Short Communication

Efficient production of biolipids by crude glycerol-assimilating fungi

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Abstract

The aim of this study was to isolate microorganisms utilizing crude glycerol as a carbon source efficiently and to evaluate their lipid productivity. *Fusarium oxysporum* W1 grew well on medium containing 20% crude glycerol as well as 50% pure glycerol. The dry cell weights and total fatty acids of *F. oxysporum* W1 reached 24.5 g/L and 12.4 g/L. *Penicillium* sp. N1 and *P. citrinum* N3 were found to accumulate free fatty acids to as much as 56.2 % and 48.5 % of total fatty acids, respectively, on cultivation in the crude glycerol-containing medium. These strains grew well on medium containing crude glycerol only heat-treated at 80-105°C without autoclave sterilization.

Key words

Crude glycerol; Biolipid; *Penicillium*; *Fusarium*

1. Introduction

Biodiesel, a mixture of long-chain mono-alkyl fatty acid esters derived from vegetable oils or animal fats, as an alternative to petroleum, has attracted increasing attention in recent years. The predominant process in biodiesel production is a transesterification phase, which consists of a chemical reaction between lipids and an alcohol (methanol or ethanol) in the presence of an alkali catalyst (Leoneti et al., 2012). However, this reaction also yields approximately 1 kg of crude glycerol, which comprises not only glycerol but also soap, salts, methanol, alkaline catalyst residues, and plant-derived organic matter, for every 10 kg of biodiesel production (Johnson and Taconi, 2007; Thompson and He, 2006). The increase in crude glycerol emissions resulting from the increase in biodiesel production is a serious

problem for the biodiesel industry, and is a financial and environmental responsibility.

The purification process for crude glycerol involving nanoparticle, enzyme catalytic system, supercritical, and membrane filtration methods yields pure glycerol (Wan Isahak et al., 2015; Ilham and Saka, 2016; Chol et al., 2018), but its attractiveness has been reduced in terms of high purification costs. Therefore, fermentation systems in which oleaginous microorganisms such as *Aurantiochytrium* sp., *Yarrowia lipolytica*, *Rhodotorula* sp., and *Cryptococcus* sp. produce biolipids with crude glycerol as a carbon source are becoming promising (Chang et al., 2015; Gao et al., 2016; Polburee et al., 2015; Poli et al., 2014). To use crude glycerol efficiently for fermentation, it is desirable to isolate microorganism strains exhibiting excellent glycerol utilization. We evaluated the lipid productivity of bacteria, yeast, and filamentous fungi growing in a medium containing crude glycerol. Here, we isolated three filamentous fungi that are superior in growth and lipid productivity on cultivation with high concentrations of pure glycerol as a carbon source. Furthermore, these strains grew well in a medium containing crude glycerol as a carbon source. They are expected to be useful breeding stocks for the biolipids production process involving pure and crude glycerol.

2. Materials and methods

2.1. Materials

Unrefined crude glycerol, which contains approximately 45% (w/w) glycerol, 13% (w/w) lipids/soap, 13% (w/w) methanol, 2.7% (w/w) potassium and other impurities, was obtained from the Kyoto Municipal Waste Edible Oil Fuel Production Facility, Japan. Pure glycerol and yeast extract were purchased from Nacalai Tesque Inc. (Japan) and Oriental

Yeast Co., Ltd. (Japan), respectively.

2.2. Isolation and identification of highly concentrated pure glycerol-assimilating fungi

Various soil and sewage samples were collected in Tokushima, Japan. The samples were streaked on to selection plates [20% (w/v) pure glycerol, 0.5% (w/v) yeast extract and 1% (w/v) agar] and then incubated at 28°C. Strains appearing on the plates were isolated and then cultivated in a medium containing 50% (w/v) pure glycerol and 0.5% (w/v) yeast extract at 28°C and 300 rpm for 7 days.

The strains isolated were identified by sequencing of the internal transcribed spacer and 5.8S ribosomal DNA region (ITS-5.8S rDNA) amplified with pair of primers; ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). The amplified PCR products were sequenced (Macrogen Inc., Kyoto, Japan) and analyzed using the BLASTn algorithm of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/).

