APPENDICES

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APPENDIX A

Media

A1: Yeast malt-extract medium (YM) agar slant (per liter)

Yeast extract	4.0	g
Malt extract	10.0	g
Glucose	4.0	g
Agar	15.0	g

YM agar slant preparation

Dissolved components of yeast-malt extract agar medium, adjusted to a final volume of 1,000 mL with distilled water and adjusted pH to 6.0 with H_3PO_4 or 1.0 N KOH, boiled at 100°C until the agar completely melt, then autoclaved at 121°C for 15 min.

A2: Yeast malt-extract medium (YM) (per liter)

Yeast extract	4.0	g
Malt extract	10.0	g
Glucose	4.0	g

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YM preparation

Dissolved all components of yeast-malt extract medium, adjusted to a final volume of 1,000 mL with distilled water and adjusted pH to 6.0 with H_3PO_4 or 1.0 N KOH and then, autoclaved at 121°C for 15 min.

A3: Basal medium supplemented with pure glycerol (BMP)

Yeast extract	1.0	g
Pure glycerol	20.0	g
Potassium di-hydrogen phosphate (KH_2PO_4)	5.5	g
Ammonium sulfate ($(NH_4)_2SO_4$)	5.3	g
Di-potassium hydrogen phosphate (K_2HPO_4)	3.7	g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.5	g
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.2	g
Sodium chloride (NaCl)	0.5	g

BMP preparation

Dissolved all components of basal medium supplemented with pure glycerol, adjusted to a final volume of 1,000 mL with distilled water and adjusted pH to 6.0 with H_3PO_4 or 1.0 N KOH and then, autoclaved at 121°C for 15 min.

A4: Basal medium supplemented with crude glycerol (BMC)

Yeast extract	1.0	g
Crude glycerol	20.0	g
Potassium di-hydrogen phosphate (KH_2PO_4)	5.5	g
Ammonium sulfate ($(NH_4)_2SO_4$)	5.3	g
Di-potassium hydrogen phosphate (K_2HPO_4)	3.7	g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.5	g
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.2	g
Sodium chloride (NaCl)	0.5	g

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BMC preparation

Dissolved all components of basal medium supplemented with crude glycerol, adjusted to a final volume of 1,000 mL with distilled water and adjusted pH to 6.0 with H_3PO_4 or 1.0 N KOH and then, autoclaved at 121°C for 15 min.

A5: The optimized basal medium

Yeast extract	1.0	g
Demethanolized crude glycerol	55.0	g
Potassium di-hydrogen phosphate (KH_2PO_4)	5.5	g
Ammonium sulfate ($(NH_4)_2SO_4$)	5.3	g
Di-potassium hydrogen phosphate (K_2HPO_4)	3.7	g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.5	g
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.2	g
Sodium chloride (NaCl)	0.5	g

The optimized basal medium preparation

Dissolved component of basal medium supplemented with demethanolized crude glycerol, adjusted to a final volume of 1,000 mL with distilled water and adjusted pH to 5.63 with H_3PO_4 or 1.0 N KOH and then, autoclaved at 121°C for 15 min.

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APPENDIX B

Crude glycerol analysis

B1: Glycerol concentration analysis by HPLC (Kusdiyantini et al., 1998)

System controller	:	SCL-10Avp (Shimadzu, Japan)
Column	:	Aminex (HPX-87H 300x7.8 mm; Bio-Rad, USA)
Detection	:	Refractive Index Detector (RID)
Oven temperature	:	40°C
Flow rate	:	0.75 mL/min
Mobile phase	:	5 mM sulphuric acid in deionized water
Injection volume	:	20 µL
Run time	:	20 min

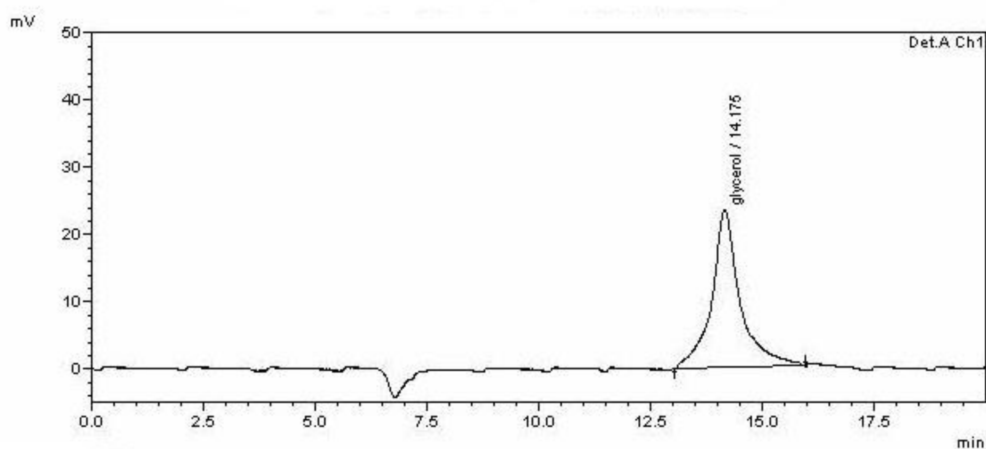

Figure B1 Chromatogram of glycerol analyzed by HPLC. The retention time of glycerol was 14.175 min

