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Xylose-assimilating oleaginous yeasts from mangrove forest

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ABSTRACT

The objectives of this research were to isolate and screen for oleaginous yeasts from leaves and soil samples at Mangrove Forest Conserve and Natural Study Center, Chonburi, Thailand. Investigation of biomass and lipid production of a selected isolated yeast was also conducted to explore the correlation of biomass and lipid production at different time courses. A total of 19 yeast isolates with xylose-assimilating ability were isolated and screened for their potential to accumulate lipid by Sudan black B staining and Nile red fluorescence assay. The results of Sudan black B staining showed that 9 yeast isolates had lipid accumulation in the cytoplasm of yeast cells, and isolate S3-2 was the most promising isolate for lipid accumulation based on the highest level of fluorescence intensity according to Nile red fluorescence assay. Thus, this yeast isolate was selected for the time-course study of biomass production, lipid production and lipid content. Biomass and lipid production achieved from isolate S3-2 were 175.56±1.13 mg/L and 42.59±0.33 mg/L, respectively. The maximum lipid content was determined to be 24.26±0.29% of dry cell weight. These results implied that oleaginous yeasts that could utilize xylose were distributed in mangrove ecosystem, and the strains isolated in this study could be used as a valuable lipid source for future relevant application.

Keywords: oleaginous yeast; microbial lipid; lipid production; xylose; mangrove forest

1. INTRODUCTION

Currently, fuel consumption demands have been increased around the world. However, petroleum fuels derived from fossils are diminishing, which necessitates the search for alternative energy sources, such as biodiesel, which is produced using lipid as a substrate. Microbial lipid has attracted attention as a renewable source for biodiesel production because it is considered as a non-food feedstock. Microbial lipid is also known as single cell oil, while lipid-accumulating microorganisms are called oleaginous microorganisms. Microbial lipids are synthesized and accumulated intracellularly, and their fatty acid profiles are similar to those of plant oils. Oleaginous microorganisms have several advantages compared to plants; specifically, they have a shorter growth cycle, they require less area to culture, and their growth conditions are easier to control (Gao et al., 2016).

Oleaginous microorganisms are capable of lipid accumulation with more than 20% of dry cell weight (Patel et al., 2019). This research focused on oleaginous yeasts because they are usually non-pathogenic and can produce triacylglycerol, the main type of lipid that is stored in lipid droplets with a concentration ranging from 20% to 76% of dry cell weight, depending on culture conditions (Lamers et al., 2016).

Oleaginous yeasts can assimilate different types of carbon sources (e.g., simple sugars to complex carbohydrates) for their growth (Sitepu et al., 2014). Lipid production from glucose in oleaginous yeast, specifically, *Cryptococcus curvatus*, *Lipomyces starkeyi, Rhodosporidium toruloides* and *Rhodosporidium glutinis*, has been shown to be able to use xylose as a carbon source (Yamada et al., 2017).

Mangrove forests are bioresources with a diverse range of plant species. *Rhizophora* is the major type of

plants found in mangrove forest. Physical and nutritional factors of each area of mangrove forests affect microbial diversity including yeasts (Chanklan et al., 2012). Yeasts can be distributed in water and soils; yeasts are frequently present in several parts of plants (e.g., leaves, flowers and stems) because plants synthesize different types of sugars and polysaccharides (Siranonthana et al., 2015) that serve as nutrients for yeasts. In addition, plant cell wall is composed of xylose, which is a pentose sugar that is presented in hemicellulose fraction (Aristilde et al., 2015). Therefore, it is assumed that plant-inhibiting oleaginous yeasts can assimilate xylose as a carbon source.

Hence, this research aimed to isolate and screen for oleaginous yeasts that are capable of utilizing xylose as a carbon source from local mangrove ecosystem and to investigate biomass and lipid production at different time courses.

