Selection of Oleaginous Yeasts with Lipid Accumulation by the Measurement of Sudan Black B for Benefits of Biodiesel

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***Abstract*—Microbial lipids possess significant potential which can be explored for biodiesel production. This study attempted to screened oleaginous yeasts and selected the strain that accumulated the largest quantity of lipid for lipid production from glucose. Among the 24 yeast strains isolated from canteen wastewater, 10 oleaginous yeasts were selected by Sudan Black B test. Sudan Black B staining revealed the presence of lipid inclusion bodies in the cells during cultivation under nitrogen-limiting conditions. Total oleaginous strains that accumulated quantities of lipid higher than 20% of their biomass when cultivated in glucose. It was found that accumulated the CTWY07 highest lipid content, up to 71.59% of its biomass (4.904 g/L lipid and 6.85 g/L biomass), while CTWY17 grew the fastest by shaking flask cultivation in glucose. The results show that the isolated yeasts could be promising candidates for biodiesel production.**

***Index Terms*—biodiesel, lipid accumulation, oleaginous, screening, single cell oil, Sudan Black B**

I. Introduction

Concern for the increase in energy demand and the depletion of fossil fuel reserves has resulted in a rapid rise in crude oil prices, and therefore, securing alternative sources of energy is urgently required. One of the most promising renewable energy resources is biodiesel, which is produced from renewable biomasses by transesterification of triacylglyceols, yielding monoalkyl esters of long-chain fatty acids with short-chain alcohols, for example, fatty acid methyl esters and fatty acid ethyl esters [1]. Biodiesel, with the properties of low pollution, high fuel value which is safer than fossil diesel, is becoming the most promising alternative energy source for crude oil. And the research of biodiesel is also becoming a key direction in energy research [2]. Biodiesel is usually produced from oleaginous crops, such as rapeseed, soybean, sunflower and palm, through a chemical transesterification process of their oils with short chain alcohols, mainly methanol. Besides addressing environmental concerns related to greenhouse gasses, biodiesel offers new income to farmers. However, traditional oil-rich crops are limited by land availability, as well as environmental and social issues regarding the use of feed and food crops for fuel. In comparison to other vegetable oils and animal fats, the production of microbial oil has many advantages: microbes have a short life cycle as compared to plants so the time to harvest is shorter, there is less labor required, microbial oil production is less affected by venue, season and climate, and scale-up is easier. Oleaginous microorganisms are defined as oleaginous species with oil contents excess of 20% of biomass weight. Microbial oils, also called Single Cell Oils (SCO), are produced by some oleaginous microorganisms, such yeast, fungi, bacteria and microalgae. While the eukaryotic yeast, fungi and microalgae can synthetize triglycerol (TAG), which are similar with the composition of vegetable oils, prokaryotic bacteria can synthetize specific lipids [3]. Yeasts can grow on a variety of substrates, even inexpensive materials such as wastes of agriculture and industry, and thus oleaginous yeast strains that can efficiently produce lipid from low-cost raw materials are of great interest. Biolipids, including triacylglycerol produced by oleaginous yeast, have been confirmed to be one of the most important raw materials for biodiesel production. The quality of biodiesel depens upon the fatty acid composition of the biolipids [4]. In general, biolipids produced by oleaginous yeast are suitable feedstock for biodiesel, because the fatty acid composition satisfies important criteria i.e., chain length and saturation degree. However, the fatty acid composition of biolipids is strain specific, it is therefore important to select oleaginous yeast strains to ascertain their suitability for biodiesel production [5]. Among the oleaginous yeasts, *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Trichosporon* and *Lipomyces* have been studied for their microbial oil properties. The oil accumulated by the yeasts are in the form of triacylglycerols that are predominantly oleic (18:1), linoleic (18:2), stearic (18:0), palmitic (16:0) or palmitoleic (16:1) [6]. In this study an attempt has been made to screening of lipid accumulated yeast strains from glucose as a carbon sources.

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II. Materials and Methods

A. Yeast Strains

Total 26 yeast strains were isolated from canteen wastewater (CTWY) in Rajabhat Maha Sarakham

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University, Maha Sarakham Province, Thailand, in June-August 2015. The strains were stored on Malt extract-Glucose-Yeast extract-Peptone (MGYP) agar slants (containing g/L : glucose, 10; peptone, 5; yeast extract, 5; malt extract, 3; KH_2PO_4, 1 and $MgSO_4.7H_2O$, 0.5) at 4°C for futher study.

