) cellulose, a homopolymer of glucose, (ii) hemicellulose, a heteropolymer of pentoses (*eg*. xylose, arabinose, mannose and galactose) and hexose (*eg*. glucose), and (iii) lignin, a heteropolymer of aromatic compounds that covers the cellulose and hemicellulose (Cheng *et al*. 2008). The hydrolysis of cellulose and hemicellulose into their simple sugar components requires the separation of these polymers by pretreatment, typically performed as an acid-pretreatment.

Thailand was the second largest exporter of cane sugar and, for example, produced 100 million tons of sugarcane on 11 million rai (17,600 km^2) of plantation area in 2016 (Office of the Sugar Cane and Sugar Board, 2016). Around 250 kg of sugarcane leaves are obtained per rai of sugarcane, but these are mainly burnt to ease harvesting of the canes, which wastes their biomass energy value and creates air pollution problems with an adverse effect on the respiratory system (Cancado *et al.* 2006). Consequently, it is of economic and environmental benefit to create value for sugarcane leaves, which are a rich lignocellulose source. Acid pretreatment of the leaves at 6% (w/v) in 1.5% (w/v) H_2SO_4 at 121 °C, 103 kPa for 30 min and then treatment of this slurry by cellulase (160 FPU/g DW) digestion at 50 °C, pH 5.0 for 6 h, yielded the simple sugars glucose and xylose at 9.8 and 9.0 g/L, respectively, in the obtained sugar leaves hydrolysate (SLH) (Jutakanoke *et al*. 2012).

The objective of this work was to screen for high oil accumulating oleaginous yeast strains that can assimilate glucose and xylose, the major monomer sugars derived from lignocellulose, and maximize its oil production using SLH as the raw material.

EXPERIMENTAL

Yeast Isolation and SLH preparation

Yeasts were isolated from 71 samples derived from soil, water, plant debris and insect frass that had been collected aseptically from three sampling sites. These were the mangrove forest resource development station 11 (26 samples), mangrove forest research center 1 (20 samples) and Ngao waterfall national park (25 samples), all in Ranong province, Thailand. Leaves of sugarcane (*Saccharum officinarum* L.C SB06-2-15) were obtained from the Sugarcane and Sugar Industry Promotion Center, Region 3, Chonburi province, Thailand. The sugarcane leaves were dried at 60 ºC until at constant weight and then hammer milled and sieved to obtain a 20–40 mesh particle size. The chemical composition of the sugarcane leaves, determined by TAPPI T211 om-12, T222 om-15 and T203 cm-09, is summarized in Table 1.

Table 1. Chemical Composition of Sugarcane Leaves

Component	Composition (%(w/w DW))
Holocellulose	62.3
Alpha cellulose	38.8
Beta cellulose	10.8
Gamma cellulose	12.7
Lignin	13.8
Ash	11.4

Yeast Isolation and Preliminary Screening for High Oil Accumulating Yeast Strains by Nile Red Staining

Yeasts were isolated from each respective sample by selective culture in YM broth containing 0.001% (w/v) chloramphenicol and 0.025% (w/v) sodium propionate at 30 °C, 200 rpm for 3 d. Purification was then performed by the streak plate method to single colonies, which were maintained on a YM slant at 4 °C. Preliminary screening for high oil accumulating strains was performed by growing the yeast isolates in a high carbon/nitrogen ratio (C/N) medium (all (w/v): 5% glucose, 0.01% yeast extract, 0.01% $(NH_4)_2SO_4$, 0.05% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2$, pH 5.5) at 30 °C, 200 rpm for 6 d. The resultant culture was stained with Nile red (Sitepu *et al*. 2012) for 10 min and observed under a fluorescence microscope (Olympus BX 51, USA) using a U-WNB 2 filter at excitation and emission wavelengths of 470–490 and 520 nm, respectively. Yeast cultures that showed a bright golden yellow spot (oil droplet) larger than half of the cell space was selected for further determination of the xylose assimilating capability.

Molecular Identification of Yeast Isolates by $(GTG)_5$ Fingerprint Profiles and 26S rDNA (D1/D2 domain) Sequence Analysis

For each yeast isolate, the genomic DNA was extracted as reported (Endoh *et al.* 2008). In brief, yeast grown in YM broth (20 µl) was mixed with 200 µl of lysis solution (0.1 M Tris buffer, 0.03 M EDTA, 0.5% (w/v) SDS, pH 8.0), boiled for 15 min, mixed with 2.5 M potassium acetate (200 µl) and then kept at 4 °C for 60 min. After centrifugation (4 °C, 16,089 ×g, 5 min) the supernatant was harvested and subjected to chloroform/isoamyl alcohol extraction, and then the genomic DNA was precipitated by the addition of isopropanol to 50% (v/v) and centrifugation. After sequential washing with 70% (v/v) and 99.8% ethanol, the DNA was dried at 37 °C and dissolved in 20 µL distilled water.

