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Optimization of Lipid Production by *Mortierella isabellina* Using Glycerol, A By-product of Biodiesel Production as a Carbon Source

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The aim of this work was to evaluate the lipid accumulation potency of oleaginous fungus *Mortierella isabellina* using glycerol, a by-product of palm oil-biodiesel production, as an alternative carbon source. Three fungal strains of *M. isabellina*, NBRC 7874, NBRC 7884, and NBRC 105998, were selected for use in this study. The results identified *M. isabellina* strain NBRC 105998 as the best lipid accumulation strain among the three tested. This strain could accumulate lipid at 35.02% based on cell dry weight with a total biomass of 0.71 g/L after 12 days when using a concentration of glycerol in the medium at 30 g/L. Furthermore, the optimization of cultivation conditions for the best oil-producing strain obtained was evaluated using response surface methodology (RSM). A 5-level 2-factor central composite design (CCD) was used to build the statistical model. The optimum cultivation condition for *M. isabellina* NBRC 105998 found in this study included glycerol concentration in the medium (44.14 g/L), inoculum size [2 fungal discs; $3.74x10^6$ spores] and incubation at 30 °C for 12 days. This optimum condition provided 45.21% of the lipid content, most of which was composed of long chain saturated fatty acids (78.28%).

Keywords: Biodiesel production, Glycerol, Lipid production, *Mortierella isabellina*, Optimization, Response surface methodology.

In recent years, much attention has been paid to the exploration and development of alternative oil sources from oleaginous microorganisms such as yeasts, fungi, bacteria, and microalgae[1]. Oleaginous microorganisms are able to accumulate more than 20% of their dry biomass as oil (single cell oil-SCO)[2,3], which is viewed as a possible alternative for commercial oil producers as food and energy resources due to their fatty acid compositions being similar to that of common plant oils[4,5,6,7,8]. Among the group of oleaginous microorganisms, fungi (molds) have attracted considerable attention because they are able to be cultivated on a wide range of substrates, particularly waste such as glycerol, as well as lignocellulosic biomass (rice hull and corn stover) hydrolysates[9,10], have short life cycles, and display rapid growth rates, as well as being unaffected by space or climatic variations and allowing production to be easily scaled up in the fermentation process to produce more lipid biomass[8]. The oleaginous filamentous fungus *Mortierella isabellina* has been reported as one of the potential oleaginous fungal species due to its ability to accumulate large amounts of lipids by up to 80% of their cellular dry weight[11]. It also has a high tolerance for cultivation on different residual materials as substrates[9,10] and short generation times with minimal nutrient requirements[11].

Biodiesel is one of the most promising renewable energy sources receiving significant attention worldwide. The rapid increase in demand for renewable fuels has also substantially increased biodiesel production[12]. In Thailand, oil palm (*Elaeis guineensis*) is used as the major

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feedstock for biodiesel production. From the production of biodiesel via a transesterification reaction, approximately 10% by weight of glycerol is created as a major by-product, most of which is disposed of as a low-value product because refining glycerol is not feasible, especially for medium and small biodiesel plants. Therefore, several methods have been investigated for utilizing this abundant waste. The use of crude or partially-purified glycerol as a carbon source for cultivating oleaginous microorganisms is promising for the utilization of such abundant glycerol waste. It could also assist in reducing the cost of microbial oil production[13].

In this work, three strains of *M. isabellina* oleaginous fungus were screened for their ability to utilize glycerol, a by-product partially purified from palm oil-biodiesel production, as a carbon source for biomass and lipid production. The optimization of lipid production for the most promising oleaginous strain was conducted using response surface methodology (RSM).

MATERIALS AND METHODS

Glycerol sample

The glycerol sample (93.97% purity), a by-product of palm oil-biodiesel production, was obtained from Patum Vegetable Oil Co., Ltd., Thailand.

Microorganisms and culture conditions

Three fungal strains of *M. isabellina*, NBRC 7874, NBRC 7884, and NBRC 105998 were obtained from the Department of Biotechnology, National Institute of Technology and Evaluation (Chiba, Japan) and preserved on potato dextrose agar (PDA, Difco, USA). The growth medium was comprised of (per 1 L distilled water) a glycerol sample from biodiesel production 30 g, yeast extract 1 g, KH_2PO_4 7 g, Na_2HPO_4 2 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, $FeCl_3 \cdot 6H_2O$ 0.08 g, $ZnSO_4 \cdot 7H_2O$ 0.001 g, $MnSO_4 \cdot 5H_2O$ 0.0001 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $CuSO_4 \cdot 5H_2O$ 0.0001 g, $Ca(NO_3)_3 \cdot H_2O$ 0.0001 g, $(NH_4)_2SO_4$ 0.5 g. All experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml of growth medium and sterilised in an autoclave at 121 °C for 15 min. Five 7 mm fungal discs (9.35×10^6 spores) from a *7-day*-old culture of each fungal strain was added to each flask. All cultures were incubated in a rotary shaker at 180 rpm and 30 ± 1 ºC.