2. MATERIALS AND METHODS

2.1 Materials

All of the chemicals and dehydrated products for culture media preparation were purchased from distributors in Thailand. Information about the manufacturing companies are given as follows; yeast extract, peptone and agar were produced by Hardy Diagnostics (California, USA). D-(+)-xylose and Sudan black B were produced by TCI Co., Ltd. (Tokyo, Japan). Chloramphenicol, Nile red, safranin O and DMSO were produced by Sigma Co. (Missouri, USA). Potassium dihydrogen phosphate (KH_2PO_4) and magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) were produced by Merck KGaA (Darmstadt, Germany).

2.2 Sample collection

Soil samples were randomly collected from 5 different areas at Mangrove Forest Conserve and Natural Study Center, Chonburi, Thailand. Fresh leaves from the same mangrove forest were picked from 2 *Rhizophora apiculata* Blume trees and 3 *Rhizophora mucronate* Poir trees. Each sample was stored in a sterile plastic bag and brought to the lab for the isolation of yeasts.

2.3 Isolation of xylose-assimilating yeasts

A total of 5 g of each soil sample or 2 g of each leave sample was separately placed in a 250-mL flask containing 100 mL of a yeast extract peptone xylose (YEPX) broth (10 g/L yeast extract, 20 g/L peptone and 20 g/L xylose); 0.05 g/L chloramphenicol was added to inhibit bacterial growth. All flasks were incubated at 25°C with 150 rpm shaking for 5 days. Each sample was diluted with 0.85% NaCl, and 0.1 mL of each dilution was spread on YEPX agar supplemented with chloramphenicol (YEPX broth, 20 g/L agar and 0.05 g/L chloramphenicol). Pure yeast cultures were obtained by picking different morphological colonies and restreaking those on YEPX agar plates.

2.4 Screening of oleaginous yeasts by Sudan black B staining and Nile red fluorescence assay

Yeast isolates were primary used for Sudan black B staining to visualize intracellular lipid accumulation. Each yeast culture was grown in a 250-mL flask containing 100 mL of a xylose magnesium sulphate yeast extract (XMY) broth (3 g/L yeast extract, 8 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 40 g/L xylose) with some modification (Munch et al., 2015) and incubated at 25°C, 150 rpm. After 5-day incubation, yeast cultures were smeared, air-dried and heat-fixed. All slides were stained with Sudan black B for 15 min followed by staining with 0.5% safranin O for 30 s and rinsing with water (Burdon, 1946; Jape et al., 2014). Lipid accumulation was observed with a 100× light microscope objective lens (Olympus, Japan).

Potent yeast isolates were also confirmed by Nile red fluorescence assay (Sitepu et al., 2012) to select the most capable yeast for lipid production. A fresh yeast culture growing on XMY broth for 5 days, at 25°C, 150 rpm (250 µL) was mixed with a mixture of DMSO and XMY broth (25 µL, 1:1 v/v ratio). Nile red solution was subsequently added to the final concentration of 5 µg/mL. A combination without Nile red solution was used as a blank. All reaction mixtures were transferred to a 96-well black microplate (Corning, USA), and fluorescence intensities were determined at excitation and emission wavelengths of 530 and 590 nm, respectively, in a fluorescence spectrophotometer (Agilent Technologies, USA).

2.5 Biomass and lipid production in a selected yeast isolate

A potent isolate was grown on 100 mL of XMY broth and incubated at 25°C, 150 rpm for 7 days. Yeast cells were collected at 24-h interval and dried at 60°C until constant weight. According to Tapia et al. (2012) and Castanha et al. (2013), dried biomass was processed as follows: a mixture of dried biomass (0.3 g) and 2M hydrochloric acid (4 mL) were incubated at room temperature for 1 h and centrifuged at 6,000 ×*g* for 15 min. Cell pellet was resuspended in a mixed solution of sterile distilled water, chloroform and methanol (4:5:5 mL). Chloroform layer was collected and dried at 60°C until constant weight. Biomass concentration (mg/L), lipid concentration (mg/L) and lipid content (% w/w) were calculated and reported.

