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ORIGINAL PAPER

Diversity and characterization of cultivable oleaginous yeasts isolated from mangrove forests

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Abstract
A total of 198 yeasts were isolated from 140 samples collected from 7 mangrove forests in 4 provinces of Thailand, and were found to belong to 30 genera, 45 described species and at least 12 undescribed species based on their 26S rRNA (D1/D2 domain) gene sequence. The most prevalent species was *Candida tropicalis*, followed by *Candida pseudolambica* and *Rhodosporidium paludigena*. Lipid accumulation, as determined by Nile red staining, of the isolated yeasts revealed that 69 and 18 strains were positive and strongly positive, respectively, while quantitative analysis of the intracellular lipid accumulated in the latter indicated that 10 of these strains, *Pseudozyma tsukubaensis* (YWT7-2 and YWT7-3), *Rhodotorula sphaerocarpa* (YWW6-1 and SFL14-1SF), *Saitozyma podzolica* (YWT1-1, NS3-3 and NS10-2), *Prototheca zopfii* var. *hydrocarbonea* OMS6-1 and *Prototheca* sp. (YMTW3-1 and YMTS5-2), were oleaginous. In this study we found that under nitrogen depletion condition (155 C/N ratio) *Pseudozyma tsukubaensis* YWT7-2 accumulated the highest level of intracellular lipid at 32.4% (w/w, dry cell weight), with a broadly similar fatty acid composition to that in palm oil.

Keywords Intracellular lipids · Oily yeast · Oleaginous yeast · *Pseudozyma tsukubaensis* · Triglyceride

Introduction

Some yeast strains can accumulate a high intracellular lipid level, as oil droplets, when grown under a high carbon but low nitrogen condition (Thanh 2006). Any yeast that accumulates more than 20% (w/w, dry cell weight; DW) oil is defined as an oleaginous yeast (Ratledge 1989). Several yeast genera have been reported as having oleaginous yeast members, such as *Cryptococcus, Rhodosporidium, Rhodotorula* (*R.*), *Lipomyces, Prototheca and Yarrowia* (Li et al. 2008; Ageitos et al. 2011; Sitepu et al. 2014). Since the fatty acid profile of yeast oil is similar to those of vegetable oils, they can likely be transformed to biodiesel by transesterification (Galafassi et al. 2012). The quality of the obtained biodiesel depends on the fatty acid composition of the yeast oil, where a higher saturated fatty acid content increases the ignition quality (cetane number) of the biodiesel but decreases its cold flow properties. On the other hand, a higher level of unsaturated fatty acids improves the cold flow properties but decreases the ignition quality and reduces the oxidative stability (Knothe 2009). However, a high unsaturated fatty acid composition is potentially beneficial from a medical point of view. For example, *Pichia* (*P.*) *segobiensis* SSOH12 oil contains 16% (w/w) palmitoleic acid (C16:1) (Schulze et al. 2014), an omega-7 mono-unsaturated fatty acid, that was reported to have a positive effect against obesity (Yang et al. 2011a) and the potential for prevention of brain and cardiovascular diseases (Matsunga et al. 1995). However, since yeast oleaginousity (Khot et al. 2012) and the fatty acid composition of the accumulated oil (Tanimura et al. 2014) are strain-specific, screening for novel oleaginous yeast strains is a necessity to explore for new strains producing a high quality yeast oil suitable for either biodiesel production or a potential health-related nutritional value.

Mangrove forests are a convergent area of terrestrial, estuary and marine ecosystems with a high diversity of microorganisms that play an important role as decomposers,

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nutrients recyclers and feed for invertebrates and zooplanktons in the ecosystem (Chen et al. 2015). Within Thailand, which has tropical savanna climate and an average annual temperature of 27.6 °C, mangrove forests have been reported as a source of several novel yeast species, such as *Candida* (*C.*) *thaimuengensis, C. andamanensis, C. phangngensis, C. laemsonensis, C. ranongensis* and *Kluyveromyces siamensis* (Limtong et al. 2007, 2008; Am-In et al. 2008, 2011). Therefore, it is an interesting sampling source for exploring novel oleaginous yeast strains. In this study, yeasts were isolated from the sediment, water and decaying biomaterial samples collected from seven mangrove forests in Thailand. The isolated yeasts were characterized by molecular phylogenetics to molecular operational taxonomic units (MOTUs) using their 26S rRNA (D1/D2 domain) gene sequence, and then converted to likely species by comparison of sequence identity to known (designated) yeast species and by standard morphological and biochemical characterization. The presence of oleaginous yeasts was screened from strong Nile red stained strains that belonged to the undescribed species and genera known to contain oleaginous yeasts.

Materials and methods

Yeast isolation

In total, 140 samples comprised of 59 sediments, 32 decaying biomaterials and 49 water samples were collected from seven mangrove forests located at Samutsakorn (13°30′11.0844″N, 100°16′16.9464″E), Trat (12°10′23.7″N, 102°24′24.0″E and 12°12′34.9″N, 102°33′07.3″E), Chantaburi (12°22′37.1″N 102°21′16.6″E) and Ranong (9°53′06.9″N, 98°33′43.4″E, 10°10′24.1″N, 98°42′43.4″E and 9°51′18.4314″N, 98°37′49.7748″E) provinces in Thailand. For sediments and decaying matters, 1 g of the sample was inoculated into 10 mL modified YM broth (mYM; 30 g/L glucose, 0.6 g/L yeast extract, 0.6 g/L malt extract and 0.6 g/L peptone, pH 5.0) supplemented with 150 mg/L chloramphenicol and incubated at 30 °C, 200 rpm for 72 h. For water samples, 5 mL was filtered through a 0.2 µm pore size membrane filter, and then the membrane was placed on mYM agar and incubated as above. The resultant cultures were purified to single colonies by the streak plate method on YM agar (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, 20 g/L agar, pH 5.0). Pure cultures were initially grouped based on their colony morphology and kept on a YM agar slant at 4 °C, and subsequently grouped by similar $(GTG)_5$ fingerprint profiles.