B. Sudan Black B Staining for Oleaginous Yeasts

Yeast culture grown till early stationary phase from the slant were stained using original Sudan Black B staining protocol and the modified protocol [7]. Heat fix the smeared on a microscopic slide and air dry, flood the smear with Sudan Black B stain, keep for 15 minutes till the stain turns greenish blue. Wash the slide remove the stain and counter stain with 0.5% saffranin for 30 seconds, wash, dry and observe under microscope.

C. Extraction of Lipids and Biomass from Isolated Strains

The yeast strains *which* showed positive response with Sudan Black B staining, were cultivated in Erlenmeyer flasks, containing 30 ml of inoculation media (MGYP) and incubated in an incubator shaker, at 150 rpm and 28±2°C for 24 hours. After 24 hours, initial cell concentration determined by optical density (OD) at 600 nm, 5 ml of culture was transferred to 45 ml of nitrogen limited fermentation media (containing (in g/l)*:* glucose, 40; $(NH_4)_2SO_4$, 2; KH_2PO_4, 7; NaH_2PO_4, 2; $MgSO_4.7H_2O$, 1.5 and Yeast extract, 1) in 250 ml Erlenmeyer flask on a rotary shaker (Lab Companion IS-971R, Korea) at 150 rpm and incubated at 28±2°C for 120 hours. All media were sterilized by autoclaving for 15 min at 121°C. 50 ml of this culture was harvested by centrifugation at 5,000g for 5 minutes, and dried at 60°C to constant mass. The biomass harvested biomass was washed twice with water was then determined gravimetrically. Followingly, 10 ml of 4M HCl was added and incubated at 60°C for 2 hours. The acid hydrolysed mass was stirred with 20 ml of hexane: methanol mixture (1:1) at room temperature for 3 hours followed by centrifugation at 2,000g for 5 minutes at room temperature to separate organic upper phase and aqueous lower phase [8]. All experiments were performed in triplicate. Followingly, upper phase containing lipids were recovered and evaporated using nitrogen gas and dry lipids were weighed. The yield of extracted material was determined and expressed as grams of extractable lipid per gram of dry solid.

III. Results and Discussion

A. Screening and Characterization of the Isolates for Lipid Accumulation

The lipid accumulation capacity of the isolated colonies was revealed by Sudan Black B staining of the cultures grown in MGYP medium. The yeast strain were stained with Sudan Black B, the oleaginous cells show lipid bodies stained blue with the pink cytoplasmic background (lipids appearing as dark spots within lighter cytoplasm). Whereas, the non-oleaginous cells are stained completely pink (uniformly stained) as indicated in Fig.1.

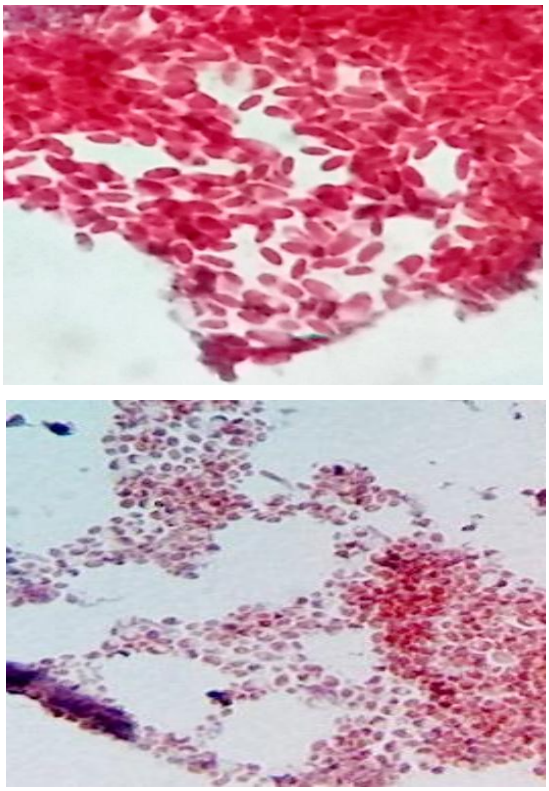


Figure 1. Cells stained by original Sudan Black B technique stained lipid matter surrounding the cytoplasmic membrane of non-oleaginous (A) and oleaginous yeasts (B) observed with microscope.

