

LIPIDS ACCUMULATION BY OLEAGINOUS YEASTS USING MOLASSES MEDIUM AS BIODIESEL PRODUCTION

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Abstract

Oleaginous yeasts show promise as a feedstock to satisfy the current demand for biodiesel production. They can utilize a large number of cost-effective renewable substrates for growth and lipid accumulation. The lipid production potential of eight isolated oleaginous yeasts was investigated under molasses medium (12%w/v molasses concentration) with pH 5.5 at 30°C for 144 h *Rhodotorula mucilaginosa* BJMK43 gave highest lipid content was determined as 65.48%w/v. Main cellular fatty acids of the yeast were oleic, palmitic, linoleic and stearic. Yeast lipids are a promising feedstock for biodiesel production due to their high content of C16 and C18 fatty acids. Lipid composition was found to be similar to that of vegetable oils. Results demonstrated the feasibility of simultaneous biovalorization of volatile fatty acids and glycerol, as cheap industrial by-products.

Keywords: Biodiesel, lipid, molasses, oleaginous yeast

Introduction

Burning fossil fuels, including petroleum, coal and natural gas, increases CO₂ levels in the atmosphere and leads to global pollution. Nowadays, interest is focused on alternative energy which is clean, renewable and biodegradable, to reduce greenhouse gases and CO₂, especially biodiesel (Karatay and Donmez, 2010). Biodiesel fuel as fatty acid mono alkyl (methyl, propyl or ethyl) is the most sustainable and renewable alternative to fossil diesel fuel (Patel *et al*, 2017). Animal fat, vegetable oils, and microorganisms can produce lipids. Lipids produced by microorganisms such as microalgae,

Yeast, bacteria and fungi found as single-cell oils that can replace plant oils and show 80-90% of triacylglyceride capacity for biodiesel production (Bagy *et al*, 2014). Some oleaginous microorganism have been reported to accumulate large amounts of lipid up to more than 20% of their biomass under a few parameter limitation such as nitrogen, phosphorus or iron. Microalgae, yeasts, molds and some bacteria present lipid content at up to 70% of their biomass (Fontanille *et al.*, 2011). Several oleaginous yeasts and fungi have been confirmed as important for biodiesel producers, while it is known

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that many lipids and their fatty acid components are similar to those of vegetable oils (Fontanille *et al.*, 2011; Bagy *et al.*, 2014).

Species contributing over 70% of observed lipids accumulation are considered as promising feedstock for biodiesel production and include *Candida tropicalis*, *Candida lipolytica*, *Cryptococcus curvatus*, *Lipomyces starkeyi*, *Rhodospiridium toruloides*, *Rhodotorula mucilaginosa*, *Yarrowia lipolytica* and *Trichosporon fermentans* (Karatay and Donmez, 2010; Xavier *et al.*, 2016; Patel *et al.*, 2017). Thus, biodiesel from oleaginous yeasts is considered as a potential candidate to replace fossil diesel fuels because of rapid growth rate, high oil accumulation, and use of agricultural waste to synthesize polyunsaturated fatty acid triacylglycerol (TAG) similar to vegetable oils (Bagy *et al.*, 2014; Tanimura *et al.*, 2014).

Molasses is a by-product from the sugar industry and considered as one of the most promising feedstocks in the production of biofuel. Black strap molasses is another by-product of the sugar industry, obtained during sugarcane refining and, like beet molasses, has a high sugar content, at around 50% (36% sucrose, 6% fructose, 3% glucose), 23-26% water, 9-14% minerals (Mg, Mn, Al, Fe, and Zn) and 8-12% nitrogenous compounds (amino acids, proteins, etc.), making it a promising substrate for biodiesel production. Sugarcane molasses is also an important organic waste due to its high sugar content (55%) and high volume of production. It is even more viscous than beet molasses and the total sugar content is higher. High availability and low cost of sugarcane molasses make it an attractive feedstock for use in many countries (Tanimura *et al.*, 2014).

Here, a cheaper raw material for biodiesel production was suggested using potential oleaginous yeasts. Molasses was used as a carbon and energy source in the experiments to reduce the cost of lipid production. To the best of our knowledge, this is the first report concerning the screening of oleaginous yeasts and lipid accumulating properties of oleaginous yeasts cells in media containing molasses.

