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Research

The Potential of *Mucor irregularis* Isolated From Fruits in Producing Microbial Lipid

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ABSTRAK

In this present study, potential oleaginous Mucorales fungi that have been isolated from fruits from local markets in Sleman, Indonesia, were screened for lipid production. A total of six fungal cultures were isolated and screened using a semisynthetic medium with glucose as a carbon source and a limited nitrogen supply. The highest lipid content was observed in isolate JR 1.1, up to 43.46% and 3.28 g/L lipid yield. Therefore, it was selected for molecular identification and fatty acid analysis. The result showed that JR 1.1 was identified as *Mucor irregularis*. The fatty acid profile of JR 1.1 showed 16.89% palmitoleic acid, 4.85% oleic acid, 45.22% linolenic acid, 30.79% gamma-linolenic acid, and 2.25% other fatty acids. It can be concluded that *M. irregularis* JR 1.1 is a potential strain to be used as a lipid producer for biodiesel feedstock. Further studies are recommended to optimize lipid productivity and improve fatty acid composition.

Key words: Mucorales, oleaginous, biodiesel, lipid, identification

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INTRODUCTION

The increase in population leads to excess fossil fuel consumption, thereby causing energy crises and environmental pollution. Energy crisis and pollution can be reduced by developing renewable energy sources such as biodiesel (Huang *et al*., 2016). Raw material for biodiesel production can be obtained from plants, animals, and microorganisms. Currently, biodiesel is mostly produced from vegetable oils such as palm, rapeseed, and soybean, or animal fat such as tallow. The use of edible oils is criticized because of their sustainability, human food consumption, and the need for high requirements of water and fertilizer (Khot *et al*., 2012).

Microbial lipid extracted from filamentous fungi, yeast, microalgae, and bacteria is deemed as a better alternative oil source due to its high productivity and less land requirement. Microorganisms capable of accumulating more than 20% of their total dry biomass are called oleaginous microorganisms (Chi *et al*., 2011; Bhanja *et al*., 2014). Out of various types of oleaginous microorganisms, filamentous fungi are considered good sources for several reasons such as short life cycles, less labor requirement, less affected by climate, easier to harvest, and higher variety of fatty acids (Huang *et al*., 2016).

One of the oleaginous mold groups is Mucorales which is a saprophytic fungus that lives in soil, decaying organic material, and waste foods such as fruits (Hoffmann *et al*., 2013). There have been reports on high lipid-producing Mucorales belonging to the genera *Mucor*, *Cunninghamella*, and *Rhizopus* that can accumulate 20-25% lipid of their dry biomass. *Mucor circinelloides* and *Mucor rouxii* have been reported to accumulate 39.7% - 42.6% lipid in their dry biomass (Jangbua *et al*., 2009; Bhanja *et al*., 2014).

Oleaginous filamentous fungi in this research were isolated from rotten fruits from Sleman, D.I. Yogyakarta, Indonesia. Research on oleaginous Mucorales in Indonesian fruits is currently lacking. Therefore, more study is needed on oleaginous Mucorales. The purpose of this study is to obtain and identify potential oleaginous Mucorales fungi from fruits in Sleman, D. I. Yogyakarta, Indonesia, and analyze its fatty acid profile.

MATERIALS AND METHODS

Sample collection

Fresh fruits (oranges, *Citrus* sp., and strawberries, *Fragaria* sp.) were collected from traditional markets and supermarkets in Sleman. The fresh fruits were left in shady areas to rot and contaminated by mold for 3 – 7 days at room temperature. Samples were taken to the laboratory in a container box and were promptly isolated.

Fungal isolation

Filamentous fungi were isolated from rotten fruits using a direct isolation method. The selected filamentous fungi were white in color and cotton-like texture which are the characteristics of Mucorales filamentous fungi. The waste fruit part was cut using a sterile scalpel and surface sterilized using 70% ethanol. The fruit piece was then transferred to a PDA medium. The plates were incubated at 30 °C for 3-5 days. Fungal colonies that showed different colony colors and textures were purified by transferring a small portion of mycelium onto a separate PDA medium (Garg *et al*., 2010).