Molecular characterization

Yeast genomic DNA was extracted by the glass bead method (Endoh et al. 2011). Cells were suspended in 200 µL lysis buffer (300 mg yatalase and 30 µL RNase A in 30 mL yatalase buffer) and incubated at 37 °C for 2 h. Then 0.3 g glass beads (0.8 mm diameter) and 67 µL of 8% (w/v) sodium dodecyl sulfate were added, vortex mixed for 2 min and then incubated at 60 °C for 10 min before 87 µL of 3 M sodium acetate was added and the mixture chilled on ice for 5 min. After centrifugation (4 °C, 20,600×*g*, 5 min) the supernatant was harvested and 70 µL was mixed with 110 µL isopropanol and transferred to an AcroPrep 96 Multi-Well Filter Plate (PALL, USA) and vacuum filtered discarding the flow through. The resultant DNA precipitate was rinsed twice with 200 µL of 70% (v/v) ethanol by centrifugation (16 °C, 1580×*g*, 1 min) and eluted twice by 60 µL TE buffer into new plate by centrifugation (16 °C, 1580×*g*, 5 min). The extracted DNA was used as template for $(GTG)_5$ amplification and 26S rRNA (D1/D2 domain) gene sequencing.

For the $(GTG)_5$ amplification by PCR, the thermal cycling consisted of 40 cycles of 93 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min (Endoh et al. 2011). The PCR products were resolved by 1% (w/v) agarose gel-electrophoresis and the product pattern, or $(GTG)_5$ fingerprint pattern, was used for grouping the isolated yeasts based upon similar profiles. A representative strain of each $(GTG)_5$ fingerprinting pattern was then selected and its 26S rRNA (D1/D2 domain) gene sequence was amplified using the NL1 (5′-GCATATCAATAAGCGGAGGAA AAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGAC GG-3′) primer pair (Kurtzman and Robnett 2003). The PCR thermal cycling was performed at 95 °C for 5 min followed by 40 cycles of 93 °C for 45 s, 50 °C for 1 min and 72 °C for 1 min, and then followed by 72 °C for 6 min.

For each reaction, the PCR product was rinsed twice with 60 µL deionized water, vacuum filtered (discard flow through), and then directly sequenced using BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Stafford, USA) and analyzed by auto-sequencer ABI Prism 3100 (Applied Biosystems, Stafford, USA). The DNA sequence data was edited manually using BioEdit v. 7.2.5 (Hall 1999). The complete 26S rRNA (D1/D2 domain) gene sequence was compared to those available in the DDBJ/EMBL/GenBank database using the BLASTn program (http://www.ncbi.nlm. nih.gov/blast), and related sequences were then aligned with Muscle (Edgar 2004). The representative strains and accession number of gene sequences were showed in Table 1. Neighbor-joining (NJ) analysis was performed using the MEGA 6 software (Tamura et al. 2013) with the Kimura-2-parameter model

Table 1 Identified species of yeast isolated from mangrove forests in Samutsakorn, Trat, Chantaburi and Ranong provinces (Thailand)

