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Indonesian oleaginous yeasts isolated from *Piper betle* and *P. nigrum*

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Abstract

In this study, 71 strains of yeast were isolated from *Piper betle* and *P. nigrum*. Isolates were identified using sequence analysis of the D1/D2 region of large 26S ribosomal subunit rDNA. They belong to 25 species in 11 genera. Strains *Cryptococcus luteolus* InaCC Y-265, *Candida orthopsilosis* InaCC Y-302, and *Candida oleophila* InaCC Y-306 could accumulate more than 40% of lipid per g of cell biomass on a dry weight basis. The fatty acids observed were primarily palmitic acid (C18:1), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2). The fatty acid profiles suggest that these yeasts may be good candidates for biodiesel production, as they are similar to the fatty acids of plant oils currently used for biodiesel.

Key words – *Candida orthopsilosis* – *Candida oleophila* – *Cryptococcus luteolus* – fatty acid

Introduction

While oleaginous yeasts have been studied for many decades (Meng et al. 2009), the search for new oleaginous yeast strains has intensified in recent years (Peng et al. 2013). This is due a global growing awareness of the environmental, social and political hazards of relying on fossil fuels. Renewable, sustainable alternatives such as biofuels are under development. Intensive work is underway by several groups to explore the economic feasibility of producing microbial oil-based biodiesel from microalgae (Roleda et al. 2013), bacteria, yeasts or filamentous fungi (Meng et al. 2009, Lian et al. 2013). Oleaginous yeasts are strong candidates as some of them have the capacity to accumulate high levels of storage lipids, primarily triacylglycerides (Ratledge 1982). Plant associated microbes are a promising target for various bioprospecting studies (Arnold 2007). The work on searching for oleaginous yeast was started with the isolation of suspected potential microbes from various microbial resources including soil, litter, and marine biota (Abraham & Srinivasan 1984, Harllen et al. 2012, Meng et al. 2007, Ratledge 1993).

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A small number of oleaginous yeast species have been examined to date. These include basidiomycetous yeast species *Cryptococcus albidus, C. curvatus, C. magnus, C. terreus, Guehomyces pullulans, Rhodosporidium diobovatum, R. toruloides, R. colostri, R. glutinis, R. gramins, R. mucilaginosa* and *Trichosporon guehoae,* and ascomycetous yeast species *Lipomyces lipofer, L. starkeyi, L. tetrasporus,* and *Yarrowia lipotica* (Wu et al. 2010, Lin et al. 2011). Several researchers have recently reported discovery of new oleaginous species (Lian et al. 2013, Huang et al. 2013, Wang et al. 2013), whose genetic and physiological diversity will aid in development of industrially viable biofuels technologies. Oleaginous yeasts belong to many taxonomic clades, with some clades such as *Lipomyces/Myxozyma* being rich in oleaginous species.

Oleaginous yeast are clearly polyphyletic. Of the 1500 known yeast species, most have never been examined to determine whether they are oleaginous. Furthermore, only 5% of yeast species have been described (Cowan 2000). A search for new oleaginous yeast strains and species should focus on traits oleaginous yeasts have in common. Because many of these species were isolated from plant surfaces (Clément-Mathiu et al. 2008) and soil (Kitcha & Cheirsilp 2011, Saengea et al. 2011) we chose to focus first on plant surface-associated yeasts.

Yeasts inhabit various ecological niches including living plant, animal, deteriorated wood, soil, litter, and water (Spencer & Spencer 1997). A search for new oleaginous yeasts is best carried out utilizing skills of both the taxonomist and the biochemist. Yeasts isolated from plant surfaces often are able to utilize a broad variety of carbon sources (Kurtzman & Fell 2011), which makes these species valuable for conversion of plant biomass hydrolysates to biofuels.

Piper betle and *P. nigrum* are plants native to South and South East Asia that are used traditionally in cooking, medicine or stimulants, and are high in phenolic compounds. The microorganisms associated with this plant could have unique physiological properties. Aravind et al. (2009) isolated 74 strains of bacteria from these two plant species, demonstrating that this plant supports significant microbial diversity on the plant surface. We used conventional staining techniques to rapidly identify potential oleaginous yeasts including the Sudan Black staining technique for observing intracellular lipid bodies (Thakur et al. 1989), and Nile Red fluorescence staining of lipid bodies (Kimura et al. 2004).