The extracted genomic DNA was PCR amplified using a GTG_5 (5'-GTGGTGGTGGTGGTG-3') primer with thermal cycling at 95 °C for 5 min, followed by 40 cycles of 93 °C for 45 sec, 50 °C for 1 min and 72 °C for 1 min, and then a final 72 °C for 6 min (Endoh *et al*. 2011). The PCR products were resolved by 1.5% (w/v) agarose gel electrophoresis and visualized and recorded under UV transillumination following ethidium bromide staining. The size pattern of the resolved amplicons (GTG_5 fingerprint) was used to group the isolated yeasts based upon identical/different fingerprints.

The 26S rDNA (D1/D2 domain) of one representative isolate (randomly selected) of each GTG_5 group was PCR amplified using the NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3) and NL4 (5'-GGTCCGTGTTTCAAGACGG-3) primers (Kurtzman and Robnett 2003). The thermal cycling was performed at 94 °C for 3 min followed by 36 cycles of 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 30 sec, and then followed by a final 72 °C for 5 min. The PCR product was purified using a GenepHlow™ PCR cleanup kit (Geneaid, Taiwan) and commercially direct sequenced at Bioneer Corporation, South Korea. The DNA sequence data was manually edited using the Bioedit program version 7.2.5 software (Hall 1999) and compared to sequences deposited in GenBank using the BLASTn algorithm (https://www.ncbi.nlm.nih.gov). Homologous sequences were aligned in the muscle program (Edgar 2004) and phylogenetic trees were constructed using the Neighbor-joining (NJ) and maximum-parsimony (MP) methods in the MEGA 6 program version 7.0 (Tamura *et al.* 2013). Nucleotide-sequence divergence was calculated using the Kimura-2-parameter model, and bootstrap support values for nodes were obtained from 1,000 replicates.

Screening for Xylose Assimilating Yeast Strains

The strongly staining Nile red yeast strains were then examined for their capability to assimilate xylose by streaking on medium containing xylose as the sole carbon source (all (w/v): 0.67% yeast nitrogen base, 1% xylose, 2.0% agar (w/v), pH 5.5) and incubating at 30 °C for 2 d. Each resultant colony (and so xylose assimilating yeast) was then further quantitatively analyzed for its ability to accumulate intracellular oil.

Analysis of Accumulated Intracellular Oil

The level of accumulated intracellular oil of each xylose assimilating yeast strain produced by two stage fermentation (Ryu *et al*. 2013) was analyzed. First, the xylose assimilating yeast was grown in YM broth (25 mL) at 30 °C, 200 rpm for 48 h, harvested by centrifugation (4 °C, 9,820×g, 10 min) and washed with distilled water. Secondly, the washed cells were resuspended in the high C/N medium (50 mL) and incubated as above for the indicated time (0–6 d in 1 d intervals). The cells were then harvested by centrifugation, washed with distilled water and dried by lyophilization. The amount of accumulated intracellular oil was analyzed as reported (Kitcha and Cheirsilp, 2011). In brief, the dried cells (0.5 g) were suspended in 10 mL of a 2:1 (v/v) ratio of chloroform: methanol, sonicated at 37 kHz (15 min), and then 4 mL of 0.73% (w/v) NaCl was added to precipitate non-oil impurities (Axelsson and Gentili 2014). After centrifugation at 4 °C, 614×g for 10 min, the oil containing lower phase was harvested and solvent removed by evaporation at room temperature to leave the oil. Yeast which accumulated lipid at ≥ 20% (w/w DW) were selected as oleaginous. *Lipomyces starkeyi* JCM 5955, a known oleaginous yeast strain, was used as a positive control.