Phylogenetic placement, related genus	Isolate/sampling site[a,b,c,d]	Representative strain (GenBank Accession no.)	Relative species (GenBank Accession no.), sequence identity of LSU D1/D2 (identity)	Source W	S	D
Ascomycota						
Pezizomycotina						
Dothideomycetes						
Aureobasidium	OMS19-1[a], OMS19-3[a], OMS19-6-1[a], YMTW9-1[b], YWW6-2[c]	YWW6-2 (LC387259)	*Aureobasidium melanogenum* (FJ150926), 99% (570/571)	■		■
Saccharomycotina						
Saccharomycetes						
Blastobotrys	FRN1-3[d]	FRN1-3 (LC387260)	*Blastobotrys chiropterorum* (NG 055412), 99% (572/578)		■	
Dipodascus	YMTW4-2[b], SRM6-1[d]	SRM6-1 (LC387261)	*Dipodascus geotrichum* (KY107740), 99%, (541/545)		■	
Kloeckera	FRN2-2[d]	FRN2-2 (LC387262)	*Kloeckera hatyaiensis* (DQ404528), 99% (562/565)	■		
Kluyveromyces	YWS2-1[c], YWS3-1[c], FRM5-2[d], YMTS6-1[b]	YWS3-1 (LC387263)	*Kluyveromyces aestuarii* (U69579), 98% (534/547)	■	■	
	FRL10-1[d], FRM 3-1[d], FRM7-2[d], FRM 8-3[d]	FRL10-1 (LC387264)	*Kluyveromyces siamensis* (AB330824), 99% (542/543)	■		
Kodamaea	FRL2-2[d], NS7-1[d], SRN6-3[d], OMS7-2[a], NS9-1[d], SFL2-1W[d], SFL6-3[d]	FRL2-2 (LC387265)	*Kodamaea ohmeri* (NG 042527), 99% (492/493)	■	■	■
Kuraishia	FRL20-5[d]	FRL20-5 (LC387266)	*Kuraishia piskuri* (NG 055227), 99% (569/571)	■		
Kurtzmaniella	FRN1-4[d]	FRN1-4 (LC387267)	*Candida natalensis* (U45818), 99% (564/566)	■		
	FRN1-5[d], NS3-1[d], NS3-2[d], NS3-4[d], NS5-1[d], NS5-2[d]	NS3-1 (LC387268)	*Candida zeylanoides* (U45832), 99% (564/570)	■	■	
Metahyphopichia	FRL2-7[d]	FRL2-7 (LC387269)	*Metahyphopichia laotica* (JX515975), 99%, (467/472)	■	■	
Meyerozyma	YMTW3-2[b], YWW3-1[c], BW2-5[b], FRL2-15[d], OMS24-5[a], FRM2-1[d], FRM8-2[d], PS1-6-2[d], BW5-2[b],FRL2-11[d]	YMTW3-2 (LC387270)	*Meyerozyma caribbica* (AY187283), 99% (567/571)	■	■	■
	SRM 6-3[d],PS1-8-1[d], SFL12-1[d]	SRM6-3 (LC387271)	*Candida carpophila* (U62311), 100% (570/570)	■	■	■
Metschnikowia	NS13-1[d], NS13-2[d]	NS13-1 (LC387272)	*Candida akabanensis* (EU100744) 100%, (540/540)	■	■	
	FRM2-5[d]	FRM2-5 (LC387273)	*Candida heveicola* (NG055367), 99% (507/513)	■	■	
Nakazawaea	FRL1-2[d], FRL2-10[d], SFL17-1[d], SFL11-1S[d], SFL1-1S[d], SFL3-1W[d], SFL1-3W[d], SFL10-2[d], SFL3-4S[d]	FRL1-2 (LC387274)	*Nakazawaea siamensis* (AB772177), 99% (566/569)	■	■	■
Nakaseomyces	OMS12-1[a], OMS12-3[a]	OMS12-2 (LC387275)	*Candida nivariensis* (AY627305), 95% (523/550)	■	■	■
	SFL16-1[d], SFL16-2[d]	SFL16-1 (LC387276)	*Candida uthaithanina* (AB588752), 98% (569/581)			■
Ogataea	FRN2-3[d]	FRN2-3 (LC387277)	*Ogataea wangdongensis* (AB734091), 99% (558/566)	■		
	BT2-1[b], BT2-4[b]	BT2-4 (LC387278)	*Candida maris* (NG 055138), 99% (561/565)			■
	BT2-5[b]	BT2-5 (LC387279)	*Candida maris* (NG 055138), 97% (552/567)			■
	PS1-8-5[d],PS1-6-5[d],PS1-6-1[d],PS1-8-4[d]	PS1-8-4 (LC387280)	*Candida cylindracea* (U45823), 93% (525/563)		■	
Pichia	YMTT3-5[b], YMTT3-7[b]	YMTT3-5 (LC387281)	*Pichia chibodasensis* (LC126429), 98% (548/559)			■
Saccharomycotina						
Saccharomycetes						
Pichia	OMS14-3[a],OMS15-5[a], OMS19-5[a],OMS24-4[a],SFL9-1[a], SFL11-4W[d]	SFL9-1 (LC387282)	*Pichia kudriavzevii* (EF550222), 100 (564/564)	■	■	■
	SRN6-1[d], FRM8-1[d], FRL10-3[d], SFL11-2W[d], YMTW6-1[b] NS7-2[d]	SRN6-1 (LC387283) NS7-2 (LC387284)	*Pichia occidentalis* (NG055110), 100% (559/559) *Pichia terricola* (EF550233), 99% (560/564)	■	■	■
	OMS13-1[a], OMS5-1[a], OMS13-2[a], OMS15[a], OMS17-1[a], OMS18-1[a], OMS21-3[a], OMS22-2[a], OMS24-2-1[a], SRM9-4[d], SFL11-1W[d]	OMS5-1 (LC387285)	*Candida pseudolambica* (U71063), 100% (556/556)	■	■	■
	FRL2-1[d], SFL6-2S[d]	FRL2-1 (LC387286)	*Candida thaimueangensis* (AB264009), 99% (555/560)	■		
Lodderomyces/ spathaspora	FRL3-1[d], FRN3-1[d]	FRN3-1 (LC387287)	*Candida maltosa* (U45745), 99% (567/572)	■		
	SFL5-3[d], SFL11-6W[d], SFL12-1S[d], SFL14-2[d], BW8-1[c], BW6-1[b],YMTW1-1[b],YMTW4-3[b], YMTW5-1[b], YMTW8-2[b], BW10-1[b],YMTW11-1[b],YWW4-1[c], YWW8-1[c], BW4-1[b], BW5-2[b], BW7-1[b],BW16-1[b], BW17-3[b], BW18-2[b], BW21-1[b], OMS1-1[a], SFL8-4[d],OMS2-1[a], OMS22-1[a], OMS23-1[a], OMS24-1[a], SFL4-1[d], SRM9-3[d] SFL20-1W[d], FRM2-4[d], SRN6-2[d], SRM6-2[d],	SFL11-6W (LC387288) YMTW1-1 (LC387289)	*Candida orthopsilosis* (KJ451719), 100% (570/570) *Candida tropicalis* (U45749), 100% (570/570)	■	■	■
	YMTW12-1[b], YMTW12-2[b],	YMTW12-2 (LC387290)	*Candida viswanathii* (U45752), 99% (561/570)	■	■	■
Saccharomycopsis	YWS4-1[c]	YWS4-1 (LC387291)	*Saccharomycopsis amapae* (U69880), 99% (562/569)		■	
Scheffersomyces	BT2-2[d], BT2-3[d]	BT2-2 (LC387292)	*Scheffersomyces illinoinensis* (NG 042637), 96% (538/563)			■
Schwanniomyces	YMTT3-1[b]	YMTT3-1 (LC387293)	*Schwanniomyces pseudopolymorphus* (U45845), 99% (569/570)			■
Starmera	PS1-2-1[d], FRM7-1[d]	FRM7-1 (LC387294)	*Candida andamanensis* (AB334210), 100% (530/530)	■	■	
	FRM2-2[d]	FRM2-2 (LC387295)	*Suhomyces atakaporum* (NG042488), 99% (556/558)	■		
Torulaspora	YMTT3-4[b], YMTT5-1[b]	YMTT5-1 (LC387296)	*Torulaspora delbrueckii* (U72156), 100% (573/573)			■
Tanzawaensis	FRL2-6[d]	FRL2-6 (LC387297)	*Candida canberraensis* (NG 054781), 98% (545/558)	■		
Wickerhamomyces	OMS14-1[a], OMS14-4[a]	OMS14-1 (LC387298)	*Wickerhamomyces anomalus* (EF550341), 99% (570/573)			■
	YMTW10-4[b]	YMTW10-4 (LC387299)	*Candida quercuum* (U70184), 98% (550/581)	■		
	FRM4-3[d], FRL1-4[d], FRL20-1[d], SFL1-1W[d], SFL10-2W[d]	FRL1-4 (LC387300)	*Wickerhamomyces sydowiorum* (KY110148), 99% (515/516)	■		
Saccharomycotina						
Saccharomycetes						
yamadazyma	SFL12-2[d]	SFL12-2 (LC387301)	*Candida gorgasii* (NG 042495), 99% (547/548)	■		
	FRL1-3[d], FRL3-2[d]	FRL1-3 (LC387302)	*Candida insectorum* (U45791), 99% (528/530)	■		
Zygoascus	PS1-8-2[d], NS10-1[d], SRM6-5[d]	SRM6-5 (LC387303)	*Zygoascus bituminiphila* (NG 055308), 99% (559/570)	■	■	■
unaffiliated	YWW5-1[c]	YWW5-1 (LC387304)	*Candida incommunis* (U62303), <90%, (431/498)	■		
Basidiomycota						
Agaricomycotina						
Tremellomycetes						
Saitozyma	YWT1-1[c], YWT1-2[c], NS3-3[d], NS10-2[d]	YWT1-1 (LC387305)	*Saitozyma podzolica* (KY107265), 100% (597/597)		■	■
	SFL6-1S[d], SFL10-1W[d], SFL11-3W[d], FRM2-3[d]	SFL11-3W (LC387306)	*Trichosporon asahii* (AB831058), 100% (597/597)		■	■
	SFL3-2W[d]	SFL3-2W (LC387307)	*Trichosporon mycotoxinivorans* (AJ601388), 99% (591/596)	■		
Pucciniomycotina						
Microbotryomycetes						
Naganishia	FRL20-4[d]	FRL20-4 (LC387308)	*Naganishia diffluens* (AF075502), 98% (572/582)	■		
Rhodotorula	YMTW9-2[b], YMTW8-1[b],YMTW10-1[b], YMTS6-1[b], YWW7-1[c],FRL2-1[d], FRM5-2[d], FRL10-2[d],YWS2-1[c],YWS3-1[c],FRL11-1[d]	YMTW9-2 (LC387309)	*Rhodotorula paludigena* (NG 042383), 99% (538/539)	■	■	■
	FRL1-1[d], FRL2-8[d], NS10-4[d]	FRL1-1 (LC387310)	*Rhodotorula mucilaginosa* (AF070432), 99% (569/570)	■	■	■
	YWW6-1[c], SFL14-1SF[d]	YWW6-1 (LC387311)	*Rhodotorula sphaerocarpa* (KY109161, 100% (571/571)	■	■	■
	FRL2-3[d], FRL2-5[d]	FRL2-3 (LC387312)	*Rhodotorula taiwanensis* (GU646863), 99% (570/571)	■	■	■
	FRL2-4[d]	FRL2-4 (LC387313)	*Rhodotorula toruloides* (AF207884), 99% (569/573)	■	■	■
Ustilaginomycota						
Ustilaginomycetes						
Pseudozyma	OMS13-5[a]	OMS13-5 (LC387314)	*Pseudozyma siamensis* (AB117963), 100% (581/581)		■	
	YWT7-2[c], YWT7-3[c]	WT7-2 (LC387315)	*Pseudozyma tsukubaensis* (AB089373), 99% (580/582)		■	■
Myxomycota						
Myxomycocetes						
Prototheca	YMTW3-1[b], YMTS5-2[b]	YMTW3-1 (LC270821)	*Prototheca cutis* (AB470469), 98% (558/571)	■	■	
	OMS6-1[a]	OMS6-1 (LC387316)	*Prototheca zopfii* var. *hydrocarbonea* (AB097095), 100% (571/571)		■	