Materials and Methods

Screening of Lipid-Producing Yeasts on a Molasses Medium

A total, of 45 yeast strains from the private culture collection of the Yeast Research Laboratory of the Department of Biology, Faculty of Science and Technology, Rajabhat Mahasarakham University (RMU) were used in this study. All yeast strains were reported to accumulate high lipid content under shaking flask cultivation in a nitrogen limited

medium. The strains were cultured on yeast extract-malt extract (YM) agar slants (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 20 g/L agar) and incubated at 30°C for 24-48 h. The working stock was maintained on a YM agar slant at 8°C and sub-cultured every two months.

This study focused on isolating yeasts, which were capable of growing on 12% molasses agar as well as utilizing the molasses sucrose as the carbon source. The medium was composed of molasses (8-24%), 20 g/L agar and mineral salts. The mineral salts were 3.0 g/L ammonium sulfate ((NH₄)₂ SO₄), 1.5 g/L potassium dihydrogen phosphate (KH₂PO₄), 1.0 g/L magnesium sulfate (MgSO₄), 0.3 g/L calcium chloride (CaCl₂) and 0.03 g/L ferrous sulfate (FeSO₄). The pH of the medium was adjusted to 5.0. All the Petridishes were incubated at 30°C for 72 h after inoculation (Tanimura *et al.*, 2014). Yeast cells were harvested by centrifugation at 5,000×g for 15 min, washed twice with distilled water, frozen at -80°C and freeze dried overnight to constant weight. The dry biomass was determined gravimetrically. Molasses were analyzed for their total carbohydrate concentration by the phenol/ sulfuric acid method (DuBois *et al.*, 1956) and then diluted with distilled water to an appropriate final carbohydrate concentration.

Cultivation Conditions

In previously study, 45 yeast isolates screened on 8-12% molasses medium. Eight yeast strains were grow on 12% (w/v) molasses agar used for lipid production in molasses medium. The yeast cells were grown on YM medium. Batch experiments were performed under aerobic conditions in a molasses medium using an orbital shaker at an agitation rate of 150 rpm and incubation temperature of 30°C. The composition of the growth medium was 12% (w/v) molasses solution (almost equivalent to 40 g/L sucrose), 3.0 g/L (NH₄)₂ SO₄, 1.5 g/L KH₂PO₄, 1.0 g/L MgSO₄, 0.3 g/L CaCl₂ and 0.03 g/L FeSO₄. The pH of the medium was adjusted to 5.0. All the Petri dishes were incubated at 30°C for 144 h after inoculation in shake flask (Karatay and Donmez, 2010).

Effect of Cultivation Time

The high performance of isolated strains were cultivated in 250 ml conical flasks containing 50 ml of 12% (v/v) molasses medium. Each flask was inoculated with 10% (v/v) and incubated at 30°C for 6 days in a shaker at 150 rpm. During incubation, samples were withdrawn at 120, 144, and 168 h to determine cell growth as dry cell biomass (g/l) and lipid content (%). All shaken flask experiments were carried out in triplicate.

Determination of Fatty Acids

A 50 ml sample was centrifuged at 5,000 rpm for 10 min. After that, the cells were washed once with 50 ml of distilled water. Then, 10 ml of 4M HCl was added and incubated at 60°C for 3 h. Later, the acid hydrolyzed mass was stirred with 20 ml chloroform/methanol mixture (1:1) at room temperature for 3 h. Finally, centrifugation was performed at 5,000 rpm for 3-5 min to separate the aqueous upper and organic lower phases. The lower phase containing lipid was then recovered with a Pasteur pipette and evaporated in a vacuum. After this procedure the dry lipid was weighed (Kurtzman and Robnett, 1998). The extracted lipids were converted to fatty acid methyl esters (FAMES) by reaction with methanol and analyzed by gas chromatography (GC) using the model of Hewlett Packard with HP-Innovax polyethylene glycol column (30 m × 0.25 mm ID × 0.25 µm film thickness). Helium was the carrier gas. A 1 µl portion of the sample was injected with a 1:1 split ratio. The inlet was maintained at 260°C. The column temperature profile was as follows: 60°C for 2 min; heated to 140°C at 10°C/min; further heating to 260°C at 5°C/min and held for 7 min. A flame ionization detector was used at 250°C. The fatty acids were identified and quantified by comparison with chromatograms of standard FAMES.