Analysis of Fatty Acid Composition

The oil was extracted and transesterified as reported (Anamnart *et al*. 1998). In brief, 1 g of cells was suspended in 10% (w/v) KOH in methanol (0.8 mL) at 80 ºC for 2 h. After cooling down to room temperature, it was extracted with petroleum ether (1 mL) to separate unsaponified materials and centrifuged. The aqueous phase was harvested and acidified by 6 N HCl (0.3 mL) prior to extraction with diethyl ether and then harvesting the diethyl ether phase. Fatty acids were then recovered by solvent evaporation under nitrogen gas and transmethylated with boron trifluoride. The obtained fatty acid methyl esters were further extracted with hexane and then analyzed by gas chromatography (GC; Agilent Technologies 7890 B, USA) equipped with a capillary column (0.32 mm × 30 m, 0.25 µ thickness) and flame ionization detector. Injection and detection temperature were 150 and 250 ºC, respectively. Helium was used as the carrier gas at a flow rate of 2.3 mL/min. Temperature was programmed as follows: the 150 ºC initial temperature was sequentially increased to 180 ºC at 10 ºC/min, to 200 ºC at 5 ºC/min, to 205 ºC at 0.5 ºC/min and then held at 205 ºC for 2 min and finally to 250 ºC at 5 ºC/min and held at 250 ºC for 5 min (total 33 min runtime) (Limsuwatthanathamrong *et al.* 2012).

Production of the SLH

Preparation

Sugarcane leaves particles were suspended at 6 or 12% (w/v) in 1.5% (w/v) H_2SO_4 and autoclaved at 121 °C, 103 kPa for 30 min. The resultant slurry (pretreated sugarcane leaves suspended in pretreatment hydrolysate) was adjusted to pH 5.0 and hydrolyzed by

AccellulaseTM[1500] **(**2,500 CMC U/g**)** at 50 °C, 125 rpm for 6 h. The solid residue was then removed by stainless steel mesh filtration and centrifugation (4 °C, 9,820×g, 20 min) leaving the SLH supernatant that was harvested, adjusted to pH 5.5 and filter-sterilized through a 0.22 μ membrane filter. The SLH produced from the 6% and 12% (w/v) loading level are referred to as SLH-6% and SLH-12%, respectively.

Detoxification of SLH

The SLH-12% was detoxified by adjusting the pH to 10.0 with $Ca(OH)_2$ at 50 °C for 30 min with mixing at 125 rpm (Yu *et al.* 2011). The mixture was then clarified by centrifugation (4 ºC, 9,820×g, 20 min) and the detoxified SLH (DSLH) supernatant was harvested, adjusted to pH 5.5 and filter-sterilized through a 0.22 μ membrane filter.

Production of Yeast Oil from SLH

Oil was produced by the oleaginous yeasts using a two-stage fermentation as previously described using the SLH-6% containing 0.3% (w/v) peptone and 0.3% (w/v) yeast extract for cell propagation in the first stage and SLH-12% for lipid production in second stage.

Analytical Methods

Glucose and xylose concentrations were analyzed using a Biochemistry Analyzer (YSI 2700 Select, USA), while analysis of furfural and hydroxymethylfurfural (HMF) were performed by HPLC using a Varian C18 column with a 88:12 (v/v) ratio mixture of acetic acid: methanol as the mobile phase at a flow rate of 1.0 mL/min (Bhatia *et al*. 2017). Total nitrogen was analyzed by the Kjeldahl method (Bradstreet 1954).

RESULTS AND DISCUSSION

Isolation of Yeasts and Selection of Oleaginous Strains by Nile Red Staining

In total 106 yeast isolates were obtained and these could be grouped into 29 distinct GTG_5-PCR patterns (Supplementary information (SI); Fig. S1). The 26S rDNA (D1/D2 domain) was PCR amplified and sequenced from a representative isolate of each of the 29 GTG_5-PCR patterns. By comparison of the obtained sequences to those in the GenBank database revealed the 29 obtained sequences (molecular operational taxonomic units; MOTUs) belonged to 25 known species and four unknown species (MOTUs).

The 58 yeasts isolated from the mangrove forest resource development station 11 belonged to 10 genera and 16 species (Table S1; SI), of which nine species were only isolated at this site (underlined): *Candida (C.) edaphicus*, *C. carpophila*, *C. glabrata*, *C.*

pseudolambica, *C. orthopsilosis*, *C. thaimueangensis*, *C. tropicalis*, *Hanseniaspora thailandica*, *Kluyveromyces (K.) aestuarii*, *Lachancea thermotolerans*, *Nakazawaea siamensis*, *Pichia kudriavzevii*, *Rhodotorula (Rh.) mucilaginosa*, *Schwanniomyces (Sc.) vanrijiae*, *Trichosporon (T.) mycotoxinivorans* and *Wickerhamomyces (W.) sydowiorum* plus the MOTU in the genus *Candida* MG11-13.3. The most frequently isolated species was *K. aestuarii* (17.2%) followed by *C. tropicalis* (15.5%) and *Sc. vanrijiae* (13.8%).

