The oleaginous yeast *Pichia manshurica* isolated from *Lansium domesticum* fruit in Thailand and its fatty acid composition of single cell oil

SONGSAK PLANONTH, AIYA CHANTARASIRI♥

Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok. Rayong Campus, Rayong 21120, Thailand.
Tel./fax.: +66-(0)38-627000 ext.5446, ♥email: aiya.c@sciee.kmutnb.ac.th

Abstract. *Planonth S, Chantarasiri A. 2022. The oleaginous yeast* Pichia manshurica *isolated from* Lansium domesticum *fruit in Thailand and its fatty acid composition of single cell oil. Biodiversitas 23: 801-809.* Oleaginous microbes can accumulate intracellular lipids or single cell oils (SCOs) in quantities higher than 20% of their biomass. They can be a sustainable alternative to fossil fuels, biofuels, and oleochemicals. Studies concerning efficient oleaginous yeasts isolated from the natural environments remain scarce. Therefore, this study isolated and screened for efficient oleaginous yeasts from the surfaces of longkong fruit (*Lansium domesticum*) samples in Thailand. Their intracellular SCOs were extracted using an ultrasonic-assisted extraction (UAE) method and quantitatively analyzed. The SCO-accumulating yeasts (produce the amount of SCOs <20 % of their biomass) genetically identified were *Candida jaroonii, Meyerozyma caribbica, Kodamaea ohmeri,* and *Pichia* sp., while the oleaginous yeasts (produce the amount of SCOs >20% of their biomass) identified were *Pichia manshurica* and *Hanseniaspora opuntiae*. Several isolated yeasts were designated as rare oleaginous microbes. The *P. manshurica* strain Y2 was considered as the most effective oleaginous yeast with a SCO content of 43.03% (w/w). The fatty acids in the accumulated SCO of this strain were analyzed by gas chromatography (GC) that consisted of palmitic, stearic, oleic, linoleic, and palmitoleic acids. These fatty acids could be further applied in the production of third-generation biodiesel, cocoa butter equivalents, and related high-value oleochemicals.

Keywords: Fatty acid, *Lansium domesticum*, oleaginous yeast, *Pichia manshurica*, single cell oil

INTRODUCTION

Microbial lipids produced by oleaginous microbes are called single cell oils (SCOs). Microbe species that are considered oleaginous can accumulate more than 20% (w/w) of their dry biomass under suitable cultivation conditions (Jiru et al. 2016; Gientka et al. 2017; Chantarasiri 2020). These types of microbes belong to yeasts, filamentous fungi (e.g., *Aspergillus oryzae, Cunninghamella echinulate, Mortierella alpina, Mortierella isabellina,* and *Mucor* sp.), microalgae (e.g., *Arthrospira platensis* and *Chlorella zofingiensis*), and bacteria (e.g., *Acinetobacter baylyi, Bacillus megaterium, B. subtilis*, and *Rhodococcus opacus*) (Jiru et al. 2016; Patel et al. 2017; Kongruang et al. 2020; Brandenburg et al. 2021; Caporusso et al. 2021). Triglycerides (TAGs) are the major form of intracellular SCOs and are essentially composed of long-chain fatty acids (Ayadi et al. 2018). The accumulation of SCOs was studied as a promising feedstock for biodiesel production and several biotechnological applications because of their fatty acid composition similar to vegetable oils (Patel et al. 2017; Ayadi et al. 2018). Recently, third-generation biodiesel was synthesized from the microbial SCOs of oleaginous microbes (Sitepu et al. 2014; Mofijur et al. 2021). Third-generation biodiesel alleviates both the food and land conflicts related to first and second-generation biodiesels (Mofijur et al. 2021). In addition, SCOs can be synthesized for many oleochemicals, food, biopolymers, and additives for cosmetics (Ochsenreither et al. 2016; Vasconcelos et al. 2019; Caporusso et al. 2021).

Today, oleaginous yeasts represent interesting microbial cell factories (Caporusso et al. 2021). They offer numerous advantages favoring the production of lipids when compared to other mentioned microbes (Qin et al. 2017; Ayadi et al. 2018). A large variety of oleaginous yeasts can be found in several environments; such as, common surfaces, fruit products, plant surfaces, and soil (Polburee et al. 2015; Vincent et al. 2018). The effective oleaginous yeasts belong to the genera *Candida, Cryptococcus, Lipomyces, Rhodosporidium, Rhodotorula, Trichosporon,* and *Yarrowia* (Dey and Maiti 2013; Jiru et al. 2016; Gientka et al. 2017; Patel et al. 2017; Qin et al. 2017; Ayadi et al. 2018; Lopes et al. 2018; Chantarasiri 2020). The lipid content of yeasts may vary and reach up to 70%, depending on the species and certain nutrient-limited conditions (Sreeharsha and Mohan 2020). The well-known oleaginous yeast, *Yarrowia lipolytica*, can produce and accumulate SCO of approximately 30-43% (w/w), while *R. gracilis* can contain SCO of 72% (w/w) in their biomass (Kongruang et al. 2020). In the past decade, many studies have been conducted and optimized on the lipid accumulation in established species; such as, *Cryptococcus curvatus* (Cui et al. 2012; Seo et al. 2013), *Lipomyces starkeyi* (Tapia et al. 2012), *Rhodosporidium toruloides* (Saran et al. 2017), and *Y. lipolytica* (Enshaeieh et al. 2013; Robles-Rodríguez et al. 2017; Park et al. 2018). However, limited studies have been done to isolate other oleaginous