Fatty acid content was defined as the mass percentage of the fatty acids in cell dry weight (%CDW). Total lipid content was calculated as the sum of the fatty acids in the following six FAMES: methyl palmitate (C16:0), methyl palmitoleic (C16:1), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2), and methyl linolenic (C18:3). Lipid contents (% w/w) of the biomass were estimated as the above-quantified total lipids as percentage of the biomass dry weight used in the extraction (Enshaeieh *et al.*, 2015).

Genomic DNA Isolation, Sequencing and Identification

A pure culture with uniform colony characteristics and morphology was processed for DNA isolation. Genomic DNA was isolated using a commercial DNA isolation kit. Identification of the three highest yeast strains of lipid accumulation in cells was performed by sequencing a fragment of the genome. Primers used for the polymerase chain reaction (PCR) were the D1/D2 domain of 26S rDNA with forward primer NL-1 (5'-GCATATC AATAAGCGGAGGAAAG) and reverse primer NL-4 (5'-GGTCCGTGTTTCAAGACGG) (Taskin *et al.*, 2016). Amplification was performed for 36 PCR cycles with annealing at 52°C, extension at 72°C for 2 min, and denaturation at 94°C for 1 min

(Donnell, 1993). The amplified DNA was purified with GeneClean II (Bio 101, La Jolla, CA) according to the manufacturer's instructions. Visualization of the amplified DNA was performed following GeneClean II treatment by electrophoresis in 1.5% agarose in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]) and staining with ethidium bromide (8×10^{-5} µg/µL). Both strands of the compared rDNA regions were sequenced with the ABI TaqDyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems Inc., Foster City, CA). The phylogenetic tree was constructed using neighbor-joining method by CLC sequence viewer 7.6.1. Confidence levels of the clades were determined from bootstrap test (1,000 replicates) as depicted on the nodes of the branches.

Results and Discussion

The biolipid-accumulating ability of 45 oleaginous yeast strains, isolated from different rotten fruit sources and previously reported for their ability to accumulate lipids as a source of biodiesel was evaluated (Thanapimmetha *et al.*, 2017). These yeast isolates were screened for the ability to grow on molasses (8-24%, w/v) based culture medium (data not shown).

The screening flow scheme is shown in Figure 1. Eight strains with high lipid content were obtained secondary screening. During screening, a comprehensive evaluation of the lipid accumulating ability of yeast strains (a total of 45 strains) was carried out. In this experiment, 12% w/v molasses medium was employed, as almost all the selected strains could grow in this medium. Distribution of the lipid accumulation level is indicated in Figure 1. Results showed that 19.27-64.61%w/v of the tested strains and listed in Table 1. Yeast strains which accumulated more than 60%w/v were used for further analysis.

Effect of Cultivation Time

To investigate the effect of time on lipid accumulation, lipid extractions of the yeast cells

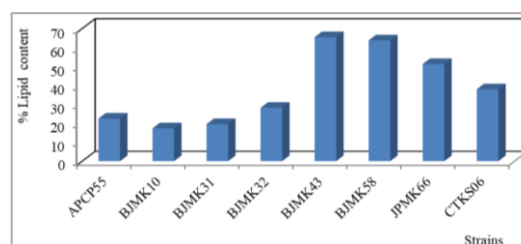


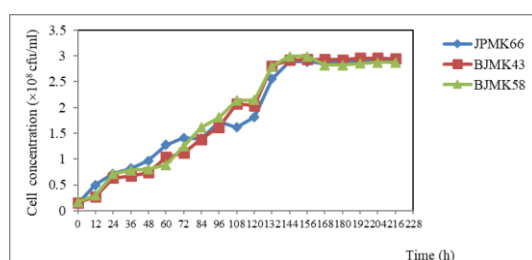
Figure 1. Lipid content profile of oleaginous yeasts in molasses medium