For the mangrove forest research center 1, the 29 isolated yeasts belonged to four genera and eight species (Table S1; SI), of which four species were only isolated at this site (underlined): *C. glabrata*, *C. orthopsilosis*, *C. sanitii*, *C. silvae*, *C. tropicalis*, *K. aestuarii*, *Kodamaea ohmeri* and *Meyerozyma (M.) caribbica*. The most frequently isolated species was *K. aestuarii* (55.2%) followed by *M. caribbica* (13.8%) and *C. glabrata* (10.3%).

From the Ngao waterfall national park the 19 isolated yeasts belonged to eight genera and nine species (Table S1; SI), of which five species were only isolated at this site (underlined): *C. akabanensis*, *C. tropicalis*, *Cryptococcus (Cr.) humicola*, *Cr. laurentii*, *Cyberlindnera (Cy.) subsufficiens*, *Saccharomycopsis fermentans*, *Sc. vanrijiae*, *T. mycotoxinivorans* and *W. sydowiorum* plus the MOTU in the genus *Kurtzmaniell* NG4.3, NG5, NG6, *Wickerhamomyces* NG10, NG12 and *Yarrowia* NG17. The most frequently isolated species was *C. akabanensis* (15.8%).

Only one species, *C. tropicalis*, was isolated from all three sites sampled. *Kluyveromyces aestuarii* was first isolated from a soil sediment at Biscayne estuary, Florida, USA (Fell 1961), but could not be isolated from mangroves impacted by neighborhood activities at Rio de Janeiro, Brazil (Hagler *et al*. 1982). That is from eight mangrove sediment samples collected in Rio de Janeiro, Brazil, *K. aestuarii* was isolated from all of them except for the one sample from a mangrove connected to a beach contaminated by garbage blown ashore by waves. Therefore, *K. aestuarii* was proposed as a potential environmental quality indicator yeast for mangroves (Araujo and Hagler 2011). Both mangrove forest sampling sites in this study are well preserved as indicated by *K. aestuarii* isolated.

Nile red staining of all 106 isolated yeasts revealed that 24 (22.6%) had an intracellular oil droplet larger than half of their cell space (Table S1; SI), and so were defined as oleaginous and selected for further study.

Xylose Assimilating Oleaginous Yeasts

Examination of the xylose assimilating capability of the 24 selected oleaginous yeast isolates indicated that 16 assimilated xylose (Table S1; SI). These were *C. tropicalis* (NG21, MG1-8, MG11-4.1, MG11-8.2, MG11-17.3, MG11-4w.2, MG11-20w.2 and MG11-20w.4), *C. orthopsilosis* MG11-11.3, *Cr. laurentii* NG22, *Cr. humicola* NG2, *Cy. subsufficiens* NG8.2, *Rh. mucilaginosa* MG11-2.3, *T. mycotoxinivorans* (NG4.1 and MG11-12.3) and *Yarrowia* NG17.

Analysis of the Level of Accumulated Intracellular Oil

Analysis of the level of accumulated intracellular oil in the 16 xylose assimilating strains produced by the two-stage fermentation revealed that five of them (*Cr. humicola*

NG2, *Cy. subsufficiens* NG8.2, *Rh. mucilaginosa* MG11-2.3, *T. mycotoxinivorans* MG11-12.3 and *Yarrowia* NG 17) were truly oleaginous. The *Yarrowia* NG 17 accumulated the highest intracellular oil level at 27.93% (w/w DW) followed by *R. mucilaginosa* MG11-2.3, *C. humicola* NG2, *T. mycotoxinivorans* MG11-12.3 and *C. subsufficiens* NG8.2 (Fig. 1). Since *T. mycotoxinivorans* has been reported as infectious to humans (Hickey *et al*. 2009) it was not studied further.

The *Yarrowia* NG17, *C. subsufficiens* NG8.2 and *C. humicola* NG2 strains were isolated from Ngao waterfall national park, while *R. mucilaginosa* MG11-2.3 was isolated from mangrove forest resource development station 11 (Table S1, SI).

