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Comparison of extraction phases for a two-phase culture of a recombinant *E. coli* producing retinoids

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Abstract To prevent degradation of intracellular retinoids through in situ extraction from the cells, a two-phase culture system was performed. Several organic solvents, including *n*-alkanes, mineral oils and cosmetic raw materials, were applied as the extraction phase. Of the *n*-alkanes, *n*-decane had the highest retinoid production as 134 mg/l after 72 h. For mineral oil, light and heavy mineral oil gave retinoid productions of 158 and 174 mg/l after 96 h, respectively. Of other materials, isopropyl myristate gave the highest retinoid production of 181 mg/l. These results indicate that many types of oils can be applied for retinoid production, and optimization of the in situ extraction process will lead to further improve of economical production for the industrial purpose.

Keywords *Escherichia coli* · In situ extraction · Retinoids · Two-phase culture

Introduction

Retinoids are lipophilic isoprenoid molecules composed of a β-ionone ring and a polyunsaturated side-chain. They include retinal, retinol, retinyl esters and retinoic acid with functional groups of aldehyde, alcohol, ester, and carboxylic acid, respectively (Sorg et al. 2006). Retinoids are synthesized from β-carotene

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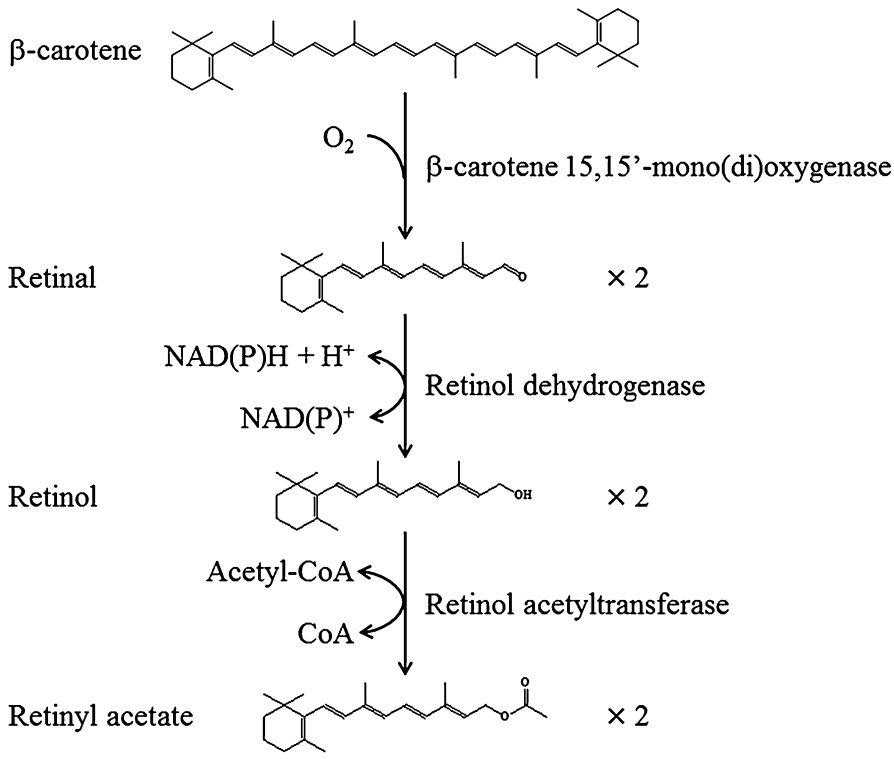


Fig. 1 Retinoid biosynthesis from β-carotene. Retinal is derived from the 15,15′-cleavage of β-carotene by β-carotene mono(di)oxygenase, which is successively converted to retinol and retinyl acetate by retinol dehydrogenase and retinol acetyltransferase, respectively

by β-carotene mono(di)oxygenase and its end-modification enzymes (Fig. 1). For commercial uses, retinoids are synthesized chemically from a pentadiene derivative; retinol is used as a cosmetic ingredient (Sorg et al. 2006; Mercier 1994). However, chemical processes have some disadvantages such as complex purification steps and undesirable by-product formation. There have been some attempts for the biological production of retinoids (Sabehi et al. 2005; Martinez et al. 2007) and we have achieved this by a two-phase culture from metabolically-engineered *Escherichia coli* (Jang et al. 2011).