[a]Samutsakorn, [b]Trat, [c]Chantaburi, [d]Ranong provinces, source: *W* water, *S* sediments, *D* decayed biomaterials

to calculate the nucleotide-sequence divergence for the NJ tree construction, where gaps were completely deleted and the bootstrap value was obtained from 1000 replications (Felsenstein 1985). Yeast strains were then ascribed to MOTUs based upon sequence identity of the D1/D2 region of the 26S rDNA gene sequence. The MOTUs were then assigned to preliminary species by comparison to those available sequences from known species in GenBank after BLASTn searching, based upon < 2% divergence being the same species (Kurtzman and Robnett 1998). Subsequently assigned species designations were checked against the yeast morphology and biochemistry (see below) in comparison to known reference strains for each nominal species.

Nile red staining and quantitative analysis of intracellular lipid

All yeasts isolated were examined for their intracellular lipid accumulation by staining the cells after growth on mYM agar at 30 °C for 5 days with 0.5 µg/mL Nile red in 25% (v/v) dimethyl sulfoxide. After incubation at 35 °C (10 min), the stained cells were examined for the presence of any intracellular lipid globules under fluorescence microscopy with excitation at 520–550 nm and emission at 580 nm (Olympus BX51, Olympus Corp., Japan). Strongly Nile red positive strains were then further quantitatively analysed for their intracellular lipid content. To this end, one loopful of yeast grown on YM agar was transferred into 150 mL YM broth in a 500-mL flask and incubated at 30 °C, 200 rpm for 48 h. The cells were precipitated by centrifugation (4 °C, 8600×*g*, 15 min) from 15 mL of culture and then washed with lipid production medium with 155 C/N ratio (LP; 50 g/L glucose, 1 g/L BD Bacto™ yeast extract (BD bioscience, USA) 0.05 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L KH_2PO4, 1 g/L $(NH_4)_2SO_4$, 0.001 g/L NaCl, 0.001 g/L $CaCl_2 \cdot 2H_2O$, pH 5.5) and inoculated into 150 mL LP medium in a 500-mL flask and incubated as above for 6 days. Yeast cells were harvested from the resultant culture by centrifugation, washed in distilled water as above and then lyophilized. The lyophilized cells (1 g DW) were suspended in 20 mL of a 2:1 (v/v) ratio of chloroform:methanol, sonicated at 37 kHz, room temperature for 30 min (Elmasonic, E60H model, Germany) and centrifuged at 4 °C, 8600×*g* for 40 min. The supernatant was harvested and dried by evaporation at room temperature. Obtained lipid were analysed by gravimetric method. The known oleaginous yeast, *Lipomyces starkeyi* JCM 5995, was used as a positive control strain.

Fatty acid composition of intracellular lipid

Fatty acids, both as free and incorporated into different cellular compounds, were extracted from whole yeast cells by direct saponification. 1 g of wet yeast cells was suspended in 0.8 mL of 10% (w/v) potassium hydroxide in methanol in a screw-capped tube, and heated at 80 °C for 2 h before being left to cool down to room temperature. After adding 1 mL of petroleum ether, mixing and allowing phase separation, to separate the unsaponified material from the mixture, the aqueous phase was harvested and acidified by adding 0.3 mL of 6 N hydrochloric acid, and then the fatty acids were extracted with 3 mL of diethyl ether. The recovered fatty acids were dried under nitrogen gas, and methyl-esterified by transmethylation with boron trifluoride (Anamnart et al. 1998). The resultant fatty acid methyl esters were analyzed by gas chromatography (GC; Agilent Technologies 7890 B equipped with INNOWAX) using a capillary column (30 m × 0.3 mm i.d., 0.25 µm film thickness) and a flame ionization detector system. The injector and detector temperatures were 150 and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 2.3 mL/min. The GC chromatographic temperature program was as follows: initial temperature of 150 °C, increasing to 180 °C at 10 °C/min, to 200 °C at 5 °C/min, to 205 °C at 0.5 °C/min and held at 205 °C for 2 min, and finally to 250 °C at 5 °C/min and held for 5 min (total run time 33 min). Individual fatty acid methyl esters were identified by comparison with reference standards (Limsuwatthanathamrong et al. 2012).