B2: Determination of lipids content in crude glycerol (Official Methods of Analysis of AOAC International, 2002)

Ten grams of crude glycerol were dissolved in *n*-hexane. The *n*-hexane extract was collected to determine of lipids content by slowly evaporated on water bath at 70–80°C for 60 min and transferred to desiccators until constant weight.

B3: Determination of ash content in crude glycerol (Official Methods of Analysis of AOAC International, 2002)

Ten grams of crude glycerol was used for the analysis of ash content in a crucible. Placed on hot plate with low heat until tested sample was black and dry and there was no danger of loss by foaming. Then, the tested sample was subjected in a furnace at 600°C to constant weight or overnight. The ash content was calculated as the formula as followed;

$$\text{Ash content (\%)} = \frac{\text{Weight of ash (g)} \times 100}{\text{Weight of sample (g)}}$$

B4: Determination of moisture content (Official Methods of Analysis of AOAC International, 2002)

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Moisture content usually is determined by the loss in weight that occurs in sample upon drying to a constant weight in an oven. The official methods involve drying a representative sample in a vacuum oven at 95–100°C of for 2 h. Ten grams of crude glycerol was used for the analysis of moisture content in a moisture can and heated at 100°C for 2 h and transferred to desiccators until constant weight. The moisture content was calculated as the formula as followed;

$$\text{Moisture content (\%)} = \frac{(A-B) \times 100}{C}$$

A = Weight of can and sample before drying in an oven (g)

B = Weight of can and sample after drying in an oven (g)

C = Weight of sample (g)

B5: Determination of methanol content in crude glycerol (Thompson and He, 2006)

Weighed ten grams of crude glycerol in moisture can heated at 85°C for 1 h to remove methanol. The methanol content in the glycerol layer was calculated as the formula as followed;

$$\text{Methanol content (\%)} = \frac{(A-B) \times 100}{C}$$

A = Weight of can and sample before heated (g)

B = Weight of can and sample after heated (g)

C = Weight of sample (g)

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APPENDIX C

Carotenoids analysis

C1: Carotenoids extraction (Manowattana et al., 2012)

1. Ten milliliters of culture broth was taken from each flask or bioreactor and then was centrifuged at 6,000 rpm (4,146 *g*) at 4 °C for 10 min (Hettich MIKRO 22R; Germany).
2. The clear supernatant was subjected to high-performance liquid chromatography (HPLC) analysis for glycerol concentration determination.
3. The cell pellet was washed twice with *n*-hexane (LabScan, Thailand) and once with distilled water.
4. After washing, the cell pellet was stored in -20°C for 24−48 h.
5. The carotenoids content of cell pellet was extracted by a method which broke the yeast cell carried out in screw cap tube (25x150 mm), containing 10.0 mL acetone (Merck, Germany) and 4.0 g of glass beads (size 3 mm).
6. The mixture was vigorously shaken in a vortex mixer for 15 min in the presence of 100 ppm ascorbic acid.
7. The broken cell was centrifuged at 6,000 rpm at 4ºC for 10 min and the clear supernatant was collected and dried by flushing it with N_2, then re-dissolved in 1.0 mL *n*-hexane.
8. The *n*-hexane phase was filtered through a nylon membrane filter (0.2 μm) and subjected to HPLC analysis.

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C2: Quantitative analysis of carotenoids by HPLC (Wang et al., 2007)

Conditions

System controller	:	SCL-10Avp (Shimadzu, Japan)
Column	:	C18 (5 µm 250x4.6 mm)
Detection	:	UV detector at 454 nm
Oven temperature	:	30°C
Flow rate	:	1.00 mL/min
Mobile phase	:	Acetonitrile/Dichloromethane/Methanol (80:10:10, v/v/v)
Injection volume	:	20 µL
Run time	:	45 min