Microbial lipid screening

Preparation of spore suspension

The spore suspension was prepared by growing fungal isolates in test tubes until the sporulation stage. A mixture of 10 mL distilled water and triton 0.01% was poured into the PDA plates containing fungal isolate (Mudrončeková *et al*., 2013). Fungal spores were then scrapped using an inoculation loop and the spore suspension was transferred into the sterile screw-capped sample bottle. The number of spores in the suspension was then counted using an Improved Neubauer counting chamber (Aldina *et al*., 2017). The absorbance of the suspension was also measured at 550 nm using a spectrophotometer to correlate it with the spore number (Schütz *et al*., 2020). The number of spores in the suspension was calculated with the following equation (Triasih *et al*., 2019):

$S= (t \times d) / (n \times 0.25) \times 10^6$

Notes:

S = number of spores

t = number of spores observed on counting chamber

d = dilution factor

n = total number of counting chambers

0.25 = hemocytometer correction factor

10^6 = conidia count standard

Biomass and lipid production

The lipid production medium consisted of (g/L): KH_2PO_4 2.5; $ZnSO_4{-}7H_2O$ 0.01; $CuSO_4{-}5H_2O$ 0.001; $MnSO_4$ 0.01; $MgSO_4{-}7H_2O$ 0.5; $FeSO_4{-}7H_2O$ 0.02; $CaCl_2$ 0.1; yeast extract 5.0; KNO_3 1.0; glucose 30.0. 50 mL of medium were placed in 250 mL flasks and were autoclave-sterilized at 121 °C, 1 atm pressure for 15 min. Each flask with sterile medium was inoculated with 10^6 spore suspension and incubated inside the incubator shaker at 28±2 °C with 200 r.p.m. agitation for 6 days (Somashekar *et al*., 2003). Pellet diameter, biomass, lipid, glucose, and nitrogen residue were determined at the 144-hr mark. All experiments were done in triplicate.

Pellet diameter measurement and biomass calculation

Fungal pellets formed after incubation were harvested by filtration method using Whatman filter paper no. 1 and spent medium was collected in an Erlenmeyer flask. The diameter of 10 representative fungal pellets was measured using a digital caliper and then the average was calculated. The biomass

dry weight was determined by washing the fungal pellets twice with distilled water and then drying them in an oven at 50 °C for 24-48 hr until a constant weight was reached (Kamoun *et al*., 2018).

Lipid extraction

Lipid extraction was performed according to Somashekar *et al*. (2003). Dry biomass was disrupted and homogenized then mixed with acid-washed sand with a 1:2 ratio. Fungal lipid was extracted by adding methanol and chloroform at a 1:2 ratio. The mixture was centrifuged at 4000 rpm for 10 min to form two layers. The top layer was collected and transferred to a glass bottle which had been weighed previously and then the solvent was evaporated. The bottle was reweighed after all the solvent completely evaporated. Lipid content (%) was determined by calculating the grams of lipid per dry biomass.

Residual glucose estimation

Residual glucose in the medium was estimated using the Dinitro Salicylic Acid (DNS) method (Miller, 1959). DNS solution was prepared by dissolving 1 g of DNS powder, 20 mL of 2 M NaOH, and 15 g of K-Na Tartrate with distilled water until the volume reached 100 mL. One millilitre of spent medium from production was transferred into the tube followed by the addition of 1 mL of DNS reagent and 2 mL of distilled water. The tube was then heated in a water bath for 5 min. The absorbance at 540 nm was measured and plotted against a standard curve of glucose.

Residual nitrogen estimation

Residual nitrogen was estimated using the Micro-Kjeldahl method (Ma & Zuazaga, 1942). Digestion was performed by weighing 5 g of spent medium from production, then transferring it to the Kjeldahl flask followed by the addition of 10 g Kjeldahl reagent and 20 mL H_2SO_4. The mixture was heated for 180 min at 350-380 °C until foamy. The distillation process was performed by adding the digested sample with 50 mL of 50% NaOH solution. Afterward, 4% boric acid and 7 drops of Tashiro indicator were added to the mixture. Titration was performed by dripping 0.25 mol/L HCl until it reached the titration equivalence point.

Fungal identification

The fungi isolate was grown on a slanted PDA medium at 30 °C for 3 days. One hundred milligrams of hyphae were harvested, and then genomic DNA was extracted from the hyphae using Zymo Research Kit (Zymo Research Corporation) following the manufacturer's instructions. gDNA purity was measured using the spectrophotometry method at λ 260/280 using Nanodrop (Maestrogen).