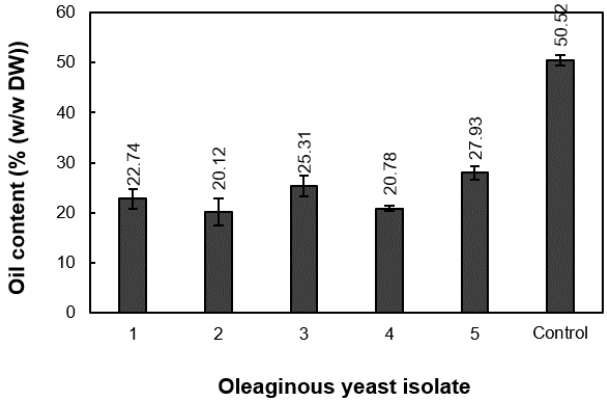


Fig. 1. Intracellular oil content of the five isolated oleaginous yeasts: *Cr. humicola* NG2 (1), *Cy. subsufficiens* NG8.2 (2), *Rh. mucilaginosa* MG11-2.3 (3), *T. mycotoxinivorans* MG11-12.3 (4) and *Yarrowia* NG17 (5), plus the known oleaginous yeast *Lipomyces starkeyii* JCM5955 as a positive reference control. Data are shown as the mean ± 1 SD, derived from three independent assays.

Fatty Acid Composition of Yeast Oils

The accumulated oil from *Yarrowia* NG 17, *Rh. mucilaginosa* MG11-2.3, *Cy. subsufficiens* NG8.2 and *Cr. humicola* NG2 produced by the two-stage fermentation using YM and then the high C/N medium was transesterified and analyzed by GC. The major fatty acids in each were oleic (C18:1), palmitic (C16:0), linoleic (C18:2) and steric (C18:0) acids. That from *Yarrowia* NG 17 and *Rh. mucilaginosa* MG11-2.3 had the highest oleic acid content at 57.6% and 25.4%, respectively, while the palmitic acid content was at 25.4% and 19.0%, respectively (Fig. 2). These results are consistent with the dominant fatty acid of oil accumulated in yeasts belonging to Ascomycota families, Basidiomycota orders and the yeast-like algal genus *Prototheca* that were reported to be oleic, palmitic, linoleic and stearic acids (Sitepu *et al*. 2013). A high proportion of palmitoleic acid (C16:1) (22.3%) was found in the oil of *Cy. subsufficiens* NG8.2 (Fig. 2), which corresponds to 445 mg/g DW. *Candida krusei* was found to produce palmitoleic acid at 430 mg/g DW when grown in medium containing a C/N ratio of 30 and a C/P ratio of six (Kolouchova *et al*. 2015).

Palmitoleic acid has several applications in medicine and cosmetics, such as to reduce the total and LDL cholesterol serum levels (Griel *et al*. 2008), prevent ß-cell apoptosis (Morgan and Dhayal, 2010), to heal and act as an anti-inflammation agent in damaged skin (Gao *et al*. 2003), inhibit the growth of Gram-positive bacteria (Wille and Kydonieus, 2003) and provide increased skin resistance against solar radiation (Hayashi *et al*. 2003).

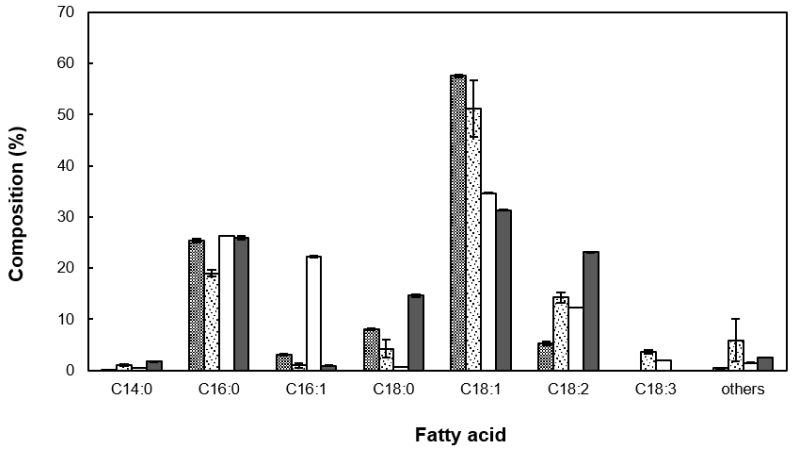


Fig. 2. Fatty acid composition of the accumulated oil from the isolated oleaginous yeasts *Yarrowia* NG17 (▨), *Rh. mucilaginosa* MG11-12.3 (⊠), *Cy. subsufficiens* NG8.2 (□) and *Cr. humicola* (■) after two-stage fermentation. Data are shown as the mean ± 1SD, derived from three independent repeats.