Morphological and physiological characteristics of the highest lipid accumulating yeasts

Morphological and physiological characteristics of the highest lipid accumulating yeasts were investigated by standard methods and compared to the type strain (Kurtzman et al. 2011). Cultures grown on 5% (w/v) malt extract agar at 25 °C for 7 days were used for the cell and colony morphological examinations. Cell characterization was examined microscopically with × 400 magnifications, while colony morphological characteristic was examined by naked eyes. Description of cell included shape, size, position of budding, and whether cells are produced single, in pairs, or aggregated in cluster. The description of agar growth colony included texture, color, surface, elevation and margin. The carbon assimilation test was performed using the ID 32 C kit (BioMerieux, France) by following manufacture instruction. The ID 32C strip consists of 32 cupules of each containing dehydrated carbohydrate substrates, D-galactose, saccharose, *N*-acetyl-glucosamine, lactic acid, L-arabinose, D-cellobiose, D-raffinose, D-maltose, D-trehalose, potassium 2-ketogluconate, methyl-α-D-glucopyranoside, D-mannitol, D-lactose, inositol, D-sorbitol, D-xylose, D-ribose, glycerol, L-rhamnose, palatinose, erythritol, D-melibiose, sodium glucuronate, D-melezitose, potassium gluconate, levulinic acid, L-sorbose, glucosamine, escullin ferric citrate, D-glucose. D-glucose is served as positive control and blank well is

served as negative control. The test cultures were grown on YM agar at 25 °C for 3 days then suspended in sterile 0.85% NaCl solution to obtain optical density equal to McFarland No. 4. Cell suspension was inoculated into ID 32C medium, 3 drops were dispensed into each well of the strip then incubated at 25 °C for 4 weeks. The starved inoculum grown in yeast carbon base broth was used for the nitrogen assimilation and vitamin requirement tests. Hydrolysis of urea (urease activity) was examined using Christensen's urea agar. Pseudohyphae or true hyphae production was examined on yeast morphology agar using the Dalmau slide culture technique and incubated at 25 °C for 7 days (Kurtzman et al. 2011).

Results

Isolation and identification of the isolated yeasts

From the 140 samples collected, 198 yeast strains were isolated. They showed 150 different $(GTG)_5$ fingerprint patterns. A representative strain of each (GTG_5) fingerprint pattern was identified to MOTU by 26S rRNA (D1/D2 domain) gene sequencing, and then ascribed to nominal species level by comparison to homologous sequences from designated species in GenBank using BLASTn searching. The results indicated that the 59 designated MOTUs (Table 1) consisted of ascomycetes (26 genera; 34 described and 12 undescribed species), basidiomycetes (4 genera; 10 described and 1 undescribed species) and yeast-like algae (1 genus; 1 described and 1 undescribed species). The 34 described ascomycetes species were *Aureobasidium melanogenum, Blastobotrys chiropterorum, C. akabanensis, C. andamanensis, C. atakaporum, C. carpophila, C. gorgasii, C. insectorum, C. maltosa, C. maris, C. natalensis, C. orthopsilosis, C. pseudolambica, C. thaimueangensis, C. tropicalis, C. viswanathii, Dipodascus geotrichum, Kloeckera hatyaiensis, Kluyveromyces siamensis, Kodamaea ohmeri, Kuraishia piskuri, Metahyphopichia laotica, Meyerozyma caribbica, Nakazawaea siamensis, Ogataea wangdongensis, P. kudriavzevii, P. occidentalis, P. terricola, Saccharomycopsis amapae, Schwanniomyces pseudopolymorphus, Schwanniomyces vanrijiae, Torulaspora delbrueckii, Wickerhamomyces anomalus* and *Wickerhamomyces sydowiorum*. The 12 undescribed ascomycetes species were closely related to *C. canberraensis, C. cylindracea, C. heveicola, C. maris, C. nivariensis, C. quercum, C. uthaithanina, C. zeylanoides, C. incommunis, Kluyveromyces aestuarii, Scheffersomyces illinoiensis* and *Zygoascus bituminiphila* with 87–97% sequence similarity (6–64 nucleotides substitution). The ten described basidiomycetes species were *Pseudozyma* (*Ps.*) *siamensis, Ps. tsukubaensis, R. paludigena, R. mucilaginosa, R. sphaerocarpa, R. taiwanensis, R. toruloides, Saitozyma podzolica, Torulaspora delbrueckii, Trichosporon asahii* and *Trichosporon mycotoxinivorans*. The undescribed basidiomycete was closely related to *Naganishia diffluens* with 98% sequence identity. The described yeast-like algae was *Prototheca zopfii* var. *hydrocarbonea* and the undescribed yeast-like algae had the closest related species of *Prototheca cutis* with 98% sequence similarity (12 nucleotides substitution). The most frequently isolated strain was *C. tropicalis* (28 strains) followed by *C. pseudolambrica* (11 strains), *R. paludigena* (11 strains) and *Meyerozyma caribbica* (10 strains). The *C. tropicalis* and *Meyerozyma caribbica* were isolated from all sampling sites, while *C. pseudolmbica* and *R. paludigena* were isolated from two and three sampling sites out of a total of seven sampling sites, respectively (Table 1).