Lipid extraction and fatty acid determination

The lipid content of each fungal biomass was extracted to determine lipid accumulation efficiency at 4, 8, 12 and 16 days according to Folch *et al.*(1957)[14] with a slight modification. The extracted lipids were measured using a calibrated balance. Lipid content per 100 g of cell dry weight was calculated. Lipid transesterification and fatty acid extraction were carried out following the procedures described by Riengsilchai *et al.* (2013)[15]. The FAMEs were analysed using a gas chromatograph (GC) fitted with a flame ionization detector (FID) (Shimadzu, Japan). A fused capillary column BPX70 (0.32 mm i.d. × 30 m, 0.25 µm film thickness; SGE, Australia) was used. GC-FID was performed using the following conditions: carrier gas He; flow rate 1.5 ml/min; injection temperature 250 °C; oven temperature programmed from 210 °C (9 min hold) to 240 °C at 20 °C/min (6 min hold); detector temperature 280 °C. Individual fatty acids were identified by comparing them to the retention time of authentic fatty acid standards obtained from Sigma Co., USA.

Optimization of cultivation conditions for the best oil-producing strain obtained by response surface methodology (RSM)

Response surface methodology (RSM) was performed using a 5-level 2-factor central composite design (CCD). There were 13 experiments involving the two variables investigated for glycerol concentration [15.86-44.14 (g/L)] and inoculum size [2-8 fungal discs (1.87×10^6 spores/disc)]. The factors and their respective levels are summarized in Table 1.

RESULTS AND DISCUSSION

Screening of potential *M. isabellina* strains for lipid accumulation

Three *M. isabellina* strains were cultured in a growth medium (using glycerol at a concentration of 30 g/L) and incubated at 30 °C on a shaker set at 180 rpm. The fungal biomass of each strain was isolated and lipid accumulation in the fungal mass was extracted at 4, 8, 12 and 16 days. The data for fungal mass (weight) and lipid content accumulation for each fungal strain at different periods of time are given in Table 2.

The results showed that *M. isabellina* NBRC 105998 was the best lipid-accumulating strain

among the three strains tested, with a total biomass of 0.71 g/L and a lipid content of 35.02% after 12 days.

Optimization of cultivation conditions for the best oil-producing strain and determination of fatty acid profiles in lipid samples

The optimum cultivation condition for the best oil-producing strain obtained (*M. isabellina* NBRC 105998) was evaluated using response surface methodology (RSM). A 5-level 2-factor central composite design (CCD) was employed for the optimization of culture conditions, as shown in Table 3. A second-order polynomial equation was used to fit the predicted model to the experimental data. The development of design matrices and analysis of variance (ANOVA) for the RSM model was conducted.

The effect of the combination of glycerol concentration and inoculum size on the lipid accumulation of *M. isabellina* NBRC 105998 is shown in Table 4. Based on these data, the equation that follows is expressed as in Equation 1, which predicts lipid accumulation in the linear regression model.

$$Y = 9.791X_1 - 2.729X_2 - 0.592X_1X_2 - 3.883X_1^2 - 0.077X_2^2 + 34.156 \quad (1)$$

where

Y represents Lipid content (%)

X_1 represents Glycerol concentration (g/L)

X_2 represents Inoculum size (fungal discs)

The optimum cultivation condition for *M. isabellina* NBRC 105998 obtained in this study (Fig. 1) was identified when the media were set as follows: glycerol concentration in the medium (44.14 g/L), inoculum size (2 fungal discs; $3.74x10^6$ spores) and incubation time at 30 °C for 12 days. This optimum condition provided 45.21% of the lipid content. The lipid content produced was analyzed further by GC-FID for its fatty acid compositions. The GC chromatogram and relative percentage of fatty acid compositions in the lipid sample are shown in Fig. 2 and Table 5, respectively.

Six different fatty acids were identified in the lipid content produced from *M. isabellina* NBRC 105998 under optimum condition. Stearic acid (57.28%) was the dominant fatty acid found,

Table 1. Experimental ranges of the two independent variables used in RSM

Variables	Levels				
	-1.414(-α)	-1	0	+1	+1.414(+α)
X_1, Glycerol concentration [g/L]	15.86	20	30	40	44.14
X_2, Inoculum size [fungal discs]*	2	3	5	7	8

* $1.87x10^6$ spores/disc

Table 2. Cell mass and lipid content accumulation during growth of *M. isabellina*

Strains	Times [days]	Biomass[g dry wt/L] *	Lipid content[% w/w] *,**
NBRC 7874	4	0.22	14.43
	8	0.45	20.86
	12	0.43	20.59
	16	0.45	20.70
NBRC 7884	4	0.44	15.25
	8	0.58	16.14
	12	0.63	24.61
	16	0.66	24.98
NBRC105998	4	0.49	18.68
	8	0.67	32.26
	12	0.71	35.02
	16	0.72	35.01