2.3. Culture conditions

Crude glycerol medium, named CG medium, that contained 2-30% (w/v) crude glycerol and 1% (w/v) yeast extract was used for microbial cultivation. CG medium containing 10% crude glycerol included 4.5% glycerol component. To evaluate the effect of heat treatment of CG medium on microbial cultivation, CG medium containing 4% crude glycerol was treated at 40, 50, 60, 70, 80, 90, 105, or 110°C for 30 min or 1 h without autoclave sterilization. All strains were cultivated in the CG medium prepared with reciprocal shaking at 300 rpm and 28°C for 7 days and analyzed as to their fatty acid productivities.

2.4. Fatty acid analysis

Mycelial cells were harvested by suction filtration, washed with distilled water, and then dried at 110°C for 3 h. The dried cells were transmethylated with 10% methanolic HCl (acetyl chloride: methanol = 1:9, v:v) and dichloromethane at 55°C for 2 h. The resultant fatty acid methyl esters were extracted with *n*-hexane, concentrated, and then analyzed by gas chromatography (GC) as described previously (Okuda et al., 2015). Tricosanoic acid (23:0) was used as an internal standard. Mycelial total lipids were extracted with the chloroform/methanol/water system described by Bligh and Dyer (1959). To separate lipid classes, extracted total lipids were spotted on to thin-layer chromatography Silica Gel 60 F254 plates (Merck KGaA, Germany) and then developed with hexane/diethyl ether/acetic acid (80:20:1, v:v:v). The lipid classes were visualized with a primulin solution [0.001% (w/v) primulin in an acetone-water solution (4:1, v:v)] and analyzed by GC, as described above.

3. Results and discussion

3.1. Crude glycerol tolerance and fatty acid productivity of the isolated strains

In this study, we obtained three filamentous fungi, *Penicillium* sp. N1, *P. citrinum* N3, and *Fusarium oxysporum* W1, which were cultured in medium containing 50% pure glycerol. These strains showed 21-33 g/L of lipid productivity in medium containing 10% pure glycerol. Compared to oleaginous fungus *Mortierella alpina* CBS754.68, three strains isolated in this study exhibited higher growth and lipid productivity on the medium containing high concentrations of pure glycerol. Strains N1 and N3 grew well in CG

medium containing 10% crude glycerol and their dry cell weights (DCW) had reached 41.0 g/L and 29.7 g/L, respectively, on the 7th day (Fig. 1). The growth of strains N1 and N3 was poor in the CG medium containing 20% or more crude glycerol (Fig. 1). On the other hand, the DCW of strain W1 reached the highest value of 24.5 g/L on cultivation in the CG medium containing 20% crude glycerol (Fig. 1). These results indicated that strain W1 is more resistant to crude glycerol than strains N1 and N3. In particular, the weight of the total fatty acids in strain W1 accounted for about 50% of its DCW (Fig. 1). The lipid productivity of three strains was higher when they were cultivated in CG medium (Fig. 1) than in pure glycerol medium, suggesting that these three strains efficiently took up lipids contained in crude glycerol into their cells.

Crude glycerol, which contains some impurities such as methanol, soap, and alkali catalysts, was reported to inhibit the growth of microorganisms and bioconversion into value-added products (Ardi et al., 2015). Studies on the fermentation process using 2-8% crude glycerol with low methanol and soap content as a carbon source have been reported so far (Chatzifragkou et al., 2011; Liang et al., 2010; Tang et al., 2009). On the other hand, in the oleaginous yeast *Rhodosporidium toruloides* 32489, the lipid production by using crude glycerol was higher than that by using glucose or pure glycerol (Gao et al., 2016). Among the impurities in crude glycerol, methyl oleate, sodium oleate, and NaCl had a promoting effect in this yeast, while only methanol had an inhibitory effect (Gao et al., 2016). The crude glycerol sample used in this study contained more impurities as described above. Strain W1 isolated in this study, which grew in the CG medium containing 20% crude glycerol, is expected to be one of promising producers of microbial lipids.