Nile red staining and quantitative analysis of the intracellular lipid level

Examination for any intracellular lipid globules by Nile red staining revealed that 69 of the 198 (34.8%) yeast strains isolated were Nile red positive, but only 18 (9.1%) strains (*Ps. tsukubaensis, R. paludigena, R. sphaerocarpa, R. toruloides, Saitozyma podzolica* and *Prototheca zopfii* var. *hydrocarbonea* and the undescribed species *Protothaca* sp.) were strongly positive. Quantitative analysis by the gravity method indicated that 10 out of these 18 strains, *Ps. tsukubaensis* (YWT7-2, WT7-3), *R. sphaerocarpa* (YWW6-1, SFL14-1SF), *Saitozyma podzolica* (YWT1-1, NS3-3, NS10-2), *Prototheca* zopfii var. *hydrocarbonea* OMS6-1 and *Prototheca* sp. (YMTW3-1, YMTS5-2), contained more than 20% (w/w DW) intracellular lipid, and hence were oleaginous (Table 2). The highest intracellular lipid level, at 32.48% (w/w DW), was accumulated by *Ps. tsukubaensis* YWT7-2, followed by *Ps. tsukubaensis* YWT7-3 (28.02%) and *R. sphaerocarpa* SFL14-1SF (26.72%). Note that *R. sphaerocarpa, Saitozyma podzolica* and *Prototheca zopfii* have previously been reported as oleaginous yeasts (Sitepu et al. 2014), while *Ps. tsukubaensis* and *Prototheca* sp. are first reported as oleaginous yeasts in this study.

Fatty acid composition of the yeast intracellular lipid of the three highest lipid accumulation yeasts

The fatty acid composition of the highest lipid accumulating yeasts (*Ps. tsukubaensis* YWT7-2 and YWT7-3, and the yeast-like algae, *Prototheca* sp. YMTW3-1) is shown in Table 3. The major fatty acids had 16 and 18 carbon atoms, which is similar to those of plant oils, such as tallow, palm, canola, corn, etc., (Ramos et al. 2009; Hoekman et al. 2012). The most abundant fatty acid was oleic acid (C18:1) at 37.94, 37.92 and 58.12% (w/w) in *Ps. tsukubaensis* YWT7-2 and YWT7-3, and *Prototheca* sp. YMTW3-1, respectively, followed by palmitic acid (C16:0) at 36.66,

Table 2 Intracellular lipid accumulation of the strong Nile red positive yeasts

Strongly Nile red stained yeast	Lipid content % (w/w DW)
Genus *Pseudozyma* (*Ps.*)	
Ps. tsukubaensis (YWT7-2)	32.48
Ps. tsukubaensis (YWT7-3)	28.02
Genus *Rhodotorula* (*R.*)	
R. paludigena (YMTW9-2)	7.16
R. paludigena (YMTW8-1)	6.49
R. paludigena (YMTW10-1)	13.17
R. paludigena (YMTS6-1)	11.82
R. paludigena (YWW7-)	7.21
R. paludigena (YWS3-1)	8.92
R. sphaerocarpa (YWW6-1)	23.61
R. sphaerocarpa (SFL14-1SF)	26.72
Genus *Rhodosporidium*	
Rhodosporidium toruloides (FRL2-4)	11.37
Genus *Saitozyma*	
Saitozyma podzolica (YWT1-1)	24.61
Saitozyma podzolica (YWT1-2)	18.37
Saitozyma podzolica (NS3-3)	21.34
Saitozyma podzolica (NS10-2)	20.05
Genus *Prototheca*	
Prototheca zopfii var. *hydrocarbonea* (OMS6-1)	20.84
Prototheca sp. (YMTW3-1)	21.12
Prototheca sp. (YMTS5-2)	21.16
Positive control	
Lipomyces starkeyi JCM 5995	41.05

Table 3 Fatty acid composition of oils produced by *Ps. tsukubaensis* YWT7-2 and YWT7-3, and *Prototheca* sp.

Fatty acid	Fatty acid composition, as % (w/w DW), in yeast oil		
	Prototheca sp.	*Ps. tsukubaensis* YWT7-2	*Ps. tsukubaensis* YWT7-3
Myristic (C14:0)	2.05 ± 0.59	2.47 ± 0.28	2.29 ± 0.13
Palmitic (C16:0)	27.95 ± 1.80	36.66 ± 1.02	37.94 ± 0.80
Palmitoleic (C16:1)	0.57 ± 0.19	4.34 ± 0.25	3.97 ± 0.17
Stearic (C18:0)	4.29 ± 0.81	7.07 ± 1.12	6.68 ± 0.61
Oleic (C18:1)	58.12 ± 3.55	37.94 ± 1.35	37.94 ± 0.43
Linoleic (C18:2)	6.31 ± 0.05	7.77 ± 0.39	9.16 ± 0.10
Other	0.72	3.75	2.01

Data are shown as the mean ± 1 SD, derived from three samples

37.94 and 27.95% (w/w), respectively. Other fatty acids were myristic (C14:0), palmitoleic (C16:1), steric (C18:0) and linoleic (C18:2) acids at a level of 2–9% (Table 3).

Morphological and physiological characteristics of the highest lipid accumulating strains

Morphological characteristics of *Ps. tsukubaensis* YWT7-2 and YWT7-3

The cell morphology of *Ps. tsukubaensis* YWT7-2 and YWT7-3 were similar. The 7 d-old cells grown on 5% (w/v) malt extract were ellipsoidal to narrowly ellipsoidal, 2–5 × 5–15 µm, with two to five oil droplets, and occurred as single cells with budding at the pole (Fig. 1a, b). The colony was raised, light brown-colored, butyrous, irregular and dull with a fringed margin. True hyphae occurred with a sterigma-like structure near the septa (Fig. 1c, d).

Physiological characteristics of *Ps. tsukubaensis* YWT7-2 and YWT7-3

Both *Ps. tsukubaensis* YWT7-2 and YWT7-3 assimilated D-glucose, D-galactose, sucrose, lactic acid, L-arabinose, D-cellobiose, D-raffinose, D-maltose, D-trehalose, L-sorbose, L-lactose, inositol, D-xylose, glycerol, palatinose, melezitose, potassium 2-ketogluconate, methyl-α-D-glucopyranoside, potassium glucuronate and *N*-acetyl-glucosamine, but did not assimilate ribose, erythritol and glucosamine, while *Ps. tsukubaensis* CBS 6389^T (type strain) assimilated ribose, erythritol and glucosamine (Table 4) (Boekhout 1995).

Based on the molecular identification (Table 1) and their morphological and physiological characteristics, they were identified as *Ps. tsukubaensis*. Although some of their physiological characteristics were different from the type strain (*Ps. tsukubaensis* CBS 6389^T), these are known to vary due to the effects of culture conditions, adaptation through passages and other unknown factors (Pizarro et al. 2008).