Materials & Methods

Yeast strains

The 71 yeast strains were used in this study were obtained from stem, leaf and flower of *P. betle* and fruit of *P. nigrum* which were collected from Bali (S 08°21’44.8”, E 114°41’00.1”) and West Java (S 06°.35’54.2”, E 106◦47’48.7”), Indonesia. Methods for isolation, maintenance and preservation were carried out based on methods developed by Kurtzman & Fell (2011). Yeast were isolated by dilution, direct inoculation, membrane filter, and ballistospore fall-methods by using isolation medium containing (in g/L) : malt extract 50, agar 20 and chloramphenicol 0.5 (MEA agar). The Yeast Malt Extract (YM) medium containing (in g/L) : glucose 10, yeast extract 3, malt extract 3, and agar 20 were used as a growth medium. Molecular identification was determined by sequence analysis. D1/D2 region of nuclear large-subunit ribosomal DNA was amplified and sequenced using primers NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTCAAGACGG), as described by Fell et al. (2000). Sequencing of D1/D2 of LSU rDNA was determined with Big Dye terminator v3.1. Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer’s instructions. The LSU gene sequences determined in this study were manually aligned with published sequences of reference strain available from the EMBL/GenBank/DDBJ databases (Altschul et al. 1990).

Evaluation of yeast strains for oleaginous properties

Purified strains were initially streaked onto YM plates and grown for 2 days at 28°C. Fresh colonies were then inoculated into 250-mL Erlenmeyer flasks containing 50 mL modified Pan et al. (2009) medium containing (in g/L) : glucose 20, $(NH_4)_2SO_4$ 5, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5,

and yeast extract 0.5 and grown at 28°C on a rotary shaker at 180 rpm for 2 days. This culture was used as inoculum. Five mL of inoculum were transferred to 45 mL (in a 250-mL Erlenmeyer flask) of screening medium contained of nitrogen-limited medium (in g/L) : glucose 40, $(NH_4)_2SO_4$ 2, KH_2PO_4 2, NaH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 1.5 and yeast extract 0.5, supplemented with a 100-fold diluted trace element solution (Wu et al. 2010). The trace element solution contained (in g/L) : $CaCl_2.2H_2O$ 4.0, $FeSO_4.7H_2O$ 0.55, citric acid H_2O 0.52, $ZnSO_4.7H_2O$ 0.10, $MnSO_4.H_2O$ 0.076, and 100 µl of 18M H_2SO_4. The medium was sterilized at 121 °C for 15 min (Wu et al. 2011). The culture was incubated on a rotary shaker at 180 rpm and 28°C for 5 days. Duplicate samples were analyzed for dry mass, percentages of lipids, and residual glucose as described below. The lipid concentration of yeast broth was determined from a standard curve obtained by plotting absorbance against the corresponding lipid concentration determined by the conventional method of acid hydrolysis followed by solvent extraction and gravimetric estimation. Forty mg samples were extracted with 3 ml of chloroform/methanol (1/2, v/v) by vortexing (1 min) and centrifugating at 2057g for 15 min at room temperature. The supernatants were collected and residues reextracted twice with 2 ml of chloroform/ methanol (1/1, v/v) by centrifugation as stated above. All the supernatants were pooled together, filtered with Whatman filter No. 1 (Whatman, USA), and washed with 2 ml of Milli-Q water, followed by centrifugation at 2057g for 5 min. The lower organic phases were collected and evaporated to dryness under nitrogen and total lipid contents were determined gravimetrically. To confirm lipid accumulating capacity, the Nile Red staining were also conducted following Sitepu et al. (2012).

Determination of lipid content

Total lipid was determined following the method of Xue et al. (2010). GC-17-A gas chromatography (Shimadzu, Japan) was used for raw lipid and biodiesel analysis. The GC was equipped with a DB-1ht capillary column (30 m × 0.25 mm; J&W Scientific, USA) and a flame ionizing detector (FID). The temperatures of the injector and detector were set at 350 and 360°C, respectively. The profile of the column temperature was as follows: raised from 100 to 180°C at 15°C/min, raised to 230°C at 10°C/min, and finally raised to 330°C at 20°C/min and maintained for 5 min. Helium was used as the carrier gas. Heptadecanoic acid methyl ester purchased from Sigma was used as an internal standard (Nie et al. 2006).

Determination of yeast dry mass

Portions of 2-mL cultures were harvested by centrifugation at 5000 × g for 5 min. Harvested biomass was washed twice with 5 mL of distilled water and then dried at 60°C to constant mass. The biomass was determined gravimetrically.