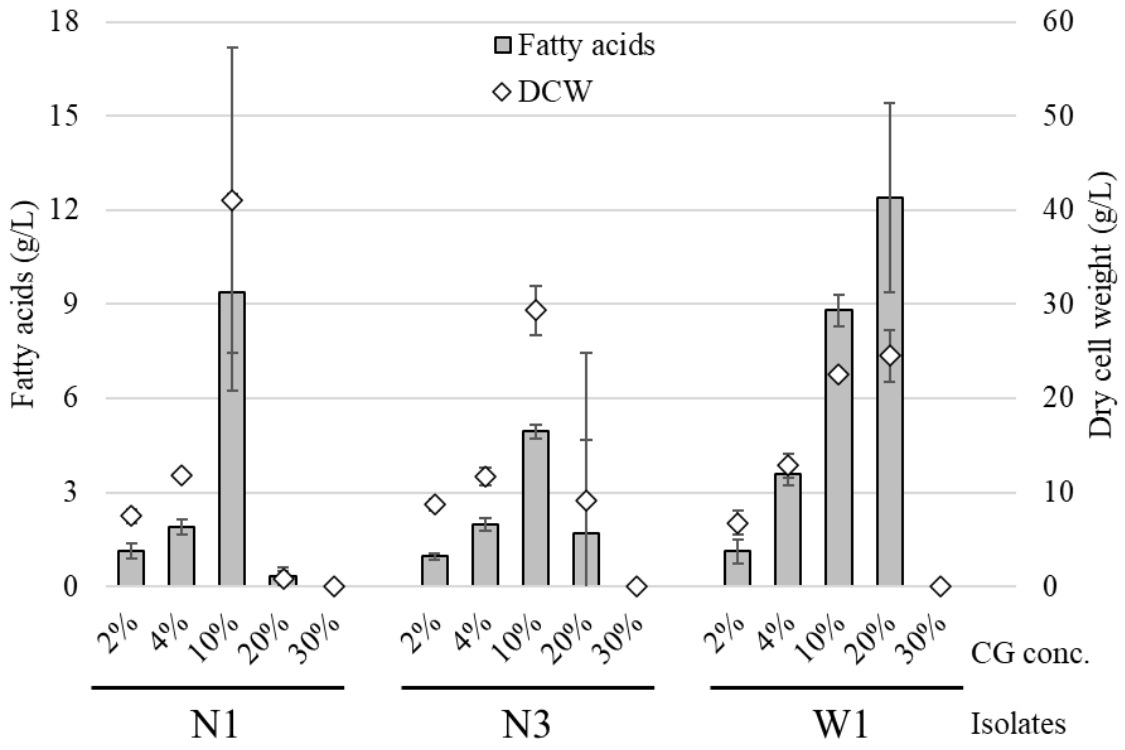


Fig. 1. The effects of crude glycerol on fatty acid production and fugal growth. The strains were cultivated in CG medium containing 2 to 30% crude glycerol for 7 days.

3.2. Analysis of fatty acid composition in each lipid class from mycelia cultivated in the CG medium

Quantitative analysis of lipid classes by TLC and GC revealed that the major lipid class in strains N1 and N3 cultured in the CG medium was free fatty acid (FFA) (Table 1). Fatty acid methyl ester (FAME) accounted for about 3% of the total lipids in strains N1 and N3. The crude glycerol used in this study contained about 13% (w/w) lipids/soap, in which FFA and FAME accounted for about 81% (w/w) and 18% (w/w), respectively (Table 1). These

results suggested that strains N1 and N3 accumulated FFAs and FAMEs derived from the crude glycerol in their mycelia. However, while the percentage of linoleic acid (18:2ω6) in the FFA fraction of crude glycerol was 27.73%, the FFA of strains N1 and N3 contained 7.74% and 6.53% of linoleic acid, respectively. Strains N1 and N3 accumulated about 20% FFAs in total lipids on cultivation in the medium containing pure glycerol as a carbon source (data not shown). FFAs induce cell death (lipotoxicity) and the inductive effect depends on the length of the carbon chain and the number of double bonds (Maia et al. 2010; Desbois and Smith 2010). In strains N1 and N3, the FFAs of linoleic and α-linolenic acid (18:3ω3), which are considered to be more toxic for mycelia, may have been selectively converted to acylglycerols such as diacylglycerols (DAGs) and triacylglycerols (TAGs) or metabolized via β-oxidation. *Penicillium* spp. N1 and N3 accumulated FFAs containing about 70% oleic acid (18:1ω9) in their mycelia. *Penicillium* spp. are known to have useful lipases and to produce fatty acids with grease waste as a substrate (Gutarra et al., 2009; Kumari et al., 2017; Lima et al., 2019). We first found in this study that *Penicillium* spp. N1 and N3 have high ability to accumulate FFAs.

The lipid composition in *F. oxysporum* W1, which included 96.98% TAG and 0.94% FFA, differed from those in *Penicillium* spp. N1 and N3 (Table 1). These results suggested that strain W1 utilized fatty acids derived from the crude glycerol for TAG synthesis as well as the crude glycerol as a carbon source. Generally, FFA is converted to acyl-CoA by acyl-CoA synthase and then bound to phospholipid, monoacylglycerol and diacylglycerol by acyltransferases. Strain W1 is expected to have high ability for lipid construction.