yeasts from the natural environments (Vincent et al. 2018). Only 3%-10% of the 1,600 known yeast species have been reported as oleaginous species (Sitepu et al. 2014). Nevertheless, screening studies are still being performed to identify new oleaginous yeast species (Lamers et al. 2016).

Lansium domesticum is an endemic fruit tree belonging to the Meliaceae family, which originated and is cultivated in Southeast Asia, particularly in Thailand, Indonesia, Malaysia, and the Philippines (Techavuthiporn 2018; Rahmawaty et al. 2020; Chantarasiri et al. 2021). According to Apridamayanti et al. (2018), *L. domesticum* can be used as a medicine for pain and fever. Longkong is the most popular of three main important cultivars of *L. domesticum* comprising langsat, longkong, and duku (Techato et al. 2005; Chantarasiri et al. 2021). Currently, there have been few studies reported on the microbial community colonizing on longkong fruit. The studies on longkong fruit and associated microbes have mainly focused on the pathogen and disease. Some new studies have shown that the oleaginous yeasts belonging to the genera *Candida, Kluyveromyces, Meyerozyma, Pichia,* and *Rhodotourula* could be isolated from other rotten fruits and fruit surfaces; such as, banana, guava, lemon, noseberry, and pineapple (Jiru et al. 2016; Diwan and Gupta 2018; Vincent et al. 2018). Therefore, isolating and screening effective oleaginous yeasts from longkong fruit could be challenging.

This study aimed to isolate, screen, and genetically identify the oleaginous yeasts dwelling on longkong fruit samples. Intracellular SCO produced from the most effective oleaginous yeast was extracted by ultrasonic-assisted extraction (UAE), and subsequently fatty acid was identified by gas chromatographic analysis. The resulting SCO was expected to be a feedstock for biodiesel production and related applications in the future.

MATERIALS AND METHODS

Sample collection

Thirty longkong fruit (*L. domesticum*) were randomly collected from orchards in Yala (6° 57′ N, 101° 16′ E) and Rayong (12° 79′ N, 101° 24′ E) Provinces, located in the Southern and Eastern regions of Thailand, respectively. The samples were kept in sterilized plastic bags at 4 °C and subjected to yeast isolation within 48 hours of collection.

Procedures
Isolation of yeast from the fruit samples

The fruit surfaces were sampled by swabbing using sterilized wet cotton swabs and subsequently placed in Yeast Malt Broth (YMB) (HiMedia, India). All inoculations were enriched by incubating at 30°C for 48 hours, shaking at 150 rpm (Daihan Labtech, South Korea). The enriched culture broths were serially diluted with 0.85% (w/v) of sterilized NaCl solution (Sigma-Aldrich, Germany) to obtain 1: 1,000,000 dilutions. One hundred microliters of diluted culture broths were spread-plated on Dichloran Rose Bengal Chloramphenicol Agar (DRBC) (HiMedia, India) and were then incubated at 30°C for 48

hours in an incubator cabinet (BINDER, Germany). The distinct morphological colonies of the isolated yeasts were selected and purified by being streak plated on Yeast Malt Agar (YMA) (HiMedia, India).

Accumulation and extraction of SCOs from the isolated yeasts

All yeast isolates were pre-cultured in YMB at 30°C for 48 hours, shaking at 150 rpm and then inoculated in a Glycerol Yeast Peptone (GYP) medium for intracellular SCO accumulation. The components of the GYP medium were 4% (w/v) of glycerol (Loba-Chemie, India), 1% (w/v) of yeast extract (i-YEAST, Japan), 1% of peptone (w/v) (HiMedia, India), and distilled water. One hundred milliliters of GYP medium were seeded with 10% (v/v) of each pre-cultured yeast and incubated at 30 °C for 120 hours with shaking at 150 rpm. The yeast cells were harvested after 120 hours of the incubation period by centrifugation at 3,500 ×g for 15 minutes (Ortoalresa, Spain) and washed three times with 0.85% (w/v) of sterilized NaCl to remove the culture medium.