Lipid composition analysis

The total lipid concentration was determined by gas chromatographic analysis of the total fatty acids directly transmethylesterified from dried cell (Kumon et al. 2002). One mL of 10% methanolic HCl and 0.5 ml methylene chloride were added to the dried biomass and placed at 60°C for 3 h for direct methylesterification. The reaction was stopped by the addition of 2 ml saturated NaCl solution and 1 ml hexane. The resultant methyl esters recovered in the hexane layer were then applied to a gas chromatograph (GCMS-QP 2010-Ultra; Shimadzu, Kyoto, Japan) equipped with a FAMEWAX capillary column (30 m×0.25 mm i.d., GL Science, Tokyo, Japan) under temperature programming (150–250°C at 5 °C/min increments). Peanut oil (Nacalai Tesque, Kyoto, Japan) was transmethylesterified and used as the reference material.

Results

Our study proved that *P. betle* and *P. nigrum* is also a good source for oleaginous yeasts. Numerous yeast species were isolated from various plant parts of *P. betle* and *P. nigrum* collected from Bali (Table 1) and from leaf and flower of *P. betle* collected from West Java (Table 2) and the list of taxa isolated (Table 3).

Fig. 1 – Morphology of *Piper betle*. A and *Piper nigrum*. B used as microbial sources, obtained from Bali and West Java, Indonesia

Table 1 Number of genera, species and strains of oleaginous yeasts isolated from *Piper nigrum* and *P. betle* collected from Bali

Sources	*Piper nigrum*			*Piper betle*		
	No. of Genera	**No. of Species**	**No. of Strains**	**No. of Genera**	**No. of Species**	**No. of Strains**
Flower	-	-	-	-	-	-
Fruit	2	2	6	-	-	-
Leaf	3	4	4	6	11	28
Stem	2	2	4	3	4	5
Total Number	7	8	14	9	15	33

Table 2 Number of species and strains of oleaginous yeasts isolated from *Piper betle* collected from West Java

Sources	*Piper betle*		
	No. of Genera	**No. of Species**	**No. of Strains**
Leaf	7	13	21
Flower	2	3	3

The isolation was immediately followed by Sudan Black staining techniques to rapidly identify potential oleaginous yeast (Thakur et al. 1989), and sensitive intracellular lipid body quantification through determination of fluorescent intensity of accumulated lipid bodies, tryacylglycerol, using Nile Red (Kimura et al. 2004; Sitepu et al. 2012)

A total number of 71 strains were analyzed for lipid content. Out of 71 isolates analyzed, 34 of them had the ability to accumulate lipids greater than 20 % (w/w) of dry cell weight. Some of them could even accumulate more than 40%, as illustrated on Fig. 2. The accumulation of lipid varies among the genera and among strains in the species group. The range of lipid accumulation is from 5.4% (*Rhodosporidium paludigenum* InaCC Y-249) to over 42.80 % (*Cryptococcus luteolus* InaCC Y-265). Lipid production is clearly growth associated. The total biomass produced (g/L fermentation broth) varies widely from 2.5 g/L (*Debaryomyces subglobosus* InaCC Y-297) to 10.9 g/L (*Candida oleophila* InaCC Y-306).

Table 3 List of genera, species and Indonesian Culture Collection (InaCC) strain number of oleaginous yeasts isolated from *Piper nigrum* and *P. betle* of Bali and West Java, Indonesia