The ITS region was amplified using PCR with ITS1 (5´-TCC GTA GGT GAA CCT GCG G-3´) as forward primer and ITS4 (5´-TCC TCC GCT TAT TGA TAT GC-3´) as reverse primer. The PCR reaction was performed in 50 µL volume and contained 4 µL DNA template, 5 µL of 10x PCR buffer, 1.5 µL 10 mmol 1-1 dNTP, 2.5 µL 10 mmol 1-1 of each primer, and 0.5 U PCR extender polymerase was used for PCR reaction. The PCR reaction was started at 94 °C for 2 min, followed by 35 cycles at 60 °C for 30 sec and 72 °C for 1 min. Finally, the reaction was ended by holding the temperature at 72 °C for 5 min. PCR reaction was run with positive control (with DNA) and negative control (without DNA). An amount of 5 µL PCR product was run on 0.7% agarose gel and dyed with ethidium bromide to confirm DNA amplification (Romanelli *et al*., 2014).

The PCR product was sequenced by PT. Genetika Science Jakarta with bidirectional sequencing method. Errors found on the sequencing result were removed with GeneStudio software (Nikolay Rovinskiy). The sequencing result was analyzed using BLAST (NCBI) to find the similarity with other strains in GeneBank.

Fatty acid analysis

The fungal lipid was converted to methyl ester by methylation. A 0.5 mL sample was added with CH_3ONa and heated at 60 °C for 5-10 min. Then, 2 mL BF_3-MeOH was added, and the mixture was heated at 60 °C for 5-10 min. The sample was then extracted using 1 mL heptane and 1 mL NaCl. The upper layer was analyzed in Gas Chromatography (Agilent 7890B) using a DB-WAX column (diameter 0.25 mm, film thickness 0.25 µm, length 30 m) with an FID detector. Helium gas was used as a carrier. First heating was performed at 50 °C and continued until it reached 230 °C and maintained for 18 min. Peak identification was done using the sequence method by Agilent ChemStation (Agilent Technologies) software and library.

RESULTS AND DISCUSSION

In this study, six different isolates of filamentous fungi were isolated from rotten fruits namely oranges and strawberries in Sleman, D.I. Yogyakarta, Indonesia. The production medium used in screening had a C/N ratio of 20.85. Glucose, yeast extract, and KNO_3 were used as carbon and nitrogen sources for the production medium. The lipid screening result showed isolate JR 1.1 as the highest lipid-producing isolate that was able to produce 43.46% lipid and 3.28 g/L yield (Table 1). Therefore, it was selected for molecular identification and fatty acid analysis.

Table 1. Lipid screening result of filamentous fungi. The fungi were grown in the semi-synthetic medium at 28±2°C with 200 rpm agitation for 6 days. The glucose consumption was measured using the DNS method, while nitrogen consumption was measured using the Kjeldahl method

Isolate	Biomass (g/L)	Lipid (g/L)	Lipid content (%)	Pellet size (mm)	Glucose consumption (%)	Nitrogen consumption (%)
JR 1.1	7.54±0.21	3.28±0.42	43.46±4.51	0.87±0.18	94.05±1.13	91.96±2.04
JR 1.2	3.36±0.01	0.49±0.03	14.73±0.87	1.72±0.24	95.58±0.77	79.48±1.50
JR 2	5.23±0.51	1.04±0.03	20.02±1.49	0.73±0.07	95.46±0.90	76.91±2.24
JR 5	6.18±0.33	1.32±0.09	21.35±2.36	0.64±0.19	81.13±1.61	71.64±2.54
SR 1.1	6.28±0.34	0.70±0.23	11.07±3.00	0.65±0.07	94.48±0.80	78.97±1.70
SR 1.2	5.53±0.22	1.25±0.15	22.68±1.81	0.93±0.06	94.68±0.73	79.97±1.90

The results of lipid screening are presented in means with ± standard deviation for triplicates of each isolate

Oleaginous Mucorales previously have been reported to produce a high percentage of lipid, namely *Cunninghamella echinulate, Cunninghamella blakesleeana, Mucor circinelloides,* and *Thamnidium elegans* which were able to accumulate up to 54% of lipid (Chatzifragkou *et al*., 2011). Oleaginous Mucorales were previously found on mulberry fruit in China that accumulated 28.8% lipid (Qiao *et al*., 2018). However, there are lack of studies on oleaginous Mucorales found in fruits, especially in Indonesia. In this study, isolate JR 1.1 which was identified as *Mucor irregularis* was able to produce a high percentage of lipid and has never been reported before to our knowledge. It is also of high interest because it was able to accumulate 43.46% lipid and grew rapidly in production medium.

The highest lipid-producing isolate, JR 1.1, was identified by sequencing the ITS region using ITS1 and ITS4 as primers. The length of the amplified JR 1.1 ITS region is 633 bp based on the electrophoresis result. Similarity values of >98% indicate the homology of one species, 95-98% similarity indicates one genus, and <94% is unclassified. Sequence data of the JR 1.1 ITS region was analyzed using the Basic Local Alignment Search Tool (BLAST). BLAST analysis showed 99.68% similarity with *Rhizomucor variabilis* which was later reclassified as *Mucor irregularis* (Table 2).