The harvested yeasts were screened for oleaginous characteristics. Primary screening of lipid accumulating yeasts was observed under an ECLIPSE E200 light microscope (Nikon, Japan) for the presence of intracellular SCO droplets. Subsequently, the secondary screening of the lipid accumulating yeasts was performed by determining the SCO content in the dried yeasts. The lipid accumulating isolates capable of the SCO storage over 20% (w/v) of their dry cell mass was considered as oleaginous yeast. The determination of the dry cell weight of the lipid accumulating yeasts was conducted by Chantarasiri (2020). Harvested yeast cells were resuspended in 0.85% (w/v) of sterilized NaCl solution and filtered through a preweighed 0.45-μm nitrocellulose filter (Millipore, USA) using a vacuum pump (Millipore, USA). Cell pellets on nitrocellulose filters were dried to a constant weight at 105°C in an oven (BINDER, Germany) and then weighed.

The extraction of intracellular SCOs from dried yeasts was conducted with minor modifications according to Byreddy et al. (2015) by UAE. Fifty milligrams of dried yeast were suspended in three milliliters of *n*-hexane solution (RCI Labscan, Thailand) and sonicated by a VCX 130PB Vibra-Cell ultrasonic liquid processor (Sonics, USA) with 90% amplitude for 10 minutes. The sonicated mixture was centrifuged at 3,500 ×g for 15 minutes to separate the SCOs and cell debris. The supernatant layer was collected and then dried under nitrogen gas to obtain the extracted SCOs.

The SCO concentration was defined as the amount of extracted SCO per liter working volume (g/L), and the SCO content was represented as a percentage of the dry cell weight (%, w/w) (Chantarasiri 2020). The positive control of this experiment was an oleaginous yeast *Y. lipolytica* strain TISTR 5212 (Thailand Institute of Scientific and Technological Research, Thailand). All experiments were performed in triplicate.

Genetic identification of the SCO accumulating yeasts

All lipid accumulating yeasts isolated from this study were identified by genetic identification. The genomic DNA was extracted from each isolated strain by a genomic DNA isolation kit (Bio-Helix, Taiwan) according to the standard protocol described by Bio-Helix. A polymerase chain reaction (PCR) of the internal transcribed spacers (ITS) region was carried out using the OnePCR reaction mixture (Bio-Helix, Taiwan) with a pair of universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplification was performed for 30 cycles in a Mastercycler Nexus Gradient thermal cycler (Eppendorf, Germany). The amplification conditions involved a preheating step at 95°C for five minutes, a denaturation step at 94°C for one minute and 30 seconds, an annealing step at 55°C for two minutes, an extension step at 72°C for three minutes, followed by a final extension step at 72°C for five minutes. PCR products from the amplifications were electrophoresed on a 1.5% (w/v) OmniPur agarose gel (Calbiochem, Germany) and subsequently visualized by Novel Juice (Bio-Helix, Taiwan). The PCR products were cleaned and nucleotide sequenced using the services of Macrogen Inc. (Seoul, South Korea). The nucleotide sequences were aligned for the species identification by the BLASTn program of the National Center for Biotechnology Information (NCBI). The phylogenetic tree of all isolated lipid accumulating yeasts was generated by the BIONJ algorithm with 100,000 bootstraps using SeaView software version 5.0.2 (Gouy et al. 2010) and FigTree software version 1.4.4.

All nucleotide sequences of the ITS region from this study were deposited in the GenBank database of the NCBI under the accession numbers MW063488, MW063524, MW063525, MW063526, MW063527, and MW063528.

Fatty acid composition of the SCO extracted from Pichia manshurica *strain Y2*

Pichia manshurica strain Y2 was concluded as the most effective oleaginous yeast in this study. It was cultured in the GYP medium for intracellular SCO accumulation and extracted to obtain the SCO by the UAE method as aforementioned. The fatty acid composition of the SCO was determined with gas chromatography (GC) as fatty acid methyl esters (FAMEs). The SCO sample was converted into a mixture of FAMEs by transesterification following the method of Lin and Lin (2017). FAME analysis was carried out with a gas chromatograph 7890A (Agilent, USA) equipped with a flame ionized detector (FID) and a SP-2560 capillary column (Sigma-Aldrich, USA). The condition of the GC analysis was conducted according to Leesing and Nontaso (2011).

Data analysis

The statistical analyses of the data in this study were performed using R software version 4.1.1. The average and standard deviation (SD) values of the data sets were analyzed. The multiple comparison analyses were determined by one-way ANOVA followed by Tukey's test with a 95% confidence interval ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation of yeast from the fruit samples

The native yeasts were swabbed from the surfaces of the longkong fruit enriched in the YMB medium and isolated on the DRBC medium. The results revealed that 16 distinct morphological colonies were isolated as the presumptive yeasts and then purified on the YMA medium. These purified yeasts were obtained from six isolates of Yala Province and 10 isolates of Rayong Province. All purified yeasts had a similar pattern of colony morphology involving white pigmentation, a shiny surface appearance, opaque, and circular shape. Otherwise, their colonies mainly differed in the diameter of the colony.