Genera	Species	Strains No. Code
Aureobasidium	*Aureobasidium pullulans*	InaCC Y-263
Bullera	*Bullera coprosmaensis*	InaCC Y-266
Candida	*Candida azyma*	InaCC Y-296
	Candida oleophila	InaCC Y-306
	Candida orthopsilosis	InaCC Y-302
	Candida parapsilosis	InaCC Y-303
	Candida saopaulonensis	InaCC Y-304 , InaCC Y-305
	Candida quercitrusa	InaCC Y-292; InaCC Y-294; InaCC Y-298; InaCC Y-257
Cryptococcus	*Cryptococcus flavescens*	InaCC Y-261; InaCC Y-262; InaCC Y-264
	Cryptococcus laurentii	InaCC Y-278
	Cryptococcus luteolus	InaCC Y-265
	Cryptococcus rajasthanensis	InaCC Y-248
	Cryptococcus taeanensis	InaCC Y-268; InaCC Y-289
		InaCC Y-275; InaCC Y-281; InaCC Y-291; InaCC Y-286 ;
	Cryptococcus sp.	InaCC Y-287
Debaryomyces	*Debaryomyces hansenii*	InaCC Y-293; InaCC Y-295; InaCC Y-301
	Debaryomyces subglobosus	InaCC Y-297; InaCC Y-299; InaCC Y-256
	Debaryomyces fabryi	InaCC Y-300
Pseudozyma	*Pseudozyma aphidis*	InaCC Y-273; InaCC Y-288
	Pseudozyma hubeiensis	InaCC Y-277; InaCC Y-274; InaCC Y-282; InaCC Y-240; InaCC Y-283; InaCC Y-270
	Pseudozyma rugulosa	InaCC Y-267; InaCC Y-269 ; InaCC Y-290
Rhodosporidium	*Rhodosporidium paludigenum*	InaCC Y-231; InaCC Y-235; InaCC Y-236; InaCC Y-249; InaCC Y-234; InaCC Y-247; InaCC Y-246; InaCC Y-280; InaCC Y-238; InaCC Y-242; InaCC Y-245; InaCC Y-252; InaCC Y-254; InaCC Y-259
Rhodotorula	*Rhodotorula sp.*	InaCC Y-279
Sporidiobolus	*Sporidiobolus ruineniae*	InaCC Y-232; InaCC Y-233; InaCC Y-237; InaCC Y-250; InaCC Y-239; InaCC Y-241 ; InaCC Y-243; InaCC Y-244; InaCC Y-251; InaCC Y-253; InaCC Y-255
Sporobolomyces	*Sporobolomyces poonsookiae*	InaCC Y-258
Wickerhamomyces	*Wickerhamomyces anomalus*	InaCC Y-260

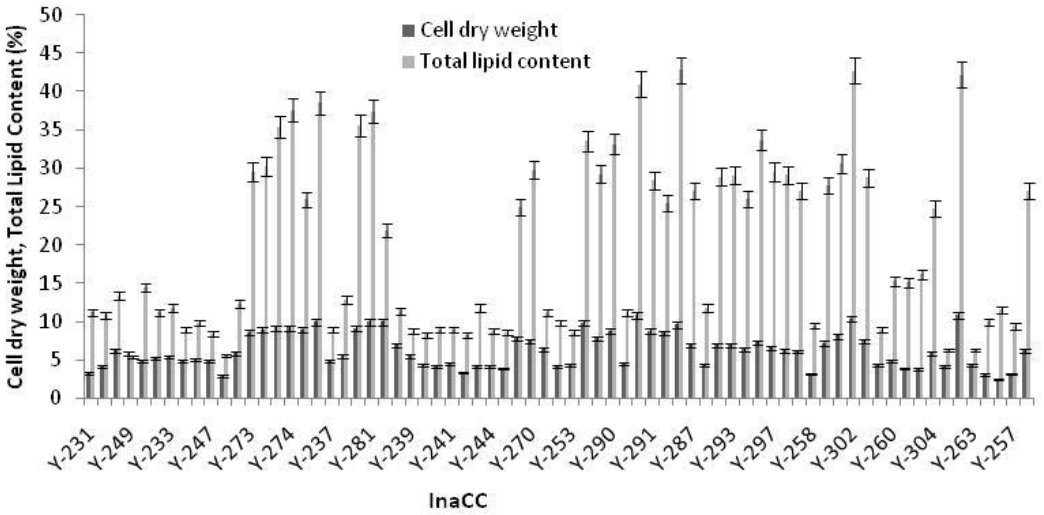

Fig. 2 – The accumulation of lipid (% , w/w; g of lipid per dry 100 g of cell biomass) and the production of cell biomass (dry weight in g/L of cultural broth) by 71 strains of oleaginous yeasts isolated from *Piper nigrum* and *P. betle* obtain from Bali and West Java, Indonesia

Production of biomass and the accumulation of lipid were diverse among species of the same genus and even strains of the same species. The range of biomass production and lipid accumulation for *Debaryomyces* are illustrated in Fig. 3, while the range of biomass production and accumulation of lipid for *Rhodosporidium* is illustrated in Fig. 4, for *Sporodiobolus* in Fig. 5, and for *Pseudozyma* is in Fig. 6.