Table 2. Strains with the highest similarity with JR 1.1 isolate based on ITS region. The database used was NCBI GenBank with the BLAST method to find the similarity between strains

Homolog species	Query coverage	Similarity value	Accession number
Rhizomucor variabilis strain CBS 103.93	99%	99.68%	HM623314.1
Mucor irregularis CBS 103.93; TYPE strain	99%	99.68%	NR_172288.1
Rhizomucor variabilis strain CBS 103.93 18S ribosomal RNA gene	99%	99.68%	DQ119006.1
Mucor irregularis strain CBS 103.93	91%	99.66%	JN206150.1
Mucor irregularis strain CBS 103.93	93%	98.82%	JX976257.1
Mucor irregularis partial 18S rRNA	89%	99.65%	FN663958.1
Mucor hiemalis f. hiemalis CBS 201.65 ITS region TYPE material	90%	89.95%	NR_152948.1
Mucor souzae URM 7553 ITS region TYPE material	90%	89.00%	NR_165210.1
Mucor sp. RO-2017b culture	90%	89.00%	KY992878.1
Mucor hiemalis f. hiemalis strain CBS 201.65	87%	89.62%	JX976246.1

Mucor has been found to grow rapidly in 3 to 7 days on fungal culture media. Filamentous fungi typically aggregate to form pellets. The pellets can vary from cottony loosely packed hyphae to tightly packed dense, compact pellets. The pellets formed in this study are dense and of the coagulating type in which the pellets are formed spores due to the coagulation of spores during the early stages of development, followed by gradual germination of spores and aggregation with other agglomerates (Xia *et al*., 2014). Other types of oleaginous microorganisms such as yeasts and algae tend to grow dispersed on the growth medium instead of forming pellets, which can be challenging to harvest and make it less suitable for the production of biodiesel (Gultom & Hu, 2013).

The highest lipid-producing isolate, JR 1.1, was analyzed using gas chromatography-mass spectrometry (GC-MS) to discover the fatty acid profile. GC is combined with MS to find the true structure of fatty acids, such as the positions of double bonds in the aliphatic chain. Fatty acids must be converted into their derivatives before gas chromatography is carried out so that they are sufficiently volatile to be eluted at tolerable temperatures without thermal decomposition. In general, functional groups containing hydrogen are substituted to form esters, thioesters, or amides. One of the most studied forms of fatty acid derivatives is methyl esters, which are produced by methylation. The ester bonds in the complex lipid are hydrolyzed to release free fatty acids, followed by transmethylation to form fatty acid methyl esters (FAME). The FAME profile is then determined by GC-MS and is referred to as the fatty acid composition of the lipid (Fisk *et al*., 2014; Chiu & Kuo, 2020).

The result showed that the JR 1.1 lipid was composed of 0.34% SFAs, 22.9% MUFAs and 76.76% PUFAs. Small percentage of SFAs was of heptadecanoic acid (C17) and arachidic acid (C20), MUFAs mainly composed of 16.89% palmitoleic acid (C16:1), 4.85% oleic acid (C18:1), PUFAs mainly composed of 45.22% linoleic acid (C18:2), 30.79% gamma-linolenic acid (Table 3, Figure 1).

Table 3. Fatty acid profile of JR 1.1 lipid. The fatty acids were measured using GC Agilent 7980B using DB-WAX column (diameter 0.25 mm, film thickness 0.25 µm, length 30 m)

Fatty acids	Fatty acid content (%)
Saturated fatty acid (SFA)	
Heptadecanoic acid (C17)	0.14
Arachidic acid (C20)	0.20
Total	0.34
Monounsaturated fatty acid (MUFA)	
Myristoleic acid (C14:1)	0.20
Palmitoleic acid (C16:1)	16.89
Oleic acid (C18:1)	4.85
11-Eicosenoic acid (C20:1)	0.42
Erucic acid (C22:1)	0.29
Nervonic acid (C24:1)	0.25
Total	22.9
Polyunsaturated fatty acid (PUFA)	
Linoleic acid (C18:2)	45.22
Gamma-linolenic acid (C18:3)	30.79
Eicosadienoic acid (C20:2)	0.26
Docosadienoic acid (C22:2)	0.24
Docosahexaenoic acid (C22:6)	0.25
Total	76.76