Table 1. Lipid class and fatty acid composition. The strains were cultivated in CG medium containing 10% crude glycerol.

Derivation	Lipid class[a]	Fatty acid content (% of TFA)	Relative fatty acid composition (%)[b]							
			16:0	18:0	18:1ω9	18:2ω6	18:3ω3	20:0	22:0	Others
N1	PL	3.04	13.21	3.81	48.44	29.43	2.05	1.07	0.53	1.46
	DAG	2.96	6.44	3.32	51.32	36.07	2.24	0.32	0.11	0.19
	FFA	56.19	3.19	12.07	69.77	7.74	2.29	3.26	1.22	0.47
	TAG	34.04	4.96	1.25	50.38	40.89	2.19	0.20	0.04	0.10
	FAME	3.43	4.89	6.22	67.27	11.78	2.07	2.84	2.99	1.93
	SE	0.34	22.45	27.06	39.52	7.52	1.67	0.74	0.73	0.31
N3	PL	2.29	15.37	5.59	47.13	25.87	2.30	1.17	0.78	1.80
	DAG	2.67	7.85	3.55	45.40	39.57	2.94	0.26	0.11	0.32
	FFA	48.52	3.68	12.04	70.94	6.53	2.21	3.00	1.15	0.45
	TAG	43.04	5.95	1.04	45.23	44.59	2.93	0.12	0.02	0.10
	FAME	3.20	7.47	7.88	60.73	15.53	1.66	2.15	2.93	1.65
	SE	0.28	27.30	28.55	31.04	10.01	1.41	0.49	0.63	0.58
W1	PL	1.34	16.33	2.90	18.92	59.52	1.72	0.06	0.09	0.46
	DAG	0.73	13.97	7.36	34.75	40.10	3.01	0.08	0.30	0.43
	FFA	0.94	15.91	9.35	27.96	20.17	9.52	0.21	12.11	4.77
	TAG	96.98	7.79	3.55	44.50	38.83	4.87	0.05	0.12	0.30
	FAME	n.d.[c]	—[d]	—	—	—	—	—	—	—
	SE	n.d.	—	—	—	—	—	—	—	—
Crude Glycerol	PL	1.19	68.81	31.19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DAG	n.d.	—	—	—	—	—	—	—	—
	FFA	81.05	11.50	3.97	52.25	27.73	4.54	n.d.	n.d.	n.d.
	TAG	n.d.	—	—	—	—	—	—	—	—
	FAME	17.75	18.57	5.61	49.74	23.00	3.09	n.d.	n.d.	n.d.
	SE	n.d.	—	—	—	—	—	—	—	—

[a] Abbreviations: PL, polar lipid; DGA, diacylglycerol; FFA, free fatty acid; TAG, triacylglycerol; FAME, fatty acid methyl ester; SE, sterol ester.

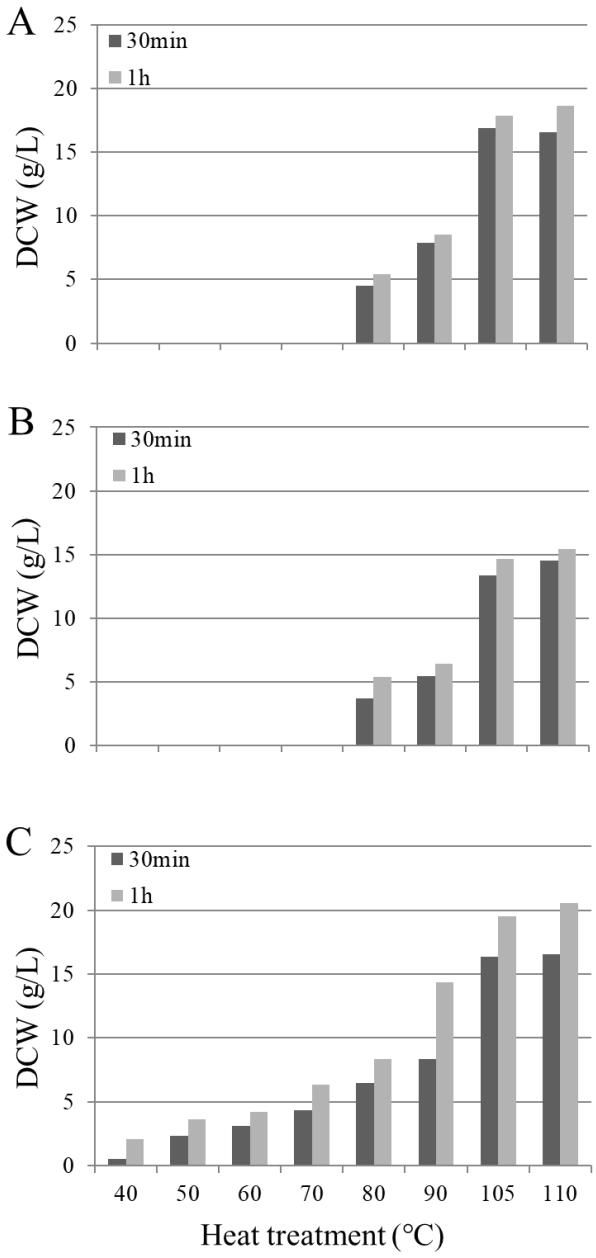
[b] Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω3, α-linolenic acid; 20:0, arachidic acid; 22:0, behenic acid.