Accumulation and extraction of the SCOs from the isolated yeasts

All presumptive yeast isolates were cultured in the GYP medium for the intracellular SCO accumulation. This medium was defined as a glucose-rich and nitrogen-limiting medium, promoting lipid accumulation. All isolates could be cultivated in the GYP medium under the laboratory conditions. The shapes and sizes of the individual cell varied slightly as observed under an ECLIPSE E200 light microscope. The primary screening by the microscopic method showed that only six yeast isolates were considered as axenic isolates of the SCO accumulating yeasts comprising isolates Y1, Y2, Y3, Y4, YB5, and YB8. They were shown to harbor noticeable intracellular SCO droplets (Figure 1). The intracellular SCO droplets appeared as transparent globules within the yeast cells. Interestingly, it was found that isolate Y2 harbored noticeable and large intracellular SCO droplets within the cells. Therefore, these six yeast isolates were used to secondary screening the SCO accumulating yeasts.

The cells of the six lipid accumulating yeasts were filtered through the nitrocellulose filters and then dried in a hot air oven. The dry weights of the yeast cells ranged widely from 0.219 ± 0.005 g/L (isolate Y4) to 1.073 ± 0.048 g/L (isolate Y3). The produced SCO concentration varied from 0.075 ± 0.004 g/L (isolate Y3) to 0.102 ± 0.005 g/L (isolate YB5). All studied yeasts could accumulate SCOs ranging from 6.984 ± 0.546% (w/w) (isolate Y3) to 43.032 ± 3.584% (w/w) (isolate Y2) according to the microscopic results of the primary screening procedure. Remarkably, the isolates Y2 and Y4 had the ability to accumulate SCOs greater than 20% (w/w) of their dry biomass. Therefore, they were confidently considered as oleaginous yeasts. Isolate Y4 accumulated SCO with the content of 33.591 ± 1.763% (w/w) under the experimental conditions. Isolate Y2 significantly attained the maximum SCO content of 43.032 ± 3.584% (w/w) under the experimental conditions and was selected for further study. Its SCO content was not significantly different from the well-known oleaginous yeast, *Y. lipolytica* strain TISTR 5212 (the positive control in this study). The dry cell weight, SCO concentration, and SCO content of the six studied SCO accumulating yeasts are shown in Table 1.
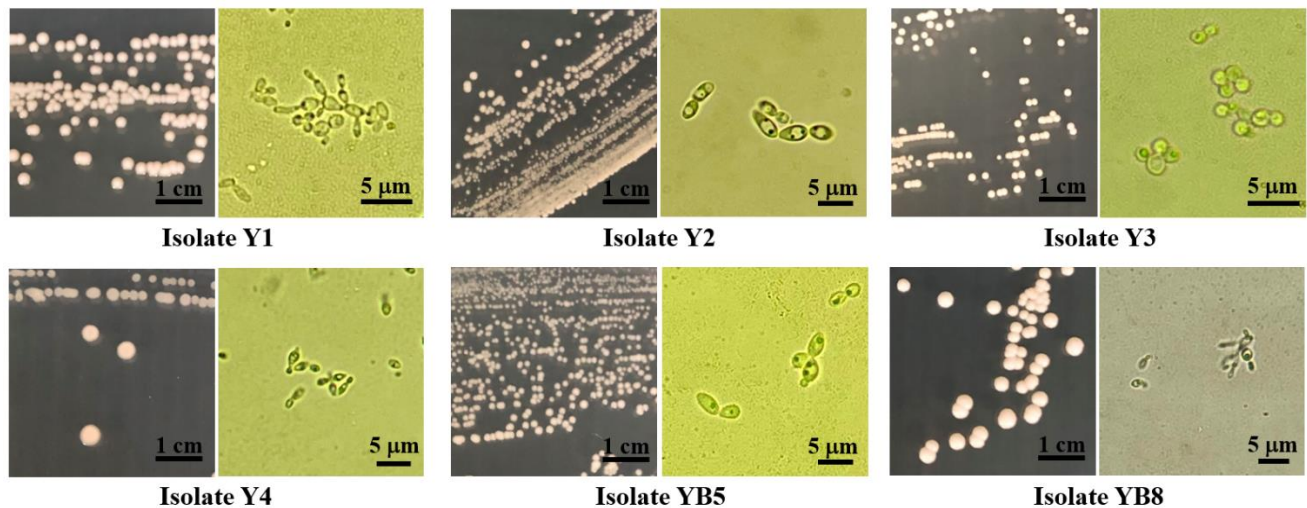
Figure 1. Colony and microscopic morphologies of the six SCO accumulating yeasts isolated from the surfaces of longkong fruit

Table 1. Dry cell weight and SCO production for the SCO accumulating yeasts.