2.6 Data analysis

All experiments were performed in triplicate. Values were presented as the mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Isolation of xylose-assimilating yeasts

A total of 19 xylose-assimilating yeasts were obtained from soil (8 isolates) and fresh leave (11 isolates) samples. Former isolates had 2 distinct colony morphologies that differed in margin and size, whereas latter isolates showed 3 different morphological types that differed in color, margin and size (Table 1). After 2 days at 25°C, it was observed that cells were spherical (5 µm in diameter) to oval-shaped (2-3 µm wide and 5-6 µm long) and occurred singly. Vegetative reproduction proceeded by multilateral budding. Pseudohypha were produced by isolates L2-3, L5-2 and S3-2 grown on YEPD medium (data not shown).

Table 1. Colony morphology of 19 yeast isolates grown on YEPD agar at 25°C for 2 days

Habitat	Frequency	Colony morphology				
		Color	Form	Elevation	Margin	Size[a]
soils	5	white	circular	convex	entire	1-3
	3	white	circular	convex	undulate	1-2
leaves	4[b]	cream	circular	convex	undulate	3
	5[c]	white	circular	convex	entire	0.5-1.5
	2[c]	white	circular	convex	undulate	2

Note: [a] diameter, mm; [b] from *Rhizophora apiculata* Blume; [c] from *Rhizophora mucronata* Poir

3.2 Screening of oleaginous yeasts

From 5-day-old cultures, 9 out of 19 yeasts (isolates L1-1, L2-1, L2-3, L3-2, L4-2, L5-1, L5-2, S1-1 and S3-2) showed lipid stained grey to black (Sudan black B) in cytoplasm of cells, whereas non-oleaginous yeasts were stained completely red (safranin O), as indicated in Figure 1. Intracellular lipid varied among yeasts because isolates L2-1, L2-3, L4-2 and L5-1 exhibited less accumulation compared to others. Isolates L2-1 and L5-2 collected several small lipid droplets, while isolates L1-1, L3-2 and S3-2 stored large lipid droplets. Lipid accumulation was especially prominent in cells and pseudohypha of soil yeast (isolate S3-2).

An attempt to demonstrate lipid accumulation in yeast cells has been previously reported. Amornrattanapan and Thongthep (2019) revealed that intracellular lipid stained grey to black in oleaginous yeasts isolated from mangrove forest. *Candida* sp. KC 966726, *Candida tropicalis* KC 966722 and *Rhodotorula mucilaginosa* KF 020690 contained grey-stained lipid droplets inside the cells (Jape et al., 2014). Sudan black B is an azo dye that is used for staining of phospholipids, sterols and neutral triacylglycerols. Visualization of lipid yeasts is performed by Sudan black B staining, which is a fast and simple method and similar to Gram staining (Gusbeth et al., 2016).

A total of 9 yeasts (isolates L1-1, L2-1, L2-3, L3-2, L4-2, L5-1, L5-2, S1-1 and S3-2) yielded positive results with Sudan black B staining in which lipid stained grey to black were observed in cytoplasm of yeast cells. Intracellular lipid accumulation of those yeast isolates were also confirmed by Nile red fluorescence assay. In 5-day-old cultures, isolate S3-2 had the strongest capability for lipid accumulation (i.e., highest fluorescence intensity, as shown in Figure 2). Therefore, it was chosen for further investigation, including biomass and lipid production.

Nile red fluorescence assay is a rapid and inexpensive method for estimating lipid content of yeasts. Nile red is a fluorescent dye that is used for detection and quantification of neutral lipids (particularly triacylglycerol) stored in oleaginous yeasts. The amount of intracellular lipid correlated with fluorescence intensity emitted from Nile red-stained lipid (Sitepu et al., 2012).