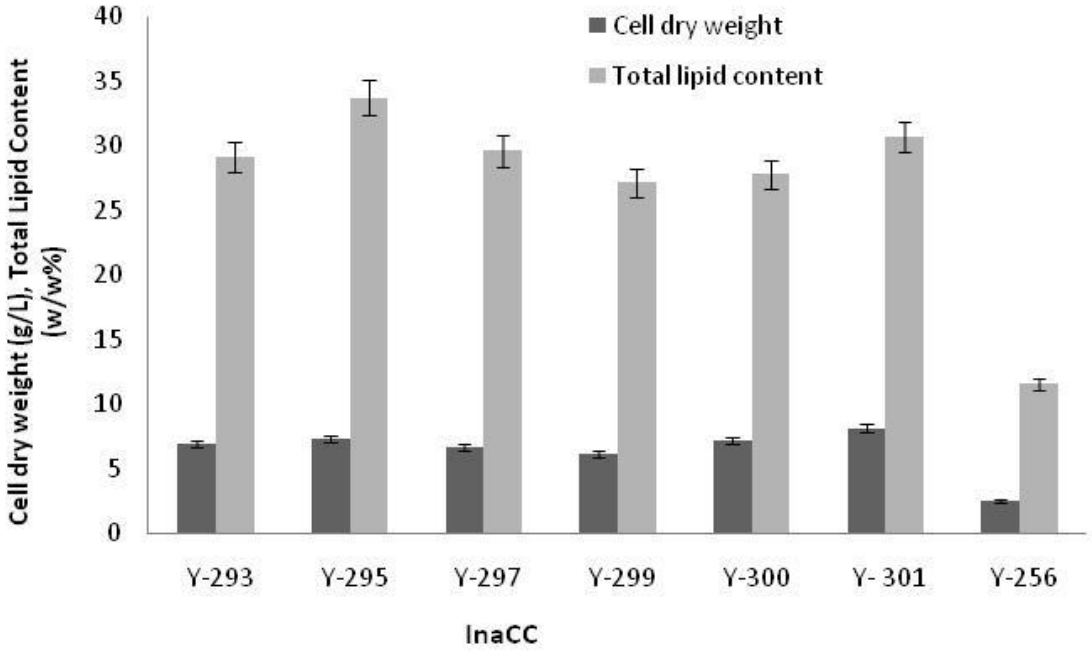

Fig. 3 – The accumulation of biomass and lipid by strains of the genus *Debaryomyces*

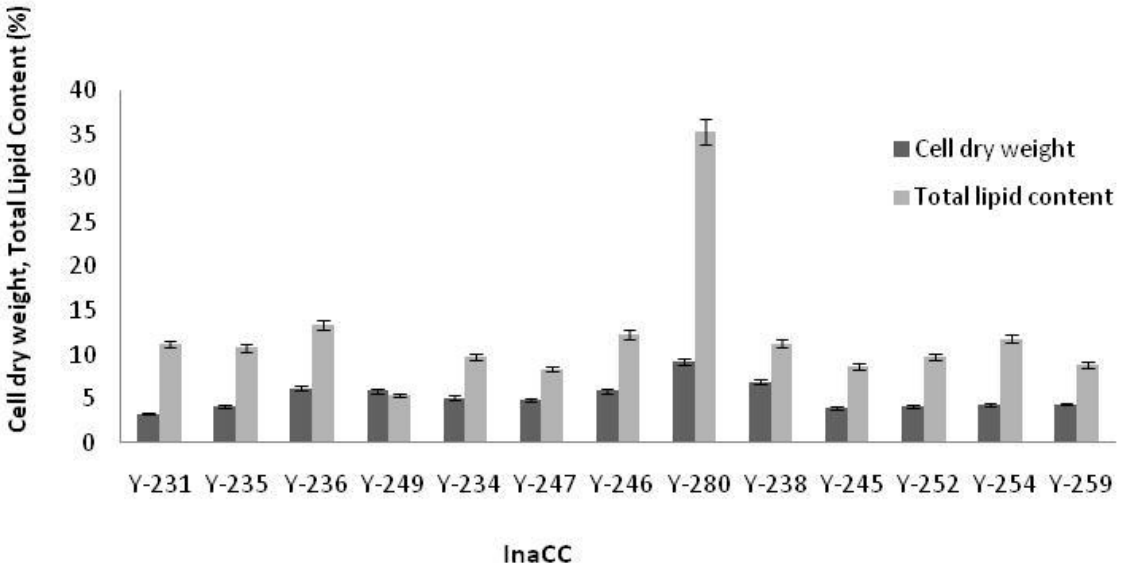

Fig. 4 – The accumulation of biomass and lipid by strains within the genus *Rhodosporidium*