Discussion

The yeasts isolated from seven mangrove forests in four provinces of Thailand, located both at the west coast of peninsula (Ranong province) and gulf (Samutsakorn, Trat and Chantaburi provinces) of Thailand, revealed that the most frequently isolated yeasts were ascomycetes (81.31%) followed by basidiomycetes (17.17%) and yeast-like algae (1.51%). Most of yeast isolated from this work (23.23%) had previously been isolated from terrestrial, mangrove and marine ecosystems: *Aureobasidium melanogenum* (Xin et al. 2017), *C. andamanensis* (Am-in et al. 2011), *C. maltose* (Loureiro et al. 2005), *C. maris* (Hagler et al. 1982; Lachance et al. 2011), *C. natalensis* (Fell 2012), *C. orthopsilosis* (Singh et al. 2012), *C. thaimueangensis* (Limtong et al. 2007), *C. viswanathii* (Fell 1967; Burgaud et al. 2010), *Dipodascus geotrichum* (Sybren de Hoog and Smith 2011),

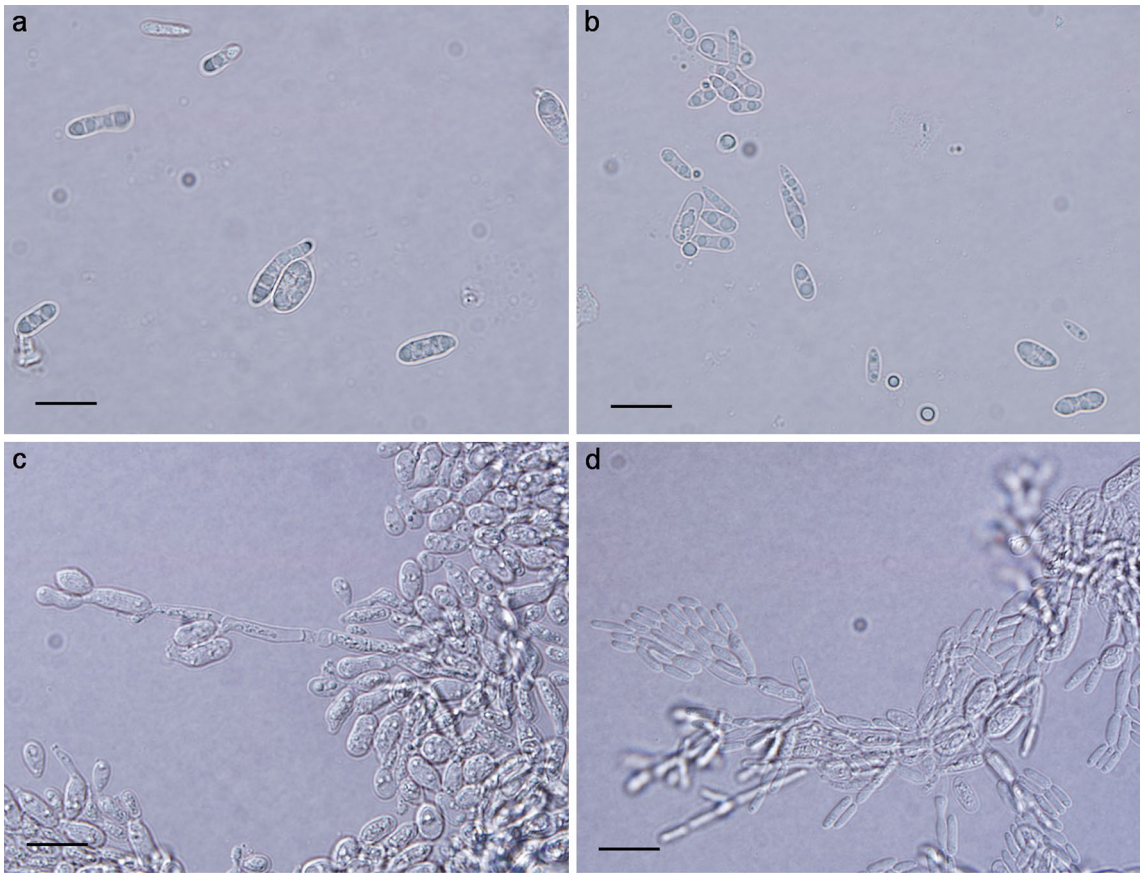

Fig. 1 The (**a, b**) cell morphology including (**c, d**) pseudohyphae of 7-day-old *Ps. tsukubaensis* strain (**a**, **c**) YWT7-2 and (**b, d**) YWT7-3 grown on (**a, b**) 5% (w/v) malt extract agar at 25 °C or (**c, d**) using the dalmau slide culture technique on yeast morphology agar under an anaerobic condition at 25 °C. Bars 10 µm. Images shown are representative of at least 12 such images per sample and two independent samples

Kluyveromyces siamensis (Am-in et al. 2008), *Kodamaea ohmeri* (Dong et al. 2015), *P. occidentalis, P. kudriavzevii, P. terricola* (Kurtzman 2011; Hagler et al. 2017; Zajc et al. 2017), *R. paludigena* (Vogel et al. 2007; Fell et al. 2011; Yang et al. 2011a, b), *R. sphaerocarpa* (Gadanho and Sampaio 2005; Sampaio 2011) and *Naganishia diffluens* (Babič et al. 2017). Mangrove forest is a convergence area of terrestrial, estuary and marine ecosystems (Pumijumnong 2014). *C. andamanensis* and *K. siamensis* likely to had specific habitat in mangrove forest since they had not yet been found in other habitat. The yeasts *C. akabaensis, Schwanniomyces vanrijiae, Kloeckera hatyaiensis, Meyerozyma caribbica, Ogataea wangdongensis, Nakazawaea siamensis, Ps. tsukubaensis, R. taiwanensis, R. toruloides, Saitozyma podzolica* and *Torulaspora delbrueckii* have all been reported as frequently isolated yeasts from the phyllosphere, especially from the phylloplane and caulosphere (Maksimova and Chernov 2004; Jindamorakot et al. 2009; Lachance et al. 2011; Duarte et al. 2016; Hagler et al. 2017; Limtong and Nasanit 2017), whereas *C. atakaporum, C. gorgasii, C. insectorum, Saccharomycopsis amapae* and *Kuraishia piskuri* have been reported as insect-associated yeasts (Morais et al. 1995; Suh et al. 2004; Endoh et al. 2008; Lachance et al. 2011). In total, 19.69% of the isolated yeasts in this study (*Blastobotrys chiropterorum, C. tropicalis, Ps. siamensis, R. mucilaginosa, Trichosporon asahii* and *Trichosporon mycotoxinivoran*) were human-associated yeasts that have been reported as important clinical pathogens in humans, especially in immunocompromised patients (Lachance et al. 2011; Sugita 2011; Mekha et al. 2014). Human-associated yeasts can serve as pollution indicators in the environment (Hagler 2006; Hagler et al. 1982; Starmer and Lachance 2011), although with caution as many human-associated yeasts have also been reported as frequently isolated yeasts from natural ecosystems (Lachance et al. 2011; Sampaio 2011; Groenewald et al. 2017).