3.3 Biomass and lipid production in isolate S3-2

After 7-day incubation in XMY broth at 25°C, 150 rpm, yeast cells were harvested at 24-h interval for quantification of biomass and lipid. Isolate S3-2 exhibited the highest values of lipid content, biomass and lipid concentration (24.26±0.29% of dry cell weight, 175.56 ±1.13 mg/L and 42.59±0.33 mg/L, respectively) at 120 h of growth (Figure 3), which may be in the stationary phase.

It is clear that the maximum lipid concentration of 0.04 g/L (or 42.59 mg/L) was lower than 0.7, 0.6 and 0.5 g/L in *Cryptococcus curvatus* NBRC 0732, *Rhodosporidium toruloides* NBRC 10033 and *Rhodotorula glutinis* NBRC 0415, respectively, grown in YPX medium containing 2% xylose after 48-h cultivation (Yamada et al., 2017).

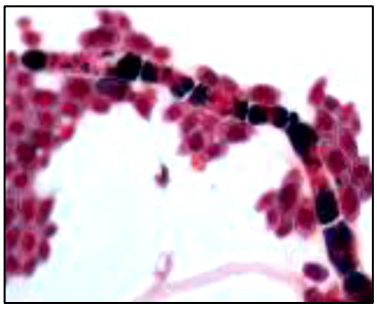
It was determined that isolate S3-2 had the highest lipid content after 120 h of growth (24.26% of dry cell weight). Levels of lipid content may vary among yeast strains. Surarit et al. (2011) reported that yeast strain BF0206 isolated from mangrove forest yielded lipid content of 26.53% of dry cell weight when grown in minimal medium containing 7% xylose after 48-h cultivation. *Rhodosporidium toruloides* DMKU-RE16 and *R. toruloides* DMKU-RE124 exhibited lipid contents of 17.7% and 16.1% of dry cell weight, respectively (Poontawee et al., 2017), whereas *Cryptococcus curvatus* showed the maximum lipid content of 40.3% of dry cell weight (Yu et al., 2014) when grown in media containing 7% xylose. Juanssilfero et al. (2018) demonstrated that *Lipomyces starkeyi* NBRC 10381 exhibited lipid content of 85% of dry cell weight when grown in medium containing 5% xylose.

It has been reported that oleaginous yeasts can accumulate the maximum lipid content during stationary phase. Similar results were observed for isolate S3-2 (24% of dry cell weight) and *Rhodosporidium fluvial* DMKU-SP314 (45% of dry cell weight), which yielded the highest lipid content after 120-h and 228-h of growth, respectively, which was possibly sufficient to reach in the stationary phase (Poontawee et al., 2017).

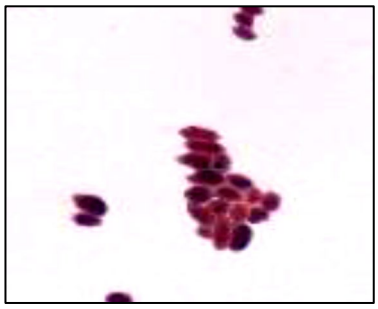
Factors affecting lipid content in oleaginous yeasts may include yeast strains, mode of cultivation, type and concentration of carbon (C) and nitrogen (N) sources as well as the C/N ratio. The C/N ratio used in this study was sufficiently high (53.33) to cultivate a promising strain because the C/N ratio of more than 20 has been suggested to be sufficient to induce lipid accumulation in oleaginous yeasts (Papanikolaou and Aggelis, 2011).