SCO accumulating yeast	Dry cell weight (g/L)	SCO concentration (g/L)	SCO content (%, w/w)
Isolate Y1	0.916 ± 0.052 [d]	0.099 ± 0.002 [c]	10.810 ± 0.755 [a]
Isolate Y2	0.236 ± 0.008 [a]	0.101 ± 0.008 [c]	43.032 ± 3.584 [c]
Isolate Y3	1.073 ± 0.048 [e]	0.075 ± 0.004 [ab]	6.984 ± 0.546 [a]
Isolate Y4	0.219 ± 0.005 [a]	0.074 ± 0.002 [a]	33.591 ± 1.763 [b]
Isolate YB5	0.772 ± 0.049 [c]	0.102 ± 0.005 [c]	13.204 ± 1.521 [a]
Isolate YB8	0.825 ± 0.034 [cd]	0.097 ± 0.003 [bc]	11.768 ± 0.317 [a]
Yarrowia lipolytica strain TISTR 5212 (Positive control)	0.444 ± 0.012 [b]	0.184 ± 0.019 [d]	41.460 ± 4.857 [c]

Note: Mean values in the same column followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$).

Table 2. Identity percentage of the ITS region sequences for the six SCO accumulating yeasts with closely related yeasts

SCO accumulating yeast	Closely related yeast	GenBank accession no. (references)	Query cover (%)	Identity (%)*	GenBank accession no. (deposited)
Isolate Y1	*Candida jaroonii* strain NBRC 103210	AB360441.1	98	98.84	MW063488
Isolate Y2	*Pichia manshurica* strain CBS 11610	KP250848.1	98	98.11	MW063524
Isolate Y3	*Meyerozyma caribbica* strain IFM 53331	LC413740.1	99	98.36	MW063525
Isolate Y4	*Hanseniaspora opuntiae* strain UniFGHO3	KT029780.1	99	99.08	MW063526
Isolate YB5	*Pichia* sp. strain TCJ3	HM044858.1	94	98.19	MW063527
Isolate YB8	*Kodamaea ohmeri* strain CBS 5367	GU246263.1	99	98.51	MW063528

Note: * Identity results were analyzed on October 23, 2021.

Genetic identification of the SCO accumulating yeasts

All SCO accumulating yeasts were genetically identified according to the PCR amplification and nucleotide aligned of their ITS region sequences. Alignment of their ITS region sequences using the BLASTn program revealed that the SCO accumulating yeasts belonged to five genera of the Phylum Ascomycota consisting of genus *Candida*, genus *Pichia*, genus *Meyerozyma*, genus *Hanseniaspora*, and genus *Kodamaea*, with a 94%-99% query coverage and 98%-99% identity. All resulting E values obtained from the nucleotide alignments were zero. The genetic identification of the SCO accumulating yeasts using the BLASTn program is shown in Table 2.

The phylogenetic tree of the SCO accumulating yeasts using the BIONJ algorithm with 100,000 bootstrap replications was generated (Figure 2). The SCO accumulating yeast isolate Y1 was phylogenetically clustered with the *Candida* species by a bootstrap value of 90. The oleaginous yeast isolate Y2 and SCO accumulating yeast isolate YB5 were grouped in the same clade of *Pichia* species by a bootstrap value of 97. The SCO accumulating

yeast isolates Y3 and YB8 were phylogenetically grouped in the clade of the *Meyerozyma* and *Kodamaea* species with a bootstrap value of 100, respectively. The oleaginous yeast isolate Y4 was phylogenetically clustered in the clade of *Hanseniaspora* species with a bootstrap value of 99. The phylogenetic tree was correlatedly conducted to the results from the BLASTn alignment. The SCO accumulating yeasts were found to be closely related based on the BLASTn alignments of the ITS region when the identity results were more than 98%. Therefore, they were designated as the *C. jaroonii* strain Y1, *P. manshurica* strain Y2, *M. caribbica* strain Y3, *H. opuntiae* strain Y4, *Pichia* sp. strain YB5, and *K. ohmeri* strain YB8. All nucleotide sequences of the ITS region were deposited in the GenBank database of the NCBI under the accession numbers MW063488, MW063524, MW063525, MW063526, MW063527, and MW063528 (Table 2).