The two-phase culture system uses a dodecane-overlay on the culture broth to minimize the intracellular degradation of retinoids. All of retinoids could be extracted directly from the cell and thus retinoids were not detected in culture broth. A number of studies have been performed on the in situ two-phase extraction for bioreactors of various hydrophobic chemicals, mostly on isoprenoid production from microalgae (Hejazi and Wijffels 2003; Leon et al. 2005; Kleinegris et al. 2011). Two-phase culture has also been used for in situ extraction of farnesol, lycopene, and retinoids from metabolically-engineered *E. coli* (Wang et al. 2010; Yoon et al. 2008; Jang et al. 2011). Extraction phases in two-phase culture for isoprenoid production are mostly alkanes, especially decane and dodecane. Major important factors for the extraction phase include low inhibition of cell viability, good immiscibility with the culture broth, and suitable density for phase separation. Furthermore, the stability of the product in the extraction phase, the economical price of the extraction phase, and the efficient usage of the product in the extraction phase could be regarded as important factors for the extraction phase. In this study, several extraction phases were applied for the in situ extraction of retinoids produced from metabolically engineered *E. coli*, including *n*-alkanes and raw materials used in the cosmetic industry, such as emollients, emulsions, and moisturizer.

Materials and methods

Bacterial strains and plasmids

Escherichia coli DH5α (F−, ϕ80d*lacZΔ*M15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1 *endA*1, *hsdR*17(rK− mK+), *phoA*, *supE*44, λ−, *thi*-1, *gyrA*96, *relA*1) was used for gene cloning and retinoid production. For

retinoid production, plasmids of pT-DHBSR and pS-NA containing the retinoid biosynthesis pathway and the mevalonate pathway, respectively, were introduced into *E. coli* DH5α, as previously reported (Jang et al. 2011). The plasmid pT-DHBSR was composed of *crtE*, *crtB*, and *crtI* from *Pantoea agglomerans*, *ipi*HP1 from *Haematococcus pluvialis*, *crtY* from *Pantoea ananatis*, *dxs* from *E. coli*, and SR gene derived from uncultured marine bacterium 66A03. The plasmid pS-NA was composed of *mvaE* and *mvaS* from *Enterococcus faecalis*, *mvaK1*, *mvaK2*, and *mvaD* from *Streptococcus pneumoniae* and *idi* from *E. coli*.

Culture conditions

The retinoid-producing *E. coli* was grown in 2YT medium (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) at 29 °C and 250 rpm. According to previous results (Jang et al. 2011), 2 % (w/v) glycerol and 0.2 % (w/v) arabinose were added as carbon sources. 100 μg Ampicillin/ml and 50 μg chloramphenicol/ml were added as required. Cell growth was determined from the OD_{600} value. For the two-phase culture, various extraction phases of volumes ranging from 1 to 5 ml were overlaid on 5 ml culture broth. *n*-Alkanes and cosmetic raw materials were used as extraction phases (see Table 1).

Quantification of retinoids

Extraction phases were separated from the culture broth by centrifugation for 10 min at 16,100×*g* and collected for analysis. In the case of alkanes, separated extraction phases were directly applied to HPLC to analyze retinoids. Retinoids in cosmetic raw materials were extracted using acetone: a small volume of the retinoid-containing extraction phase was re-suspended in 1 ml acetone and incubated at room temperature for 15 min with intermittent vortexing. The mixture was then centrifuged for 10 min at 16,100×*g*, and the upper acetone phase was applied to HPLC for retinoid analysis. HPLC analysis was carried out using a Symmetry C18 column (250 × 4.6 mm, 5 μm, Waters, Milford, USA) with a Sentry Guard C18 (15 × 4.6 mm, 5 μm, Waters, Milford, USA). The mobile phase was methanol/acetonitrile (95:5, v/v) at 1.5 ml/min. Retinal was detected at 370 nm and retinol and retinyl acetate at 340 nm. The results are presented in the mean ± SD from three independent experiments. Retinal, retinol and retinyl acetate (all from Sigma, USA) were dissolved in acetone to use as standard compounds.