4. CONCLUSIONS

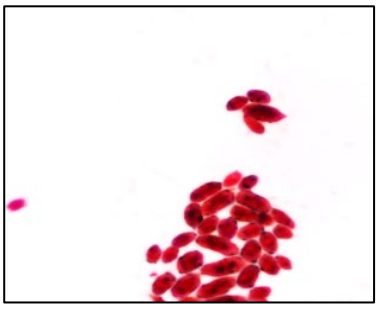
This study revealed the capability of xylose assimilation and ability of lipid accumulation of yeasts isolated from Mangrove Forest Conserve and Natural Study Center in Chonburi Province, Thailand. Among 9 isolates, isolate S3-2 was the most promising strain, which accumulated lipid content of 24.26±0.29% of dry cell weight. Further optimization of culture conditions for growth and lipid production is required. Xylose-containing substrates (e.g., lignocellulosic materials from agricultural wastes) are considered to be a cheap substrate for yeast cultivation to make this approach feasible for future application.



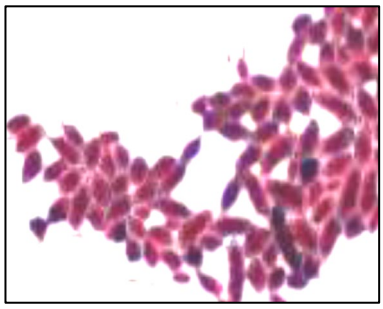
L1-1 (*R. apiculata* Blume leaves)



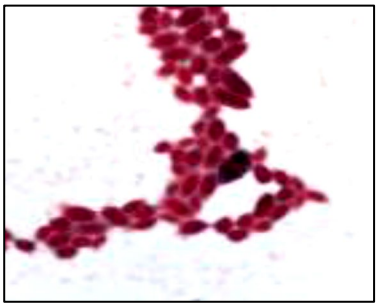
L5-1 (*R. mucronata* Poir. leaves)



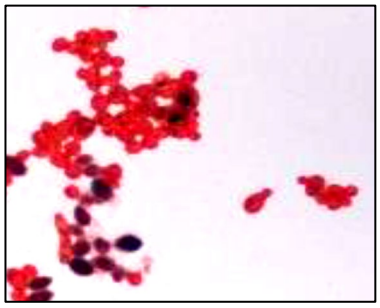
L2-1 (*R. mucronata* Poir leaves)



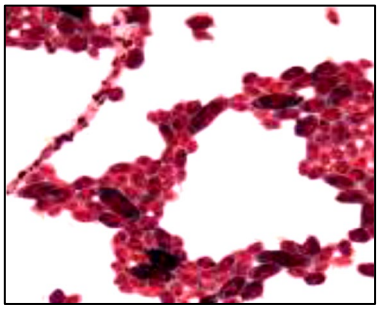
L5-2 (*R. mucronata* Poir leaves)



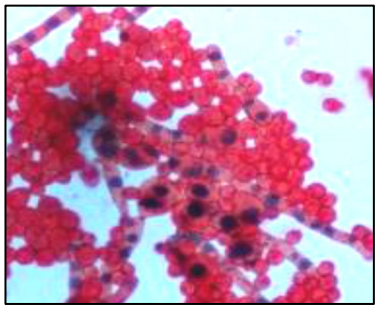
L2-3 (*R. mucronata* Poir leaves)



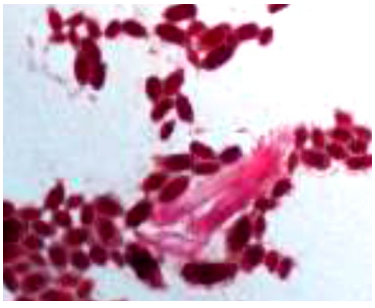
S1-1 (Soil)



L3-2 (*R. apiculata* Blume leaves)



S3-2 (Soil)



L4-2 (*R. mucronata* Poir leaves)

Figure 1. Intracellular lipid in 9 investigated yeasts stained with Sudan black B