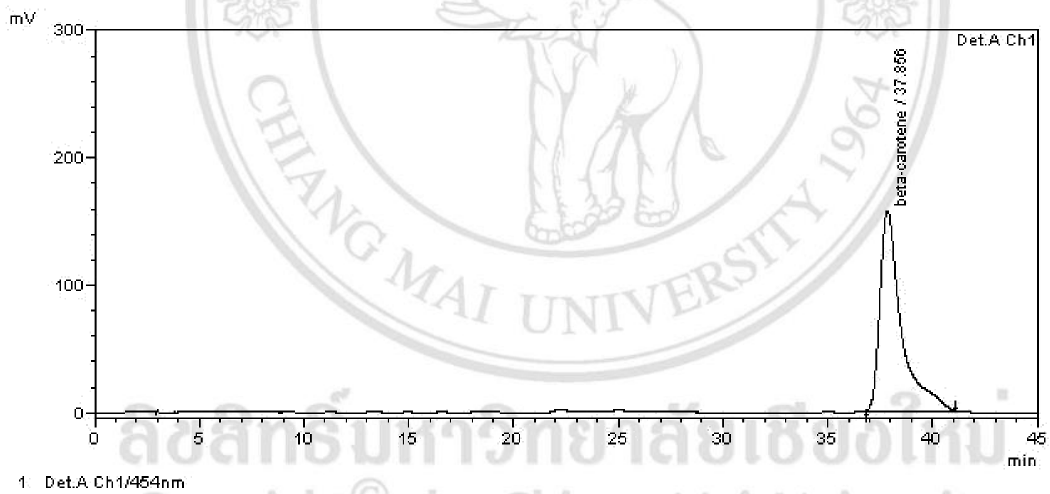

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Figure C1 Chromatogram of β-carotene analyzed by HPLC. The retention time was 37.856 min.

A standard curve of β-carotene concentration was determined. Integration of the area below the absorbance peak of β-carotene at the concentrations varied between 0−100 mg/L, resulted in the standard curve shown in Figure C2.

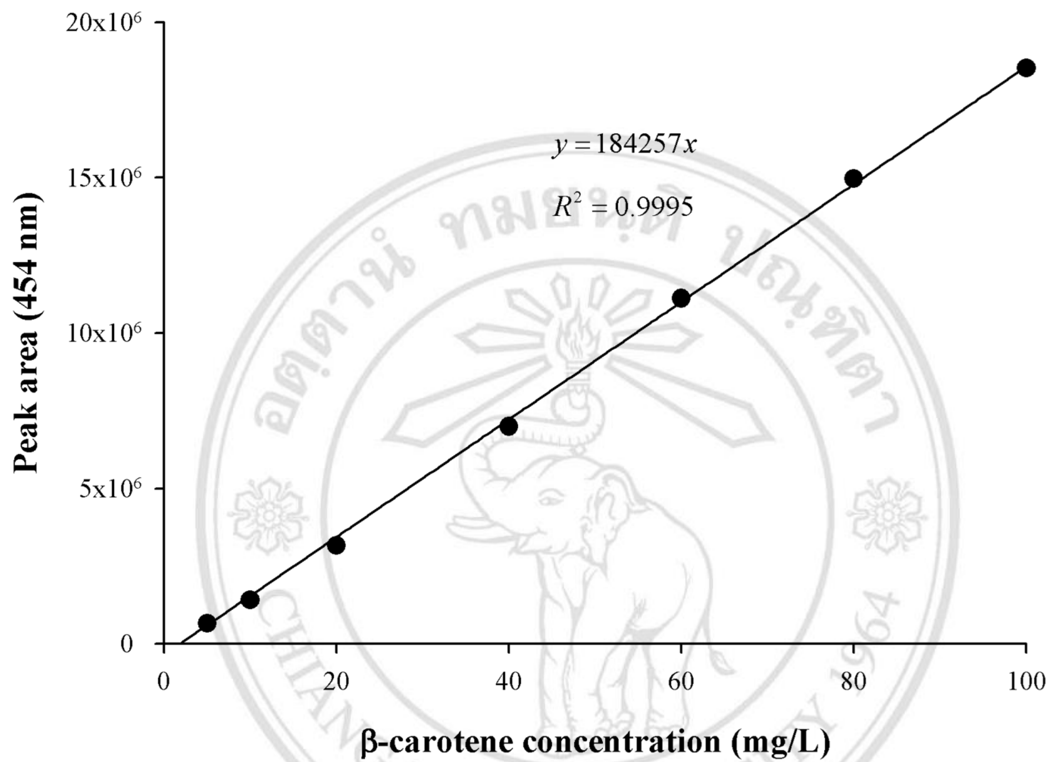

Figure C2 The β-carotene concentration standard curve

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APPENDIX D

Fatty acid methyl ester analysis

D1: Lipids extraction (Bligh and Dyer, 1959)

1. Twenty milliliters of culture broth was taken from each flask or bioreactor and then was centrifuged at 6,000 rpm at 4°C for 10 min.
2. The clear supernatant was subjected to HPLC analysis for glycerol concentration determination.
3. The cell pellet was washed twice with *n*-hexane and once with distilled water.
4. After washing, the cell pellet was stored in -20°C for 24−48 h.
5. The extracted lipids from the yeast biomass was extracted by a method which broke the yeast cell carried out in screw cap tube (25x150 mm), using a mixture of chloroform : methanol (2:1, v/v) and 4.0 g glass beads (size 3 mm).
6. The mixture was vigorously shaken in a vortex mixer for 30 min and sonicated at 70 Hz for 30 min.
7. The ruptured cells and crude extracted lipids were centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min, after that the clear supernatant was collected, and the organic solvent was removed by evaporation under vacuum of 300 mm bar.
8. The volumetric productivity of lipids was expressed as g/L of the culture broth.