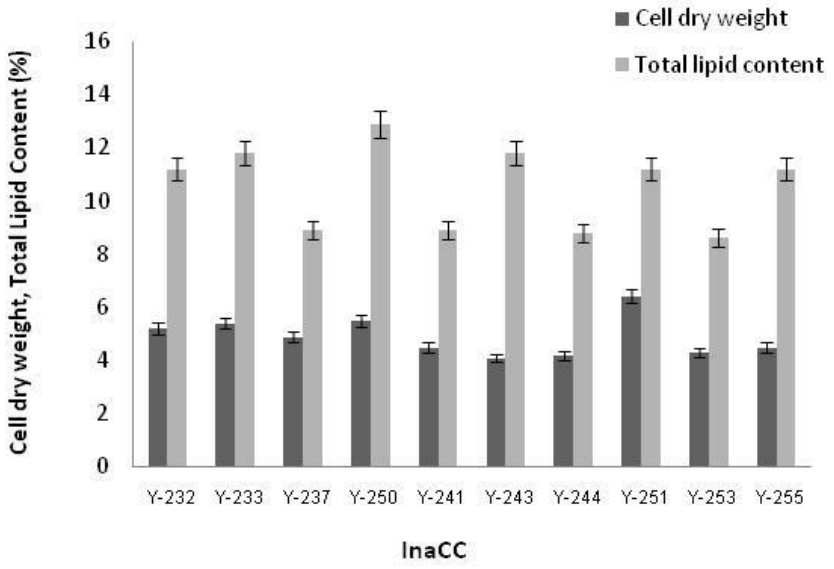

Fig. 5 – The accumulation of biomass and lipid by strains within the genus *Sporodiobolus*

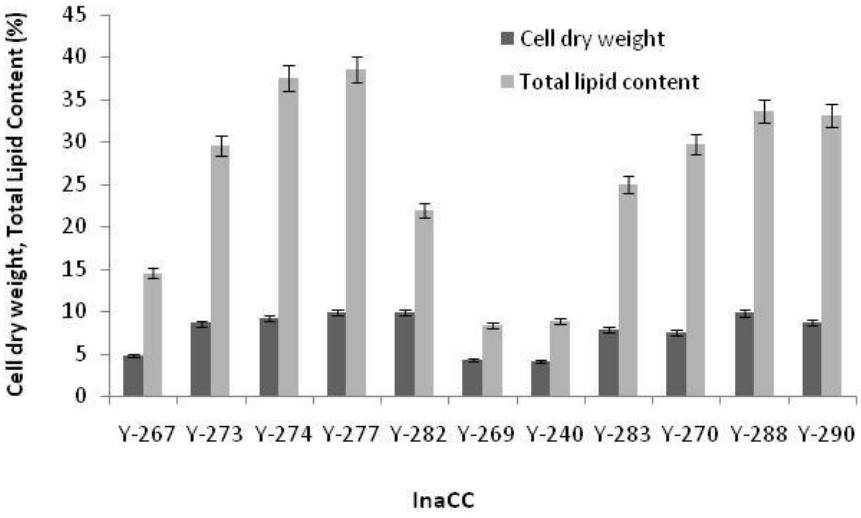

Fig. 6 – The accumulation of biomass and lipid by strains within the genus *Pseudozyma*

The lipid content of strains of the same species or genus was quite variable. Two out of ten strains of *Candida* were able to accumulate lipid more than 40% of dry cell weight, namely *Candida azyma* InaCC Y-296 and *Candida oleophila* InaCC Y- 306. The remaining eight strains were poorer at lipid accumulation. Among 13 strains of *Cryptococcus* only one strain, *Cryptococcus luteolus* InaCC Y-265 could accumulate more than 40% lipid. The remaining 12 strains accumulated lipid from 8.9 to 37.38%. Six out of seven *Debaryomyces* strains were able to accumulate lipid between 27 and 33%. The lipid accumulation by *Pseudozyma* are similar to *Candida* and *Cryptococcus*. Meanwhile, *Rhodosporidium paludigenum* InaCC Y-280 has the ability to produce 35% lipid, but the remaining strains have low capability in accumulating lipid. No strain of *Sporidiobolus ruineniae* was a good lipid accumulator (Fig. 5).