Table 1. Lipid contents produced by different yeast isolate¹

Yeast isolate	Biomass (g/L)	Lipid content (%w/v)	Sucrose utilize (g/L)
APCP55	17.31±1.78 ^{bc}	22.07±7.14 ^{cd}	22.40±1.83 ^{de}
BJMK10	21.54±3.67 ^a	19.27±6.40 ^d	17.66±0.04 ^f
BJMK31	19.88±0.26 ^{ac}	18.75±1.23 ^d	33.82±0.04 ^{ab}
BJMK32	21.17±0.57 ^a	26.94±0.82 ^c	31.59±0.19 ^b
BJMK43	10.99±0.85 ^d	64.61±0.86 ^a	19.54±0.34 ^{ef}
BJMK58	15.53±0.19 ^c	61.03±1.23 ^a	26.59±0.45 ^c
CTKS06	7.98±0.18 ^c	45.78±0.13 ^b	24.09±0.71 ^{cd}
JPMK66	17.77±0.83 ^{bc}	62.83±3.40 ^a	36.82±4.57 ^a

¹ Operational parameter were working volume: 50 ml; temperature: 30°C; pH: 5.0; shaking speed: 150 rpm.

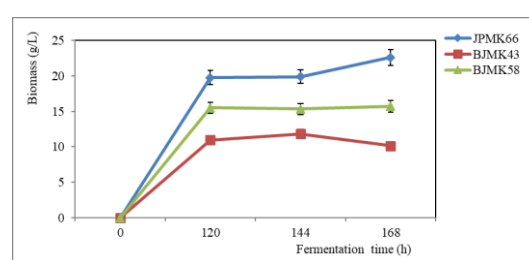
^{a,b,c} Means within row with the same superscripts are not significantly different at $P \leq 0.05$.

**Figure 2. Growth profile of oleaginous yeast strains on molasses medium**

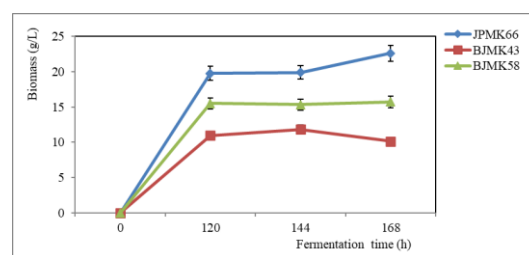
were performed at the 96, 120, and 144 h. of incubation. The isolate BJMK43 showed the maximum lipid concentration as 66.37% on the fifth day of incubation.

Batch experiments were performed under aerobic conditions in a molasses medium using an orbital shaker at an agitation rate of 150 rpm and incubation temperature of 30°C investigate every 12 h until 216 h. The cells rapidly increased in growth at the early stages of incubation. BJMK58 (cell number 3) and JPMK66 (cell number 1) cells showed the same trend with BJMK43 (cell number 2) cells, however, BJMK58 cell growth was higher than BJMK43 and JPMK66 during incubation (Figure 2).

Here, lipid-accumulating ability of the yeast strains was expressed as biomass and lipid content after 120 h of cultivation (Figure 3). All selected yeasts showed high lipid-producing ability under experimental conditions, with BJMK43 recorded the highest lipid content at 66.37% on the sixth day. However, the lipid content had significantly difference other report depended on their type strain, Paserakung *et al.* (2015) studies effect of cultural time on lipid accumulation of *T. asahii* GSY10 was cultured in molasses medium with increasing time course from 2 to 10 days. The result illustrated that maximum lipid yield and lipid content were obtained on day 8 of fermentation (4.1 g/l and 39.4% respectively). Further rise in cultural time beyond 8 days resulted in lipid yield and lipid content decreasing. This resulted from the breakdown of the



(a)



(b)

Figure 3. Time course of biomass (a) and lipid content (b) of JPMK66 (filled rhombus), BJMK43 (filled square) and BJMK58 (filled triangle)

accumulated lipid for cell proliferation due to the lack of carbon. Holdsworth and Ratledge (1998) determined the lipid turnover in oleaginous yeast by transferring cells with storage lipid into a carbon - starvation medium. The result found that the storage lipid was rapidly utilized after the carbon exhaustion.