[c] "n.d.", not detected.

[d] "—", not calculated.

3.3. Effect of heat treatment of CG medium on fungal cultivation

When crude glycerol, as an inexpensive carbon source is used for a mass culture in a bioreactor, a simpler sterilization method than autoclaving (121°C, 20 min) is desired. The effect of heat treatment of the CG medium on fungal cultivation was examined. The growth (indicated as DCW) of all strains was dependent on the temperature and time of heat treatment, as shown in Fig. 2. Strains N1 and N3 did not grow at all in the CG medium treated below 70°C, which indicated that the crude glycerol contains some growth inhibitors (such as methanol) that are decomposed or converted on heat treatment. On the other hand, growth of strain W1 was observed on cultivation in the CG medium treated above 40°C (Fig. 2), which indicated that strain W1 has strong resistance to the toxicity of the crude glycerol. The three strains were found to grow well in the CG medium treated at 105°C for one hour. The preparation of culture media is a challenge when mass culturing in large culture devices. Preparing the medium by a simpler heat treatment rather than autoclaving may lower the cost of culture. Crude glycerol, which contains alcohols, salts, and heavy metals in addition to glycerol, is a byproduct of the chemical transesterification of lipids in biodiesel production. The major impurity of methanol in the crude glycerol is very toxic for microorganisms. The culture of these fungi in CG medium subjected to simple heat treatment to produce microbial oils is expected to be new utilization method for waste crude glycerol.



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195 **Fig. 2.** Effect of heat treatment of the CG medium on fungal growth. *Penicillium* sp. N1
196 (A), *P. citrinum* N3 (B), and *F. oxysporum* W1 (C) were cultivated in CG medium
197 containing 4% crude glycerol for 7 days. The CG medium was heat-treated at 40, 50, 60, 70,
198 80, 90, 105, or 110°C for 30 min (dark grey) or 1 hour (light grey) before inoculation.

199

4. Conclusion

Utilization of crude glycerol is a serious issue in the process of generation of biodiesel. Simple and efficient methods for its regeneration are required to avoid environmental pollution. Three oleaginous fungi obtained in this study effectively produced biolipid in medium containing waste crude glycerol. Two strains, *Penicillium* spp., isolated are expected to be hosts accumulating FFAs. *F. oxysporum* W1 accumulated triacylglycerols as a major lipid and grew well on medium containing crude glycerol only heat-treated without autoclave sterilization. This study proposes a new application of crude glycerol to produce microbial oils.

CRediT authorship contribution statement

The authors' responsibilities were as follows: TS and ES conceived and designed the overall research. YK, CK, MK, and NM carried out the experimental work, analyzed, and interpreted data. TS and ES recommended and edited the paper. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material