GC-MS analysis showed that most of the lipid is composed of C 18:1 and C 18:0 (Table 4), indicating that *Bullera coprosmaensis* InaCC Y-266, *Cryptococcus luteolus* InaCC Y-265, *Candida orthopsilosis* InaCC Y-302 and *Candida oleophila* InaCC Y-306 are good candidates for biofuel development.

Table 4 Lipid composition of selected oleaginous yeasts

Strains code	Total lipid (%)	Dry weight (g/L)	Relative fatty acid composition (%, wt/wt)								
			14:0	16:0 Palmitic	16:1	18:0 Stearic	18:1 Oleic	18:2	18:3	20:0	22:0
InaCC Y-302	42.78	5.8	1.3	18.9	6.9	17.2	38.4	11.3	1.6	1.9	1.2
InaCC Y-306	42.23	5.9	1.1	16.9	7.2	24.3	41.6	6.9	1.1	1.1	1.3
InaCC Y-265	42.80	5.8	1.1	16.9	7.2	24.3	38.5	6.9	1.1	1.1	1.3
InaCC Y-266	40.98	5.7	-	14.8	6.9	19.8	47.4	5.8	1.3	1.2	1.3

*The lipid content was measured after 72 hours cell cultivation

Discussion

We report here our preliminary work on isolation and identification of yeast associated with *P. betle* and *P. nigrum* (Fig.1). A number of microbial strains grow in association with *P. nigrum* and *P. betle*. Those two plants species are known as important medicinal plants. The plants are rich in phenolic substances and traditionally used widely in Asia. Aravind et al. (2009, 2010) demonstrated that diverse bacterial species are associated with *P. betle* and *P. nigrum*, some of which produce valuable bioactives. Our study clearly indicates that several physiologically and economically important strains of yeast could also be isolated from phenolic rich plant species including *P. bettle* and *P. nigrum*.

A small number of yeast species have been the subject of study for oil production, most notably *Yarrowia lipolytica* (reviewed in Beopoulos et al. 2008), which is the subject of numerous patents and whose genome has been sequenced. Yeasts have been intensively explored for their potential in conversion of lignocellulosic hydrolysates to oil for biodiesel (Meng et al. 2009).

Overall, this study indicates that plant associated microbes are an important genetic resource for future biofuel development. Furthermore, many species, and many strains per species, must be examined to identify strains with desirable properties such as lipid accumulation and biomass production.

Oleaginous yeast strains have been obtained from diverse habitats including soil, leaf litter, fish gill, and microalgae (Starkey 1946, Li et al. 2007, Pan et al. 2009, Kitcha & Cheirsilp 2011, reviewed in Meng et al. 2009).

Rhodosporidium is quite ubiquitous, having been isolated from soil, herbaceous plants, silage, sea water, wood, woodchips, leaf, pine cone, plant litter, and air (Hamamoto et al. 2002). Li et al. (2007), studied oleaginous yeast *Rhodosporidium toruloides* Y4, using fed-batch culture cultivation after 25 days, obtained a biomass yield of 151.5 g l^{-1} and lipid content of 48% (w/w). When grown in pilot-scale fed-batch cultures in a 15-l stirred-tank fermentor for 134 h the biomass obtained was even higher (106.5 g l^{-1}) and lipid production of 0.54 g $l^{-1}.h^{-1}$. Important process parameters were culture media manipulation, and nutrient feeding mode.

Strains of species in the genus *Sporidiobolus* have also been isolated from leaf of *Malpigea coccigera*, garden soil, dead leaf *Mangifera indica*, leaf of lemon and basidiocarps of *Myxarium nucleatum* (Valerio et al. 2002). Libkind et al. (2008) evaluated the potential of strains within the family *Sporidiobolales* to produce fatty acids. They found that while the total fatty acid content of their strains was not in the oleaginous range, certain yeasts did produce valuable metabolites including omega-3 fatty acids. Furthermore, altering the growth medium greatly affected the relative abundance of fatty acids. Further investigation of our yeasts is needed to determine if they also share these valuable properties. The red yeasts, especially members of genus *Rhodotorula*, have been a target to study the metabolism of fatty acids synthesis, and studies of the link between taxonomic position, lipid accumulation properties, and environmental source (Libkind et al. 2008).