Production of Yeast Oil from SLH

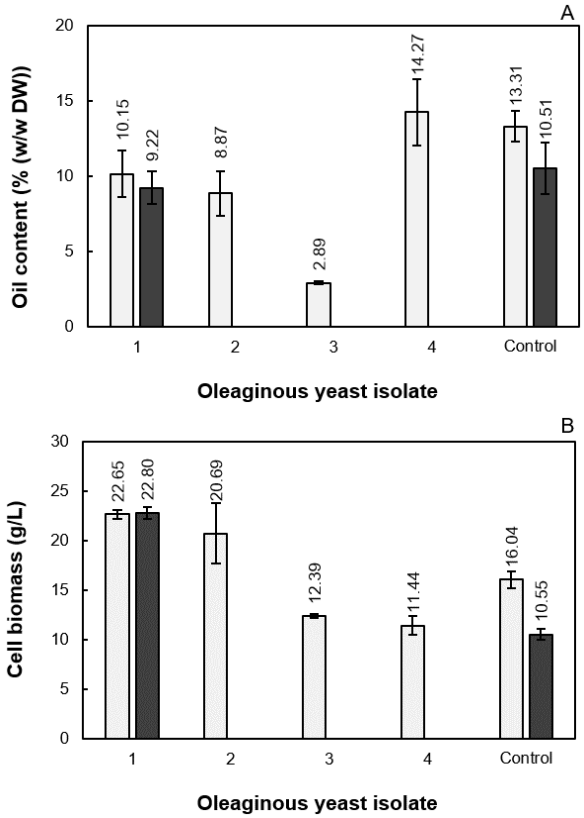
The production of accumulated yeast oil from SLH was evaluated in a two-stage fermentation using the enriched SLH-6% followed by SLH-12% as the media. The concentration of glucose, xylose, total nitrogen, furfural and HMF in the SLH-12% is shown in Table 2.

Table 2. Concentration of sugars, total nitrogen and some inhibitors in the SLH-12% and DSLH-12%

Component	SLH-12% (g/L)	DSLH-12% (g/L)
Glucose	18.7	18.0
Xylose	19.1	18.5
Nitrogen	0.6	0.6
Furfural	0.13	0.10
HMF	0.3	0.29

The accumulated oil content in the yeasts when grown in SLH-12% (Fig. 3) was highest in *Cr. humicola* NG2 at 14.3% (w/w DW) followed by *Yarrowia* NG17 at 10.2% (w/w DW), but *Yarrowia* NG17 had a higher cell biomass (22.7 g/L) than *Cr. humicola* NG2 (11.4 g/L) and so a higher oil yield was obtained from *Yarrowia* NG17 (2.3 g/L) than *Cr. humicola* NG2 (1.64 g/L). Therefore, *Yarrowia* NG17 was selected for further study.

The effect of acid-pretreatment inhibitors on the oil accumulation of *Yarrowia* NG17 in SLH was examined by comparison between its oil yield in SLH-12% and DSLH-12%. *Yarrowia* NG17 had a higher oil yield in SLH-12% (2.3 g/L) than in DSLH-12% (2.1 g/L) (pH 5.5). While there was no significant difference in the furfural and HMF concentrations in the SLH-12% and DSLH-12%, the concentration of glucose and xylose was lower in the DSLH-12% than in the SLH-12% (Table 2). This reduction in the C/N ratio of the DSLH-12% might have been the cause of the lower oil accumulation in *Yarrowia* NG17. In accord, a reduction in the glucose, xylose and furfural concentrations occurred when sugarcane bagasse hydrolysate was detoxified by $Ca(OH)_2$ (Huang *et al.* 2009). In addition, there have been several reports that a reduction in the oil accumulation in oleaginous yeasts occurs when grown in the acid pretreated hydrolysate of lignocellulosic biomasses after being detoxified by $Ca(OH)_2$. For example, when *Y. lipolytica* Po1g was grown in the detoxified HCl-pretreated hydrolysate of sugarcane bagasse (Tsigie *et al.* 2011), *Cr. curvatus* in the detoxified H_2SO_4-pretreated hydrolysate of wheat straw (Yu *et al.* 2011) and *Rh. glutinis* in the detoxified H_2SO_4-pretreated hydrolysate of corncob (Liu *et al.* 2015). The oil accumulation in *Rhodosporidium toruloides* Y4 was not affected by acetate 5-HMF and syringaldehyde, while hydroxybenzaldehyde and vanillin at 1 mM reduced the oil accumulation and cell biomass, and 1 mM furfural reduced the cell biomass by 5% (Hu *et al.* 2009).