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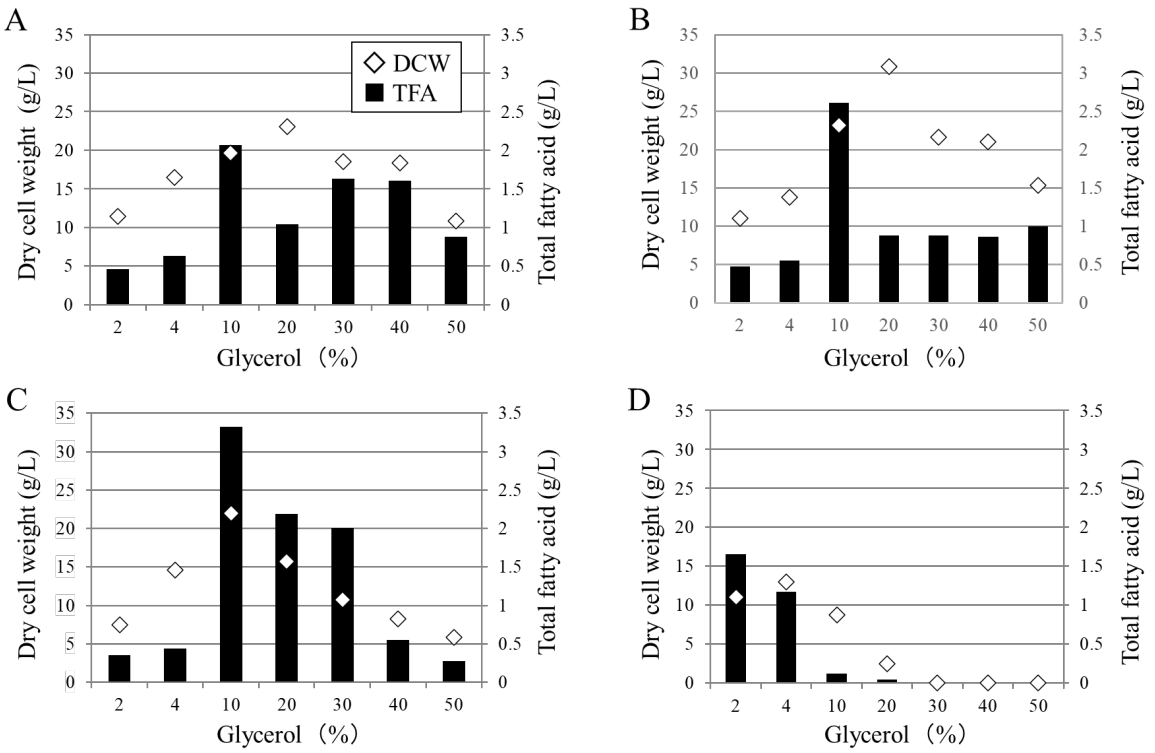

Supplemental Figure S1. Effect of glycerol concentrations on growth and lipid productivity. *Penicillium* sp. N1 (A), *P. citrinum* N3 (B), *Fusarium oxysporum* W1 (C), and *Mortierella alpina* CBS754.68 (D) as a standard strain of oleaginous filamentous fungi, were cultivated in the medium containing pure glycerol and 1% yeast extract for 7 days, at 28°C. Abbreviations: DCW, dry cell weight; TFA, total fatty acid.

Supplemental Table S1. Crude glycerol composition used as a carbon source and yield and accumulation of biolipids.

Oleaginous species	Crude glycerol component (%)[a,b]	Concentration of crude glycerol used	Lipid yield (g/L)	Lipid accumulation (% w/w)	References
Penicillium sp. N1	Glycerol (45), Lipids[c] (13), MeOH (13), Salt (2.7)	10% (w/v)	9.38	22.86	This study
P. citrinum N3		10% (w/v)	4.93	16.80	This study
Fusarium oxysporum W1		20% (w/v)	12.40	50.64	This study
Rhodosporidium toruloides 32489	Glycerol (49), Oleate (2), MeOH (18), Salt (1)	4% (w/v)	6.20	41.76	(Gao et al., 2016)
R. fluviale DMKU-RK253	Glycerol (82), NGOM (5.4), MeOH (0.1)	7% (w/v)	3.9	65.2	(Polburee et al., 2015)
Aurantiochytrium sp. TC 20	Glycerol (41), Fat (0.08), MeOH (-[c])	4% (w/v)	1.0	12.5	(Chang et al., 2015)
Yarrowia lipolytica QU21	Glycerol (83), Lipids[c] (-), MeOH (0.008), Salt (5.2)	8.3% (w/v)	1.27	18.96	(Poli et al., 2014)
Cryptococcus curvatus ATCC 20509	Glycerol (49), Lipids[c] (3), MeOH (23), Salt (-)	Total 437 g in 2 L fed-batch	17.4	52.9	(Liang et al., 2010)

[a] The numbers in parenthesis indicate percentages.

[b] Abbreviations: MeOH, methanol; NGOM, non-glycerol organics.

[c] "Lipids" contains soaps, free fatty acids, and fatty acid methyl esters.

[d] "-" indicates the components that were not described in the reference.