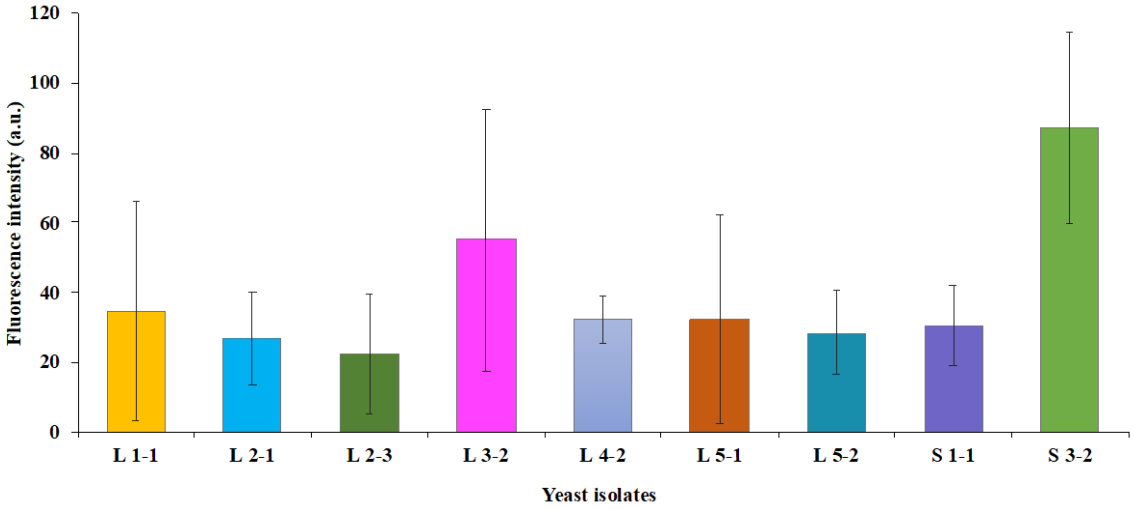


Figure 2. Fluorescence intensity measurement from Nile red-stained lipid of 9 yeast isolates cultivated in XMY broth at 25°C, 150 rpm for 5 days

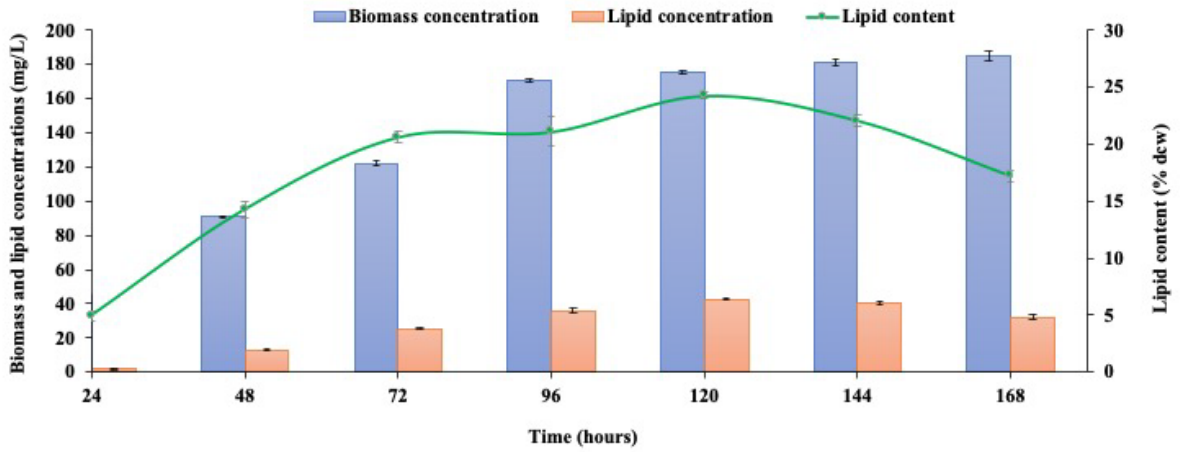


Figure 3. Biomass concentration, lipid concentration and lipid content of isolate S3-2 cultivated in XMY broth at 25°C, 150 rpm for 7 days

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