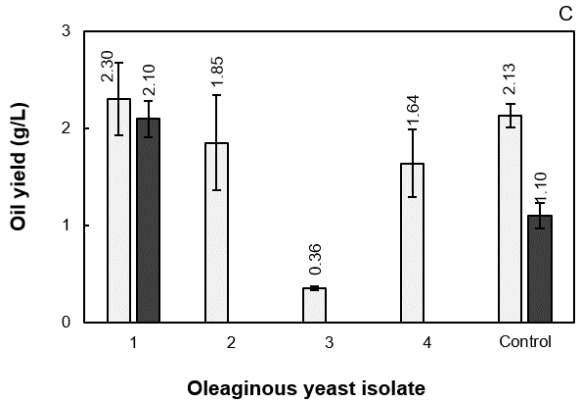


Fig. 3. Comparison of the (A) oil content, (B) cell biomass and (C) oil yield of the oleaginous yeasts *Yarrowia* NG17 (1), *Rh. mucilaginosa*, MG11-12.3 (2), *Cy. subsufficiens* NG8.2 (3) and *Cr. humicola* (4), plus the positive reference control *L. starkeyii*, when grown in SLH-12% (□) or DLSH-12% (■), pH 5.5. Data are shown as the mean ± 1SD, derived from three independent repeats.

The effect of varying the pH (5.0–6.5) of the SLH-12% on the oil production by *Yarrowia* NG17 was investigated. The highest oil content (21.0 % w/w DW), cell biomass (24.1 g/L) and oil yield (5.07 g/L) were obtained at pH 6.5. In accord, the initial pH of the medium is known to impact on the resulting oil yield and cell biomass of oleaginous yeasts (Ryu *et al.* 2013).

Yarrowia NG17 utilized glucose and xylose simultaneously, but the glucose was utilized faster, with the rate of xylose use increasing after the glucose was exhausted (Fig. 4). *Rhodosporidium kratochvilovae* HIMPA1 utilized glucose, fructose and sucrose simultaneously during accumulation of its intracellular oil by utilized glucose faster than the other sugars in the first 48 h (Patel *et al.* 2015a). *Lipomyces starkeyi* AS 2.1560 utilized glucose and mannose simultaneously for oil accumulation (Yang *et al*. 2014).

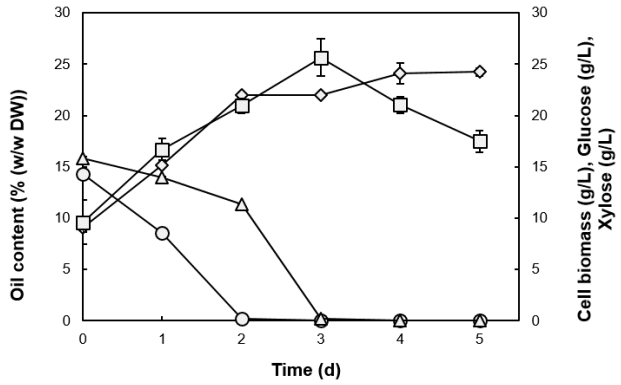


Fig. 4. Oil and biomass accumulation of *Yarrowia* NG17 in SLH-12% (pH 6.5) plus sugar content. Oil content (□), cell biomass (◇), glucose (○) and xylose (△). Data are shown as the mean ± 1SD, derived from three independent repeats.

Compared to the high C/N medium, the fatty acid composition of the oil from *Yarrowia* NG17 grown in SLH-12% had a reduced content of oleic and palmitic acids from 57.6 to 48.5% and from 25.4 to 22.1%, respectively, while the palmitoleic acid level was increased from 3.06 to 11.07% (Fig. 5). The major fatty acid composition of *Rhodosporidium kratochvilovae* HIMPA1 oil was oleic (45.4%), palmitic (21.9%) and linoleic (15.9%) acids when grown in pulp and paper industry effluent (Patel *et al*. 2017). However, the fatty acid composition changed to principally be palmitic (43.1%), steric (28.7%) and oleic (17.3%) acids when grown in the water extract of *Cassia fistula* L fruit pulp (Patel *et al*. 2015b). Given that palm and canola oils, two oils commonly used for biodiesel production, have oleic, palmitic and linoleic acids as their major fatty acids (Huang *et al*. 2012), then the oil from *Yarrowia* NG17 grown in SLH-12% has the potential to be a raw material for biodiesel production.

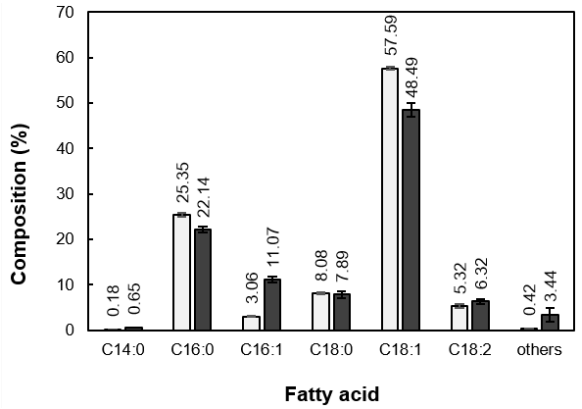


Fig. 5. Comparison of the fatty acid composition of the accumulated oil in *Yarrowia* NG17 when grown in high C/N medium (□) or SLH-12% (■). Data are shown as the mean ± 1SD, derived from three independent repeats.