A high intracellular lipid accumulation, which is a strain-specific characteristic (Sitepu et al. 2014; Dien et al. 2016), has been found in approximately 70 out of 1500 known yeast species (4.68%). In accord, 10 of the 198 strains of yeast isolated in this study (5.05%) were found to accumulate more than 20% (w/w DW) lipid. As noted earlier, the

Table 4 Carbon assimilation characteristics of *Ps. tsukubaensis* YWT7-2 and YWT7-2 and *Ps. tsukubaensis* CBS 6389^T (Boekhout 1995)

No.	Carbon compound assimilation	Strain		
		Ps. tsukubaensis CBS 6389^T	*Ps. tsukubaensis* YWT7-2	*Ps. tsukubaensis* YWT7-3
1	D-Galactose	s	+	+
2	Cycloheximide 0.01%	n	−	−
3	D-Saccharose or sucrose	+	+	+
4	*N*-Acetyl-glucosamine	n	+	+
5	Lactic acid	+	+	+
6	L-Arabinose	+	+	+
7	D-Cellobiose	+	+	+
8	D-Raffinose	+	+	+
9	D-Maltose	+	+	+
10	D-Trehalose	+	+	+
11	Potassium 2-keto gluconate	+	+	+
12	Methyl-α-D-glucopyranoside	+	+	+
13	D-Mannitol	−	−	−
14	D-Lactose	+	+	+
15	Inositol	+	+	+
16	D-Sorbitol	n	+	+
17	D-Xylose	+	+	+
18	D-Ribose	s	−	−
19	Glycerol	+	+	+
20	L-Rhamnose	−	−	−
21	Palatinose	n	+	+
22	Erythritol	+	−	−
23	D-Melibiose	−	−	−
24	Sodium glucuronate	n	+	+
25	D-Melezitose	+	+	+
26	Potassium gluconate	n	−	−
27	Levulinic acid	n	−	−
28	D-Glucose	+	+	+
29	L-Sorbose	s	+	+
30	Glucosamine	s	−	−
31	Escullin ferric citrate	n	−	−

+: positive; w: weak; s: slow; −: negative; v: variable; n: no data

highest intracellular lipid level was found in *Ps. tsukubaensis* YWT7-2 at 32.48% (w/w DW).

Lipid accumulation of oleaginous yeast in genera *Cryptococcus, Rhodotorula, Trichosporon, Lipomyces* and *Yarrowia* was in a level of 40% (w/w DW). This level could be increased to 70% (w/w DW) under an optimized condition (Beopoulos et al. 2011). Interestingly, *Ps. tsukubaensis* has been reported to produce various biocompounds, such as fungicide, glycol lipid biosurfactant, sweetener and galacto-oligosaccharides (Golubev et al. 2006; Fukuoka et al. 2007; Morita et al. 2010; Jeya et al. 2009; Fai et al. 2014). However, to the best of our knowledge, *Ps. tsukubaensis* has not been previously reported as an oleaginous yeast until this present study. The fatty acid composition of the accumulated intracellular lipid in *Ps. tsukubaensis* was similar to that in tallow and palm oils, which contained a high proportion of oleic and palmitic acids (Hoekman et al. 2012). Oleic acid is an interesting fatty acid in nutraceutical as an enhancer of drug absorption and skin wound healing, improver of immune system function for reduction of cancer risk development (mainly breast, colorectal and prostate cancer), enhancer of immune response against infectious microorganisms such as *Listeria monocytogenes, Helicobacter pylori* and *Candida albicans*, in addition, oleic acid might reduce cholesterol level, atherogenesis risk and blood pressure level (Sales-Campos et al. 2012). Palmitic acid is an important ingredient in cosmetic and pharmaceutical industries as foaming agent, humectant and cream consistency

modifiers (Mitsui 1992). This fatty acid is generally found in vegetable and animal oils, overconsumption of this fatty acid significantly raises total cholesterol and LDL levels in blood of which leads to an increase of cardiovascular disease (Williams 2002). Other fatty acids which was composition of *Ps. tsukubaensis* intracellular lipid namely myristic, palmitoleic, stearic and linoleic acids were previously reported with respect to their effects on human health elsewhere (Papandreou 2014; Calder 2015; Gairín et al. 2015). Among these fatty acids, palmitoleic gains a great interest in health benefits such as prevention of type-2 diabete (Morgan and Dhayal 2010), reduction of LDL and cholesterol concentrations in blood (Griel et al. 2008), healing of burned, scaled, wounded and radioactively damaged skin (Gao et al. 2003), and protection against skin aging (Hayashi et al. 2003). As feedstock for biodiesel production, the fatty acid composition has an influence in both quality and property of biodiesel. Feedstock with longer chain and more saturated fatty acids gives higher cetane number biodiesel where the minimum acceptable cetane number by the EU standard is 51. In contrast, the longer chain and the more saturated fatty acids are leading to poorer in cold flow property and fuel density (Ramos et al. 2009; Hoekman et al. 2012; Bonturi et al. 2015). While feedstock with long chain fatty acids, especially monounsaturated fatty acids, give more preferable biodiesel quality of ignition, melting point, kinematic viscosity and improved oxidative stability (Ramos et al. 2009; Knothe 2008; Steen et al. 2010).

Conclusion

From 198 strains of yeast isolated from seven mangrove forests in four provinces of Thailand (at the west coast and the gulf of Thailand), 10 (5.05%) were found to be oleaginous. Of these 10 isolates, *Ps. tsukubaensis* YWT7-2 accumulated the highest level of lipid at 32.48% (w/w DW) with a fatty acid composition similar to that of palm oil, and so it has the potential to be used as an alternative feedstock for biodiesel production.

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