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D2: Fatty acid methyl esters (FAME) (Chaiyaso et al., 2012).

1. After evaporation of excess solvent, 10 mg of acylglycerol was methanolized with 0.5% KOH in methanol (500 µL) and then incubated for 15−20 min at 60°C.
2. The FAME was extracted with *n*-hexane (400 µL) for 1 min.
3. The hexane phase was washed with 200 µL DI-water and dried over sodium sulfate anhydrous.

D3: Gas chromatography with a flame ionization detector (GC-FID)

Conditions EN14103:2011 method (McCurry, 2011)

System controller	:	GC-2010; Shimadzu, Japan
Column	:	HP-INNOWAX column
		(30 m × 0.25mm, 0.25 µm film thickness)
Detection	:	flame ionization detector (FID)
Inlet temperature	:	250°C
Detector temperature	:	250°C
Oven temperature	:	60°C (for 2 min), 10°C/min to 200°C and
		5°C/min to 240°C (for 7 min)
Flow rate	:	1.0 mL/min
Carrier gas	:	Helium
Injection volume	:	1 µL
Split ratio	:	100:1

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D4: Gas chromatography with a mass spectroscopy (GC-MS)

Conditions (McCurry, 2011)

System controller	:	GC 7890A: MSD 5975C (EI): Agilent; USA
Column	:	DB5-MS column
		(30 m × 0.25 mm, 0.25 µm film thickness)
Detection	:	mass spectroscopy (MS)
Inlet temperature	:	250°C
Detector temperature	:	250°C
Oven temperature	:	60°C (for 2 min), 10°C/min to 200°C and
		5°C/min to 240°C (for 7 min)
Flow rate	:	1.0 mL/min
Carrier gas	:	Helium
Injection volume	:	1 µL
Split ratio	:	100:1
Scan parameter	:	50–500 amu
MS quadrupole	:	150°C
MS source	:	230°C

The GC-MS analysis was done by the Science and Technology Service Center, Chiang Mai University (STSC-CMU), Thailand.

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APPENDIX E

Nucleotide sequence of 26S rRNA gene of *Sporidiobolus pararoseus* KM281507

Accession number: KM281507

Identify: *Sporidiobolus pararoseus*

26S rRNA sequence (501 bp):

CGNCGTCCGAGTTGTAATCTCGAGAAGTGTTTTCCGTGATAGACCGCATACAAGTCT
CTTGGAACAGAGCGTCATAGTGGTGAGAACCCAGTACACGATGCGGATGCCTATTA
CTTTGTGATACACTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAATTGGGTG
GTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGT
GAGGGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAACAGTACGTGAAATTGTTG
GAAGGGAAACACATGCAGTGATACTTGCTATTCGGGGCAACTCGATTGGCAGGCCC
GCATCAGTTTTTCGGGGCGGAAAATCGTAGAGAGAAGGTAGCAGTTTCGGCTGTGT
TATAGCTCTTTACTGGATTCGCCCTGGGGGACTGAGGAACGCAGCGTGCTTTTAGCA
TGAGCTTCGGCTTATCCACGCTTAGGATGCGGGTTTATGGCTGTATATGACCCGTCT
TGAAAACAC

BLAST result: *Sporidiobolus pararoseus* 26S ribosomal RNA gene, partial sequence

Sequence ID: AF070437.1

Length: 601

Score = 942 bits (510), Expect = 0.0

Identities = 514/516 (99%), Gaps = 1/516 (0%), Strand = Plus/Plus

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Sbjct  86   CGACGTCCGAGTTGTAATCTCGAGAAGTGTTTTCCGTGATAGACCGCATACAAGTCTCTT  145

Query  61   GGAACAGAGCGTCATAGTGGTGAGAACCCAGTACACGATGCGGATGCCTATTACTTTGTG  120
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Publications

[1] **Manowattana, A.**, Techapun, C., Seesuriyachan, P. and Chaiyaso, T. 2011. Carotenoids production from red yeasts using waste glycerol as a sole carbon source. Thai Journal of Agricultural Science. 44(5): 95-100.

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