* values are means of three replications ** based on dry mass

Table 3. Experimental design and predicted values of lipid production yield for CCD

Run no.	Code X_1	Code X_2	X_1 [Glycerol concentration (g/L)]	X_2 [Inoculum size (fungal discs)*]	Y [Lipid content (%)] Experimental values	Y [Lipid content (%)] Predicted values
1	-1	-1	20	3	22.84	22.54
2	+1	-1	40	3	45.86	43.31
3	-1	+1	20	7	17.01	18.27
4	+1	+1	40	7	37.66	36.67
5	-1.414	0	15.86	5	13.49	12.55
6	1.414	0	44.14	5	38.00	40.24
7	0	-1.414	30	2	36.11	37.86
8	0	1.414	30	8	30.60	30.14
9	0	0	30	5	35.62	34.16
10	0	0	30	5	35.03	34.16
11	0	0	30	5	34.42	34.16
12	0	0	30	5	33.10	34.16
13	0	0	30	5	32.61	34.16

* 1.87×10^6 spores/disc

Table 4. Analysis of variance (ANOVA) for the quadratic model of the lipid production yield

Parameters	Coefficients	p-value
Constant	34.156	0.000
X_1	9.791	0.000
X_2	-2.729	0.005
X_1X_2	-0.592	0.549
X_1X_1	-3.883	0.001
X_2X_2	-0.077	0.917

R-squared (R^2) = 0.956

Table 5. Relative percentage of fatty acid compositions in the lipid content extracted from *M. isabellina* NBRC 105998 when grown under the optimum condition

Fatty acid	Composition (%)
Myristic acid (C14:0)	1.07
Palmitic acid (C16:0)	19.93
Stearic acid (C18:0)	57.28
Oleic acid (C18:1)	13.15
Linoleic acid (C18:2)	5.80
Linolenic acid (C18:3)	2.77

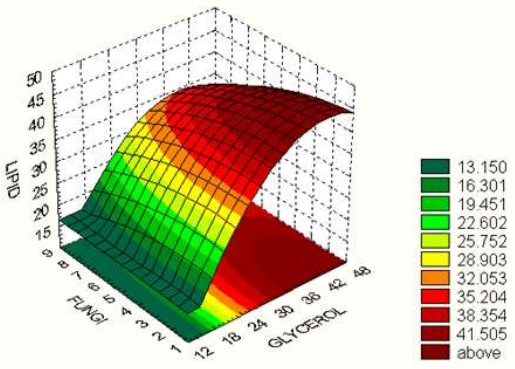

Fig. 1. Response surface and contour plot of the combined effects of glycerol concentration and inoculum size on lipid production by *M. isabellina* NBRC 105998

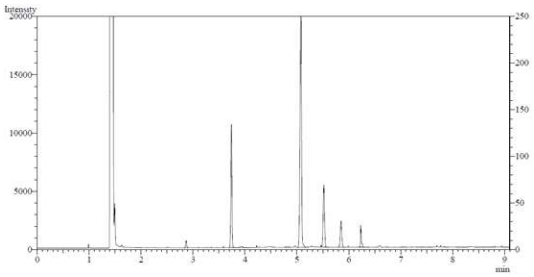

Fig. 2. GC chromatogram of fatty acid compositions in the lipid produced from *M. isabellina* NBRC 105998 when grown under optimum condition

followed by palmitic acid (19.93%), oleic acid (13.15%), and linoleic acid (5.80%). There were also small amounts of linoleic acid (2.77%) and myristic acid (1.07%). The entire fatty acid profile present in the produced lipid content showed that it possessed potential utilization for biodiesel production[10,16,17].

CONCLUSION

M. isabellina is an oleaginous fungal species with a high capability for utilization of glycerol, a by-product of palm oil-biodiesel production, as an alternative carbon source. In this study, the NBRC 105998 strain showed the most ideal lipid accumulation among the 3 strains tested, with a total biomass of 0.71 g/L and a lipid content of 35.02% after 12 days when using a concentration of glycerol in a medium at 30 g/L. Moreover, the lipid content of the best oil-producing strain (*M. isabellina* NBRC 105998) can be increased significantly by optimizing two of the principal factors in the culture, namely glycerol concentration and inoculum size using RSM. The optimum cultivation condition for *M. isabellina* NBRC 105998 identified this study included the glycerol concentration in the medium (44.14 g/L), the inoculum size (2 fungal discs; $3.74x10^6$ spores) and incubation at 30 °C for 12 days. This optimum condition provided 45.21% of the lipid content. Further, six fatty acids were found in the lipid content produced from this condition, composed mostly of long chain saturated fatty acid (78.28%), which had suitable properties for use as an alternative feedstock for biodiesel production.

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