The method of current isolation of oil fungi is Sudan Black B stain, which is cumbersome and time-consuming, and the result is greatly influenced by the staining. This method of isolating fat microorganism gives less false positive results, when compared with the results got form Sudan Black B dyeing separation. Moreover, it is faster and simpler [9], [10]. The oil accumulationg yeasts of the 10 strains is shown of Morphologcal characteristics in Table I.

B. Oil Extraction

The lipid-accumulating ability of the yeast strains is expressed as lipid content after 5 days of cultivation (Table II). The current research of oil production is mainly concentrated on the high concentration of glucose as carbon source. The best lipid concentration of strains CTWY19, CTWY24, CTWY02, CTWY07 and CTWY01 were 7.50, 6.06, 5.00, 4.90 and 4.54. The highest biomass strains were CTWY17, CTWY22, CTWY24, CTWY19 and CTWY01, which were 11.09, 10.93, 10.82, 10.47 and 8.82 g/ L, respectively, Therefore, stain CTWY, CTWY and CTWY can utilize glucose as carbon source to produce oil well. In particular, CTWY showed the highest lipid content at 37.88-71.59%.

TABLE I. MORPHOLOGICAL AND LIPID PRODUCTION BY VARIOUS MICROORGANISMS

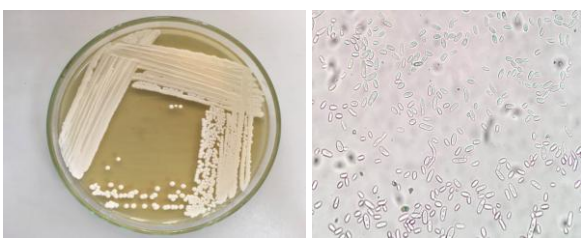
Strains	Sources	Colony color	Colony Margin	Colony Elevation	Colony Surface	Cell shape
CTWY01	Canteen Wastewater	Cream	Entire	Raised	Smooth	Oval
CTWY02	Canteen Wastewater	White	Undulate	Raised	Rough	Cylindrical-rod
CTWY05	Canteen Wastewater	White	Undulate	Umbonate	Smooth	Cylindrical
CTWY06	Canteen Wastewater	Cream	Entire	Raised	Smooth	Ellipsoidal
CTWY07	Canteen Wastewater	White	Undulate	Umbonate	Rough	Cylindrical
CTWY17	Canteen Wastewater	Salmon Red	Entire	Raised	Smooth	Oval
CTWY19	Canteen Wastewater	White	Entire	Convex	Smooth	Oval
CTWY22	Canteen Wastewater	Salmon Red	Entire	Pulvinate	Smooth	Oval
CTWY23	Canteen Wastewater	Salmon Red	Entire	Pulvinate	Smooth	Oval
CTWY24	Canteen Wastewater	Orange Red	Entire	Pulvinate	Smooth	Oval

TABLE II. THE BIOMASS, LIPID AND LIPID CONTENT OF 10 OLEAGINOUS YEAST STRAINS

Strains	Biomass (g/l)	Lipid concentration (g/l)	Lipid content (%)
CTWY01	8.82	4.54	51.47
CTWY02	7.72	5.00	64.74
CTWY05	7.00	4.38	63.55
CTWY06	6.99	4.31	61.65
CTWY07	6.85	4.90	71.59
CTWY17	11.09	3.80	34.40
CTWY19	10.47	7.50	71.44
CTWY22	10.93	4.14	37.88
CTWY23	8.40	4.23	50.36
CTWY24	10.82	6.06	55.97

By applying Sudan Black B tests, 10 strains were identified as potential lipid biomass producer (Table I). The result showed that two oleaginous strains (CTWY07 and CTWY19) accumulated the highest lipid content were 71.59 and 71.44% of their dry biomass (morphology and cell shape in Fig. 2). This amount of lipid content (71.59 and 71.44%) were greater than those taken different yeasts in other studies. For example, it had been reported that the oleaginous yeast *R. toruloides* Y4 in the flask culture reached a dry biomass of 151.5 g/l, however, the final cellular lipid content was only 48.0%[11]. In addition, Yumauchi and colleagues [12] obtained a high cell density of 153 g/l and a lipid content of 54% with *L. starkeyi* using a complicated feeding medium. When the culture medium lacks the nitrogen source, the isocitric dehydrogenase (ICDH) was suppresses, therefore the tricarboxylic acid circulation (TCA) was blocked. Extra carbon source was transformed to triglyceride (TAG) by a series of enzymes like the citric acid lytic enzyme, the malic acid enzyme, the fatty acid enzyme, thus completed the fat accumulation. In this article. Fat microorganisms were screen by the principle of high fat contents and fast growth on nitrogen limited culture medium [2].