Fatty acid composition of the SCO extracted from Pichia manshurica strain Y2

Pichia manshurica strain Y2 was considered as the most effective oleaginous yeast with the SCO content of 43.032% (w/w). The SCO extracted from this oleaginous yeast was converted into FAMEs by the transesterification reaction and analyzed for the fatty acid composition in terms of the FAME profile by the GC-FID. The GC-FID result showed that the SCO extracted from the *P. manshurica* strain Y2 contained fatty acids with C16-fatty acids and C18-fatty acids involving palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1n-9c), and linoleic acid (C18:2n-6c). It mainly produced saturated long-chain fatty acids as palmitic acid (C16:0) of 42.57% and stearic acid (C18:0) of 25.11%, respectively. The GC-FID chromatogram and FAME profile for the oleaginous *P. manshurica* strain Y2 are shown in Figure 3 and Table 3.
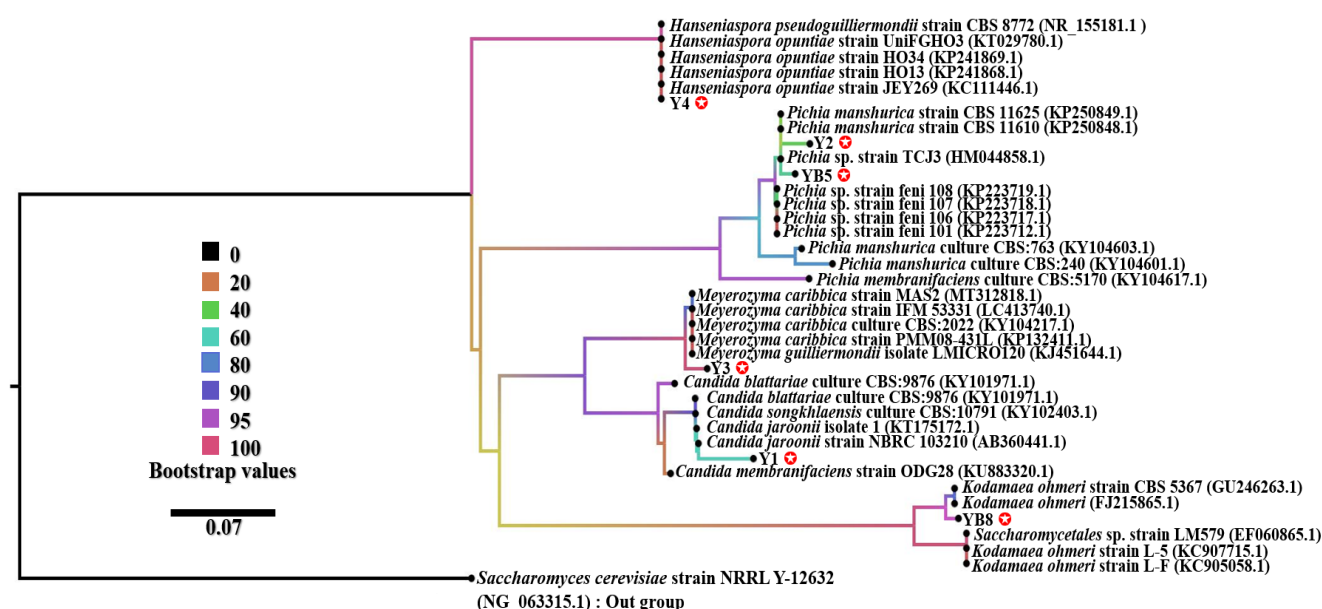

Figure 2. Phylogenetic tree of the SCO accumulating yeasts using the BIONJ algorithm with 100,000 bootstrap replications. The phylogenetic tree was generated by SeaView software version 5.0.2 and visualized by FigTree software version 1.4.4. The red star symbols represented the six SCO accumulating yeasts in this study

Table 3. Fatty acid methyl ester (FAME) profile of the SCO produced from the *Pichia manshurica* strain Y2

FAME profile	Retention time (minutes)	Peak area (pA*s)	Percentage of FAMEs in the SCO (%) of the *P. manshurica* strain Y2
Palmitic AME (C16:0)	26.824	86.570	42.57
Palmitoleic AME (C16:1)	28.642	13.386	6.69
Stearic AME (C18:0)	32.564	51.030	25.11
Oleic AME (C18:1n-9c)	34.989	27.746	13.55
Linoleic AME (C18:2n-6c)	39.414	24.423	12.08
Total			100.00
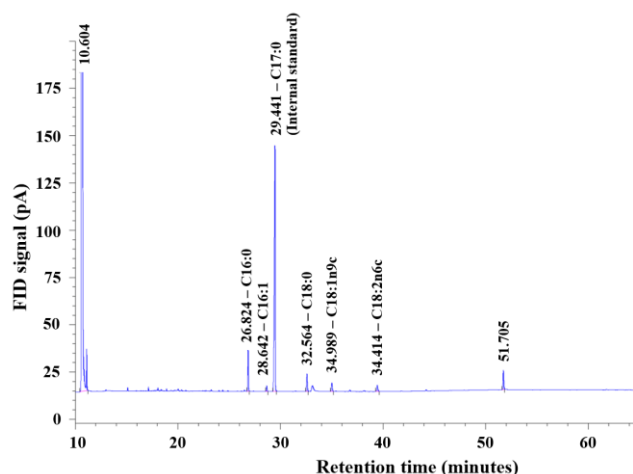
Figure 3. GC-FID chromatogram of the fatty acid composition in the SCO extracted from the *Pichia manshurica* strain Y2. Heptadecanoic AME (C17:0) was the internal standard used in this experiment

Discussion

Oleaginous yeasts are promising sources of microbial oils or SCOs (Ramírez-Castrillón et al. 2017). They are considered as sustainable alternative feedstock for third-generation biodiesel and high-value compound productions. The oleaginous yeasts can grow on various substrates, and many yeast species have been isolated from plant surfaces. However, only a small number of oleaginous yeast species have been examined to date (Kanti et al. 2013).