FAME Composition and Estimation of Biodiesel Properties

FAME profiles of accumulated lipids by different oleaginous yeast strains were analyzed with results shown in Table 2.

In our study, BJMK43, BJMK58 and JPMK66 BJMK58 cells could accumulate lipid were 65.48 and 63.76% of dry biomass, respectively in molasses medium were fatty acids analyzed by GC. The main cellular fatty acids were linolenic (C18:3) composed 49.55%, 42.15%, and 35.23%, respectively. High methyl ester yields with 16 and 18 carbon atoms

Table 2. FAME composition (%) of lipids found in oleaginous yeast strains grown on molasses medium

Fatty acid (%)	BJMK43	BJMK58	JPMK66
Myristoleic acid (C14:1)	-	7.02	6.14
Palmitic acid (C16:0)	23.67	24.48	22.08
Palmitoleic acid (C16:1)	-	6.22	6.14
Stearic acid (C18:0)	-	-	4.33
Oleic acid (C18:1)	13.85	6.94	12.35
Linoleic acid (C18:2)	-	4.07	4.76
Linolenic acid (C18:3)	49.55	42.15	35.23
Arachidic acid (C20:0)	12.93	2.87	8.99
Total C16 and C18	87.07	83.86	84.89
Methyl ester yield (%)			

Table 3. Predicted relatedness among described oleaginous yeast species with similar or identical nucleotide sequences in LSU rDNA domain D1/D2

Strains	Species pairs	nucleotide	rDNA nucleotide differences	Predicted relatedness	Sources
BJMK43	<i>Rhodotorula mucilaginosa</i>	576/576	-	Same species	Baby Jack Fruit
BJMK58	<i>Rhodotorula mucilaginosa</i>	576/576	-	Same species	Baby Jack Fruit
JPMK66	<i>Haseniaspora opuntiae</i>	572/572	-	Same species	Jambolan Plum Fruit

(total more than 80%) were achieved by transesterification of the cellular crude lipids (Table 3).

Other researchers investigated on the lipid production from molasses medium of *C. lipolytica*, *C. tropicalis* and *R. mucilaginosa* in medium containing 8% molasses solution and 1.0 g/l (NH₄)₂SO₄ at pH 5 after four days of incubation. Maximum lipid contents and methyl ester yields were measured as 59.9% and 84.9% for *C. lipolytica*, 46.8% and 93.2% for *C. tropicalis*, and 69.5% and 92.3% for *R. mucilaginosa*. The main cellular fatty acids were stearic acid (C18:0), palmitic acid (C16:0) and oleic acid (C18:1) (Karatay *et al.*, 2010). Taskin *et al.* (2016) found that the main cellular fatty acids of *R. glutinis* TR29 were oleic acid (C18:1) (63.5%), palmitic acid (C16:0) (15.4%), stearic acid (C18:0) (9.1%) and palmitoleic acid (C16:1) (7.2%). Other fatty acids produced in lower amounts were myristic (C14:0) (3.0%) and linoleic acid (C18:2) (1.8%). Thus, molasses medium can be used as a raw material for biodiesel production. Similarity of Jiru1 *et al.* (2018) found that single cell oil using *R. kratochvilovae* SY89 on cane molasses as a biodiesel feedstock, but also to characterize the biodiesel obtained from the resultant lipids. After determination of the sugar content in cane molasses, *R. kratochvilovae* SY89 was grown on the optimized cane molasses for 168 h. Under the optimized conditions, the yeast accumulated lipids up to 38.25±1.10% on a cellular dry biomass basis. This amount corresponds to a lipid yield of 4.82±0.27 g/L. The fatty acid profiles of the extracted yeast lipids were analyzed using gas chromatography, coupled with flame ionization

detector. A significant amount of oleic acid (58.51±0.76%), palmitic acid (15.70±1.27%), linoleic acid (13.29±1.18%) and low amount of other fatty acids were detected in the extracted yeast lipids. The lipids were used to prepare biodiesel and the yield was 85.30%. The properties of this biodiesel were determined and found to be comparable to the specifications established by ASTM D6751 and EN14214 related to biodiesel quality.