(A)



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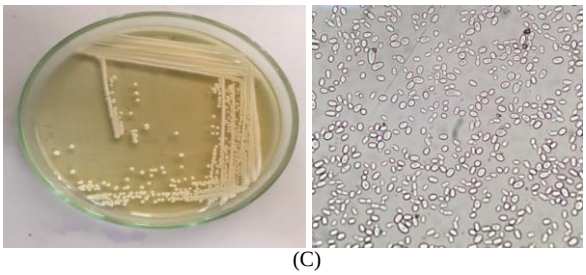
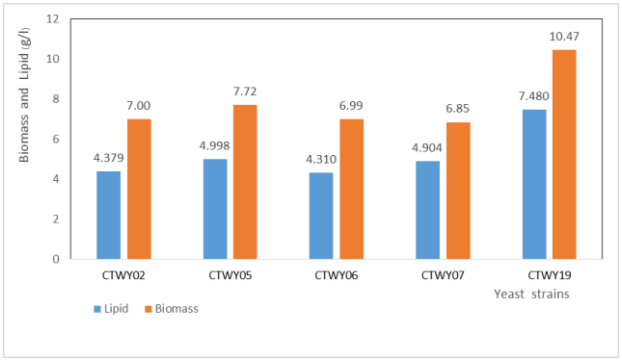
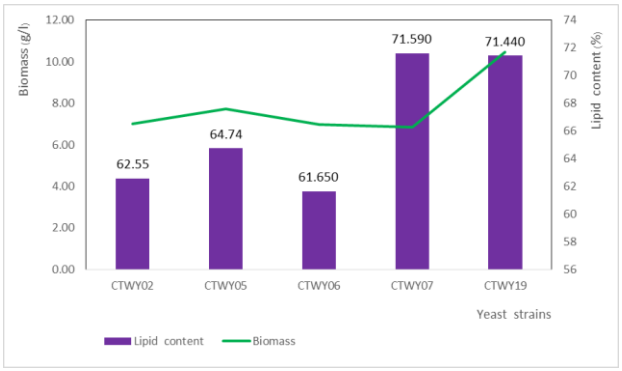


(C)

Figure 2. The colony morphology on YPD agar plate and cell shape under microscope 400X (A) CTWY02 (B) CTWY07 and (C) CTWY19.



(A)



(B)

Figure 3. The biomass and Lipid (A) and Biomass and Lipid content (B) of the five oleaginous yeast strains cultivated in glucose by shaking flask cultivation at 150 rpm and room temperature for 120 h. The data represented averaged of three replicates.

Biomass, lipid concentration and lipid content are important indicators to evaluation of microbial oils. The result indicate that 10 strains the oil content of which was more than 20%, and five strains that accumulated the high lipid levels of 61.65-71.59% of their dry biomasses (Figure 3).Lipid accumulation in oleaginous yeasts and fungi has been studied mainly with the aim to produce importance as potential source of triacylglyceride (TAG), poly-unsaturated fatty acids (PUFA) and as biodiesel precursors. In general, yeasts and molds can accumulate more lipids over bacteria and microalgae mainly due to rapid growth rate and therefore, have potential as commercial sources of oils. The accumulated lipids in yeasts get deposited as intracellular lipid bodies (LBs) [13].

IV. Conclusion

Our results show that the efficiency of lipid recovery from extraction techniques. We found both species that already been reported as oleaginous yeasts. Evaluation of lipid production from glucose of the oleaginous yeast strains revealed that CTWY07 and CTWY19 were 71.59 and 71.44% of its dry biomass, respectively. These yeast species has the ability to utilize glucose and nitrogen compounds and to grow under stressful conditions. It was found that some of those impurities could even increase biomass concentration and lipid accumulation. Therefore, it is a promising oleaginous yeast candidate for production of biodiesel production.Acknowledgment

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