The strain *Pseudozyma hubeiensis* InaCC Y-277 accumulated 38.57 % lipid by cell dry weight. This genus is known for industrial application such as production of itaconic acid (US Patent 7,479,381), biocontrol of plant pathogens (WO Patent WO/2011/151,819), and oil

production (US Patent App. 13/224,326). Morita et al. (2011) recently published a description of a novel yeast, *Pseudozyma churashimaensis*, isolated from sugarcane in Okinawa, Japan. This novel strain produced novel substances including glycolipid biosurfactants, a mixture of mannosylerythritol lipids (MELs), and tri-acetylated derivative (MEL-A2).

Cryptococcus is a polyphyletic genus, with species residing in five orders within Basidiomycota. Oleaginous *Cryptococcus* species are known within three of these orders. We isolated several strains of *Cryptococcus* that accumulated high levels of intracellular lipid. Strain *Cryptococcus luteolus* InaCC Y-248 accumulated 42.80 % lipid (w/w) with total biomass of 9.6 g (dry weight) per L of culture. The lipid accumulating capability of *Cryptococcus*, especially *Cryptococcus curvatus* O3 is well documented (Zhang et al. 2011). Using glucose and varying nitrogen sources, grown in shake flasks at 30°C, this strain was able to achieved 51.8 kg m3, with lipid content of about 651 g/kg. When the yeast was grown in a 30 L stirred fermentor for 185 h, it produced a cellular biomass containing lipid with lipid productivity of 104.1 kg m^3. Lipid composition was mainly palmitic acid, stearic acid, oleic acid, and linoleic acid, and hence *Cryptococcus curvatus* O3 was proposed as oleaginous yeast for biofuel feedstock.

Seven strains within the species *Debaryomyces* were isolated. Most species within this genus efficiently assimilate glycerol (Kurztman & Fell 2011), a by product of biodiesel production. Utilization of this by product is a desirable trait for biodiesel production.

Processes have been developed to utilize yeasts for conversion of wastewater to value-added products (Chen et al. 2012). The well known oleaginous yeasts *Lipomyces starkeyi* (Angerbauer et al. 2008), *Cryptococcus curvatus* (Chi et al. 2011), and *Rhodotorula glutinis* (Xue et al. 2008) are examples of this. Several organic substances such as volatile fatty acids were converted to phenolic compounds by the oleaginous yeast *Cryptococcus albidus* (Fei et al. 2011). The ability of several yeasts such as *Trichosporon coremiiforme* to assimilate volatile organic substances could be an important trait for treatment of wastewater containing alcohols (Xue et al. 2012), which implies that exploration of yeasts from several biotopes is worthwhile.

Microbial culture conditions affect lipid composition, and amount of lipid accumulated inside the cell. Culture conditions can be manipulated for optimization of yeast cell biomass production and lipid accumulation (Chi et al. 2011, Wu et al. 2010, Jin et al. 2012, Li et al. 2007). The information on fatty acid synthesis pathways and effects of culture conditions on lipid accumulation have been generated primarily from intensive studies of two oleaginous yeast species: *Yarrowia lipolytica* (reviewed in Beopoulos et al. 2009) and *Rhodotorula glutinis* (Xue et al. 2008).

Storage lipids are located primarly in lipid bodies (Nielsen 2009). The amount of accumulated lipid is affected by multiple factors include carbon source, C/N, C/P, and C/S ratios (Wu et al. 2011) and other abiotic factor such as dissolved oxygen, temperature, growth stage, and genetic characters (Meng et al. 2009), and we observed *Cryptococcus luteolus* InaCC Y-265, *Candida orthopsilosis* InaCC Y-302, and *Candida oleophila* InaCC Y-306 accumulated high lipid when grown aerobically at 30ºC after 5 days cultivation.

The fatty acids observed (Table 4) were primarily palmitic acid (C18:1), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2) The fatty acid profiles suggest that these yeasts may be good candidates for biodiesel production, as they are similar to the fatty acids of plant oils currently used for biodiesel (Meng et al. 2007).

There are four foci of microbial lipid research: finding novel oleaginous microbes with high TAG accumulating capacity, obtaining lignocellulosic based microbial biodiesel (Yu et al. 2011, Gong et al. 2012), microbial mediated transesterification (Lai et al. 2005), and searching for novel lipid species with pharmaceutical activities. Discovery of novel oleaginous yeasts will further enhance our knowledge in these areas. Our observations indicate that additional yeast species including *Bullera coprosmaensis* and *Pseudozyma hubeiensisis* are also promising for future biofuel industry. Discovery of these new oleaginous species is an important step in developing new knowledge in these areas.

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