Soluble phosphate was removed from the SLH during the dilute H_2SO_4 pretreatment of sugarcane leaves followed by $Ca(OH)_2$ detoxification (Wu *et al*. 2010). However, phosphorus is important for yeast growth and metabolism. In addition, oleaginous yeasts accumulate intracellular oil in a specific cell compartment encased with a phospholipid monolayer as an oil droplet (Lei *et al*. 2017), and phosphorus is an essential factor for phospholipid synthesis. Thus, phosphate deficiency might be another reason for the lower level of oil accumulation and lower cell biomass obtained when grown in the DSLH compared to in SLH.

As the DSLH had carbon/nitrogen ratio different from SLH, comparison of oil accumulation in DSLH and SLH affected by phosphate could not be carried out. But instead, a study on the effect of phosphate on oil accumulation of *Yarrowia* NG17 in SLH-12% with and without phosphate supplementation was done. In SLH-12% (pH 6.5) supplemented with 0.1% (w/v) KH_2PO_4, the oil content, cell biomass and oil yield of *Yarrowia* NG17 increased from 21.0 to 27.6% (w/w DW), 24.1 to 24.3 g/L and 5.07 to 6.67 g/L, respectively, with the same oil productivity (0.2 g/L/h) as in SLH-12% without phosphate supplementation (Fig. 6).

Thus, SLH requires phosphate supplementation to increase the oil accumulation of *Yarrowia* NG17.

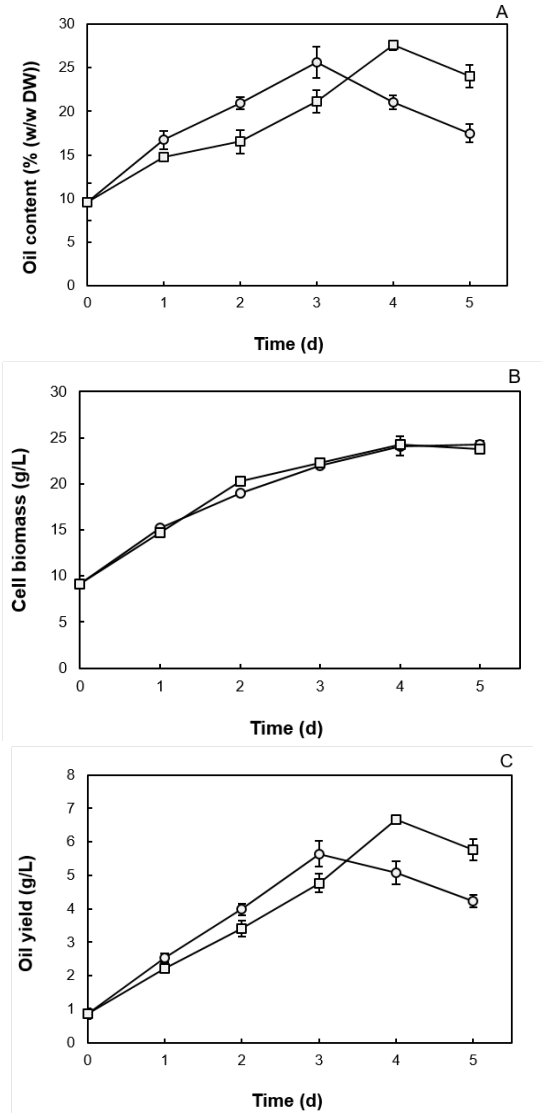


Fig. 6. Comparison of the (A) oil content, (B) cell biomass and (C) oil yield of *Yarrowia* NG17 grown in SLH-12% (○) or SLH-12% supplemented with 0.1% (w/v) KH_2PO_4 (□) (pH 6.5). Data are shown as the mean ± 1SD, derived from three independent repeats.

CONCLUSIONS

1. Intracellular oil accumulation in *Yarrowia* NG17 grown in sugarcane leaves hydrolysate (SLH) was enhanced by phosphate supplementation.
2. *Yarrowia* NG17 oil produced from SLH had a high proportion of C16 and C18 fatty acids similar to plant oil, and so has the potential to be a raw material for biodiesel production.

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