DNA Sequencing and Phylogenetic Analysis

The obtained PCR product was 572-576 bp long the same size as the region on *Rhodotorula mucilaginosa* and *Haseniaspora opuntiae* the D1/D2 domain of 26S rDNA between the two primers. An alignment of the PCR product and the *R. mucilaginosa* and *H. opuntiae* 26S rRNA gene sequences showed that they were identical. Sequencing chromatograms were assembled into one complete sequence using Bioedit Sequence Alignment Editor version 7.2.5 and the sequence was compared to all known sequences in the GenBank using the BLASTN 2.2.26+ program and deposited in the GenBank database. Yeasts strain examined in this study are listed in Table 3.

Table 3, shows the sequence analysis results of BJMK43 and BJMK58 (576 nucleotides) and JPMK66 (572 nucleotides) revealing that the amplified sequences were 100% identical to those corresponding to *Rhodotorula mucilaginosa* CBS316T and *Haseniaspora opuntiae* CBS8733T. BJMK43 and BJMK58 were, therefore identified as *Rhodotorula mucilaginosa* and JPMK66 was identified as *Haseniaspora opuntiae*.

The colony and cell morphology of the yeast strains (Figure 4), suggested that BJMK43, BJMK58 and JPMK66 belonged to the genus *Rhodotorula mucilaginosa* (BJMK43 and BJMK58) and *Hanseniaspora opuntiae* (JPMK66). The sequence of the D1/D2 domain of the 26S rRNA gene was obtained and compared to the sequence deposited in GenBank (NCBI) (Thancharoen *et al.*, 2017). The phylogenetic placements of these strains in relation to closely related species, based on the D1/D2.

Sequence are presented in Figure 5.

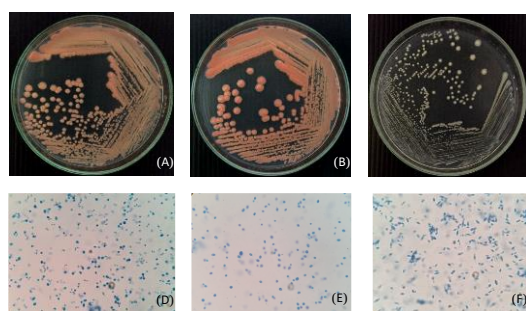


Figure 4. Morphologically characteristics (A-C) and cell shape (D-F) under microscope of BJMK43, BJMK58 and JPMK66

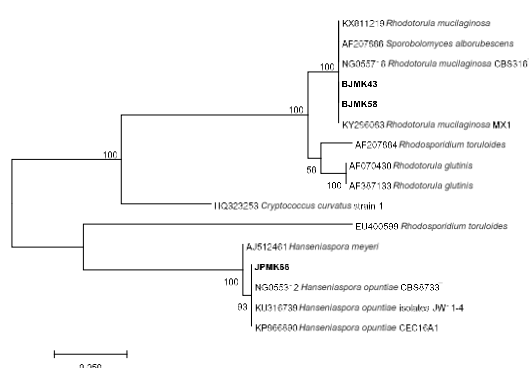


Figure 5. Phylogenetic dendrogram constructed from 26S rDNA sequence of BJMK43, BJMK58 and JPMK66 strains and their related strains in GenBank. The tree was constructed using the maximum likelihood method of MEGA 7 software. Bootstrap values (1,000 tree interactions) are indicated at the nodes

Conclusions

Our results confirmed that yeast biomass can be used as a potential biodiesel feedstock. It is known that C16 and C18 fatty acids are more suitable for biodiesel production. Hence, the cost of biodiesel production could be considerably reduced by using an agroindustrial residue such as molasses. This

strain can potentially shorten fermentation times and reduce production cost. Generally, fermentation with oleaginous yeast requires extensive time to reach the required lipid yield, as lipid accumulation in oleaginous yeast is known to occur during stress conditions in the medium. When cells begin to deplete nutrients, such as nitrogen, the excess carbon in the culture medium is converted into cellular lipid. This study indicated that high efficiency of *R. mucilaginosa* BJMK43 has a potential to use as alternative renewable source in future application.

Acknowledgments

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