This study isolated and genetically identified the oleaginous yeasts from the surfaces of a local fruit, *L. domesticum* cultivar longkong. A previous study reported numerous yeasts and other microbes associated on the surface of longkong fruit that caused fruit spoilage (Sirichote et al. 2013). A study on the baker yeast strains from a variety of Malaysian local fruits reported that *Saccharomyces cerevisiae* was isolated from *L. domesticum* cultivar duku, rambutan, mango, and bamboo shoots (Ma'aruf et al. 2012). Noteworthy, the published information of oleaginous yeasts colonizing the longkong fruit has been scarce. In this study, 16 presumptive yeast isolates were isolated from the surface of longkong fruit and cultured under laboratory conditions. Four yeast isolates (isolates Y1, Y3, YB5, and YB8) were identified as the SCO accumulating yeasts with accumulation of the SCO below 20% (w/w), and two yeast isolates (isolates Y2 and Y4) were considered as the oleaginous yeasts with accumulation of the SCO above 20% (w/w) in the GYP medium under laboratory conditions. This evidenced that oleaginous yeasts could be isolated from the surface of longkong fruit.

The isolate Y2 was shown to harbor noticeable intracellular SCO droplets observed by a light microscope in the primary screening procedure. This procedure was a simple and inexpensive method without any advanced equipment. However, the intracellular SCO droplets without staining were a little difficult to investigate and distinguish under the light microscope. This should be improved by staining the SCO accumulating yeasts with a family of lipophilic dyes before microscopic investigation; such as, Sudan III (Jiru et al. 2016), Sudan IV (Vincent et al. 2018), and Sudan Black B (Duarte et al. 2013). The secondary screening of the oleaginous yeasts showed that the isolate Y2 accumulated a maximum lipid content among the isolated yeasts of 43.03% (w/w). Its SCO content was nearby a well-known and commercial oleaginous yeast, *Y. lipolytica*. It was believed that the isolate Y2 was an interesting strain for further studies and related applications.

The isolate Y2 was genetically identified and designated as the *P. manshurica* strain Y2. In previous studies, this species was reported as an oleaginous yeast of the Phylum Ascomycota, which could be isolated from diverse environmental sources. *P. manshurica* strain CHC34 was isolated from chili sauce collected in Taiwan and considered as a SCO accumulating yeast with the resulting lipid content of 9.52% (w/w) (Areesirisuk et al. 2015). The *P. manshurica* strain DMKU-UbC9 was isolated from the soil and plant surfaces in Thailand. It was identified to be an effective oleaginous yeast with a lipid content of 64.80% (w/w) (Polburee et al. 2015). Recently, five strains of *P. manshurica* were isolated from the soil, rambutan, and pineapple in Malaysia (Vincent et al. 2018). They were shown to harbor intracellular lipid droplets after being Sudan IV stained. owever, limited literature had intensively reported on the intracellular SCO production in *P. manshurica* (Areesirisuk et al. 2015; Vincent et al. 2018). The isolate YB5 was designated as the *Pichia* sp. strain YB5 by a nucleotide sequence alignment. Its cell morphology observed under light microscope and phylogenetic tree revealed that it might evolutionarily correlate to the isolate Y2 and neighboring yeasts in the clade of *P. manshurica*. It was considered as a SCO accumulating yeast with a SCO content of only 13.20% (w/w).

The SCO accumulating yeast isolate Y1 was genetically identified as the *C. jaroonii* strain Y1 with a SCO content of 10.81% (w/w). It was a new ascomycetous anamorphic yeast, which was first isolated from various substrates in Thailand (Imanishi et al. 2008) and currently isolated from the phylloplane of rice in Thailand (Limtong and Kaewwichian 2015). Furthermore, a previous study from Thailand showed that the *C. jaroonii* strains RK92 and RK138 were screened to identify the oleaginous yeasts (Polburee et al. 2015). Accordingly, the results revealed that they were not considered yeast strains that accumulated SCO at levels higher than 20% (w/w).

Another SCO accumulating yeast found in this study was the isolate Y3. It was designated as the *M. caribbica* strain Y3 with the lowest SCO content of 6.98% (w/w). In addition, many studies have depicted the production of SCO provided by *M. caribbica*. Two strains of *M. caribbica* isolated from sugar cane in Thailand were cultured and determined for accumulation of the lipid content (Polburee et al. 2015). The result found that the *M. caribbica* strain DMKU-RK258 could accumulate intracellular SCO of 37.60% (w/w). Recently, the *M.*

caribbica strain MH267795 isolated from olive oil mill wastewater in Tunisia was identified as an oleaginous yeast (Chebbi et al. 2019). This result demonstrated that SCO accumulation of yeasts was strain dependent not species or genus dependent (Polburee et al. 2015).