Oils are deemed suitable for use as biodiesel feedstock if they have a high proportion of saturated fatty acids, moderate monounsaturated fatty acids, and low polyunsaturated fatty acids. This is related to the autoxidation of biodiesel because oils with a high proportion of PUFA are more susceptible to autoxidation which leads to an unpleasant odor (Patel *et al*., 2020). High-quality biodiesel typically contains high amounts of SFAs such as palmitic acid (C16:0) and stearic acid (C18:0), along with MUFAs such as palmitoleic acid (C16:1) and oleic acid (C18:1). PUFAs such as linoleic (C18:2) and linolenic acid (C18:3) are also commonly found in biodiesel. However, PUFAs with more than three double bonds are considered unsuitable for biodiesel production because of their low stability (Pinzi *et al*., 2013; Athenaki *et al*., 2018). Free fatty acids cannot be utilized directly as biodiesel because, when used over time, they can result in carbon deposit and engine oil gelling. Fatty acids must go under transesterification with a catalyst by methanol and be converted to fatty acid methyl esters (FAMEs) which are used for biodiesel and glycerol as a by-product (Khot *et al*., 2012).

CONCLUSION

The filamentous fungi found in waste fruits showed the presence of oleaginous fungi. The results of this study add to the knowledge of oleaginous fungi and its potential as a lipid producer for biodiesel feedstock. *Mucor irregularis* was discovered to be a new oleaginous fungal species. Its lipid productivity can be further increased by optimization of nutrition and environmental conditions during the fermentation process. The fatty acid analysis showed the lipid produced by *M. irregularis* contains a high amount of palmitoleic acid and linoleic acid. This amino acid composition has the potential to be converted into high-quality biodiesel. Thus, optimization of fatty acid composition is needed to increase the SFA

content of *M. irregularis* lipid to make it more suitable for biodiesel production, which can be achieved by genetic modification of the strain, and/or process engineering.

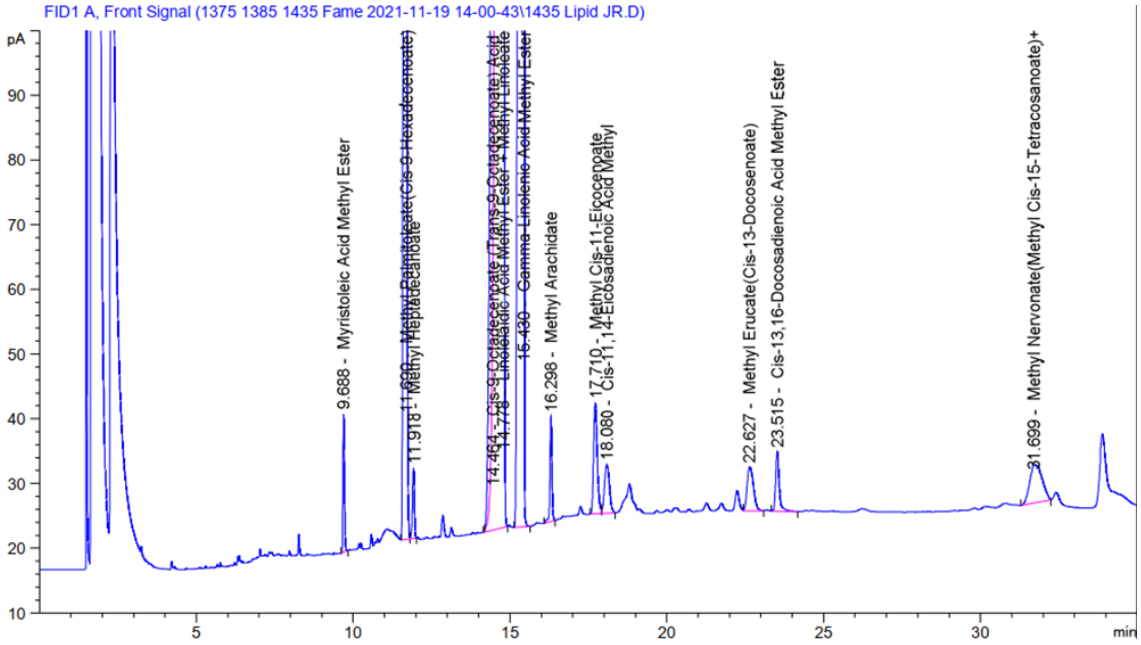

Fig. 1. GC/MS chromatogram of JR 1.1 lipid. The fatty acids were measured using GC Agilent 7980B using DB-WAX column (diameter 0.25 mm, film thickness 0.25 µm, length 30 m)

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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