The results of the current study showed that isolate Y4 was also oleaginous with the SCO content of 33.51% (w/w). It was genetically identified as the *H. opuntiae* strain Y4. The *H. opuntiae* yeasts were commonly isolated from plant surfaces and related equipment; such as, a cocoa bean fermentation box in Malaysia (Papalexandratou et al. 2013) and bark of the *Ficus* tree in Ethiopia (Koricha et al. 2019). Today, the study on oleaginous *H. opuntiae* has been limited. A previous study reported that *H. opuntiae* code UFLA BM 14.3 isolated from cocoa in Brazil was cultured in glycerol and evaluated for its growth ability and lipid production (Souza et al. 2014). The results showed that it did not show any growth in media containing commercial glycerol and was not evaluated for the lipid production. Therefore, the current study was the very first report in which *H. opuntiae* strain Y4 was considered as an oleaginous yeast.

The last accumulating yeast found in this study was the *K. ohmeri* strain YB8, which produced the SCO content of 11.77% (w/w). *Kodamaea ohmeri* was isolated mostly from pickled food, sand, and marine environment (Chakrabarti et al. 2014; Vivas et al. 2016). There were also many studies regarding its biotechnological and lipid production potential (Corbu et al. 2019). The *K. ohmeri* strain BY4-523 was isolated from palm oil related samples in Thailand (Kitcha and Cheirsilp 2011). It was found that BY4-523 accumulated lipid content of 53.28% (w/w), and the *K. ohmeri* strain DMKU-RK67 was isolated from rice and accumulated intracellular SCO of 22.90% (w/w) (Polburee et al. 2015). The SCO content of each yeast was strain dependent, likewise the explanation of the aforementioned *M. caribbica* strain Y3.

The *P. manshurica* strain Y2 was the most effective oleaginous yeast in this study. It was cultured in the GYP medium and analyzed for fatty acid composition by GC. The result revealed that the major fatty acids of its SCO were saturated fatty acids, including palmitic acid (C16:0) of 42.57% and stearic acid (C18:0) of 25.11%. The other fatty acids were unsaturated fatty acids, which were detected in a lower concentration; such as, palmitoleic (C16:1), oleic (C18:1n-9c), and linoleic (C18:2n-6c) acids. Its fatty acid profile was closely similar to a previous study on the *P. manshurica* strain CHC34 that mainly contained palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic (C18:3n-6) acids (Areesirisuk et al. 2015). However, the quantity of the resulting fatty acids was slightly different from that previous study. The findings indicated that oleic and stearic acids were the major fatty acids of the *P. manshurica* strain CHC34. Thus, it might be that the fatty acid profile of oleaginous yeast could be dependent upon the culture medium and various cultivation conditions (Patel et al. 2017). Palmitic acid and stearic acid are mostly found in various vegetable oils (Giakoumis 2018) and have been recommended for biodiesel production (Areesirisuk et al. 2015). Moreover, long-chain saturated fatty acids are

prime importance for determining the biodiesel quality, especially the cetane number, NOx emission, and kinetic viscosity (Patel et al. 2017). Therefore, the SCO of the *P. manshurica* strain Y2 could be used as an alternative feedstock for biodiesel production. A recent study informed that oleaginous yeasts could be considered as candidates for the production of bio-based oleochemicals; such as, lubricants and supplements in the food, feed, and cosmetic industries (Khot et al. 2020). Interestingly, the *P. manshurica* strain Y2 contained a high content of stearic acid of 25.11%. As such, it could be considered as a feedstock to produce the cocoa butter equivalents. Increased demands and insufficient cocoa plants have also led to a shortage of cocoa butter supply; therefore, it was interesting to find an alternative cocoa butter supply; such as, cocoa butter equivalents (Wang et al. 2020). Interestingly, several oleaginous yeasts represent promising sources for the production of several fats like cocoa butter and shea butter (Khot et al. 2020).

In conclusion, the isolation of yeast from local fruit is a challenging study to obtain novel effective oleaginous strains. This study successfully isolated and cultured six yeast isolates from the surfaces of longkong fruit (*L. domesticum*) samples. Intracellular SCO quantification confirmed the oleaginous character of the isolated yeasts that there were four SCO accumulating yeast isolates and two oleaginous yeast isolates. They were genetically identified and phylogenetic analyzed as belonging to the five genera involving *Candida*, *Hanseniaspora*, *Kodamaea*, *Meyerozyma*, and *Pichia*. The results revealed that the isolate Y2 was evidenced as the most effective oleaginous yeast with SCO content of 43.03% (w/w) and designated as the *P. manshurica* strain Y2. It was cultured for gaining the intracellular SCO and quantitatively analyzed using GC analysis. Its SCO revealed fatty acid profiles similar to vegetable oils with the potential for uses in the production of third-generation biodiesel and cocoa butter equivalents. Further studies could be conducted to examine the growth kinetics and optimize the culture conditions for improving the SCO production.

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