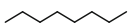
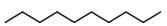
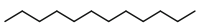
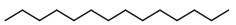
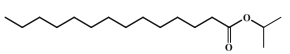
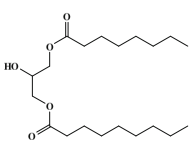
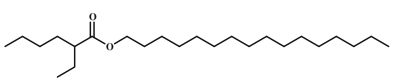
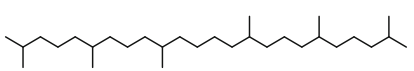
Results and discussion

Retinoid production from two-phase culture using *n*-alkanes

n-Alkanes including decane and dodecane are currently the most favored extraction phases for two-phase cultures of microorganisms due to their low toxicity, high hydrophobicity and low volatility. In this study, *n*-alkanes of 8–14 carbons were used as the extraction phase for retinoid production from two-phase cultures of metabolically engineered *E. coli*. The physicochemical properties of *n*-alkanes used in this study are presented in Table 1. Retinoid-producing *E. coli* was grown in 2YT medium containing 0.2 % (w/v) arabinose and 2 % (w/v) glycerol for 72 h at 29 °C (Fig. 2). Two-phase cultures using *n*-alkanes were performed by overlaying 5 ml *n*-octane (C8), *n*-decane (C10), *n*-dodecane (C12), and *n*-tetradecane (C14) on 5 ml culture broth in test tubes. Retinoids were effectively extracted into all of *n*-alkane extraction phase to produce 85.9–134.2 mg/l of total retinoid after 72 h. The highest retinoid production was obtained with *n*-decane, which also showed the highest cell specific productivity among the *n*-alkanes (5.9 mg/l per OD unit).

As shown in our previous results (Jang et al. 2011), when different volumes of dodecane were added, the highest level of retinoid production was obtained after 72 h with 5 ml dodecane overlaid on 5 ml culture broth. With *n*-alkane extraction phases, a larger volume of the extraction phase might be more effective due to its relatively low viscosity of 0.77–2.86 cSt (Table 1). For all *n*-alkanes, cell growth was not significantly different (22–26 OD units) at 72 h which suggested that cell viability is not affected by the extraction phase. Organic solvents with log*P* values between 1 and 5 are toxic to microbial cells because they disrupt the integrity of cell membranes (Aono 1998; Inoue and Horikoshi 1989; Nicolaou et al. 2010; Ramos et al. 2002). The solvents used in this study have log*P* values above 5, with the exception of octane (log*P* of 4.27), and are probably

Table 1 Properties of water and extraction phases used in this study

Compound	CAS number[a]	Molecular weight	Density (g cm^{-3})	Partition coefficient (log*P* or $logK_{0w}$)[b]	Viscosity (cSt at 20 °C)	Vapor pressure (Pa at 30 °C)[c]	Structure[d]
Water	7732-18-5	18.02	1	−1.38	1	0.0000396	
Octane	111-65-9	114.23	0.703	4.27	0.771	2,590	
Decane	124-18-5	142.28	0.73	5.25	1.26	320	
Dodecane	112-40-3	170.33	0.75	6.23	1.8	45.9	
Tetradecane	629-59-4	198.39	0.763	7.22	2.857	7.52	
Mineral oil, light[e]	8042-47-5; 8012-95-1	405.6	0.83–0.86 Rawlings and Lombard (2012), Nash et al. (1996)	>6.0	14.2–17.0	<100	
Mineral oil, heavy[f]	8042-47-5; 8012-95-1	405.6	0.875–0.905 Rawlings and Lombard (2012), Nash et al. (1996)	>6.0	63.6–70.4	<100	
Isopropyl myristate	110-27-0	270.45	0.853	7.17	7.62	0.178	
ODO (caprylic/capric triglyceride)	65381-09-1; 73398-61-5	372.54	0.95	5.3–6.2	23	<0.00000909	
Cetyl ethylhexanoate	59130-69-7	368.72	0.86	10.61	14	0.000287	
Squalane (phyto-)	111-01-3	422.81	0.803	14.63	37.4	0.0612	

[a] Chemical abstract service registry number

[b] Estimated from KOWWIN Program v1.68 of EPI suite v4.11 (Environmental Protection Agency, USA)

[c] Estimated from MPBPWIN program v1.43 of EPI suite v4.11 (Environmental Protection Agency, USA)

[d] PubChem Compound of NCBI (http://www.ncbi.nlm.nih.gov/pccompound)

[e] Obtained from sigma, USA

[f] Obtained from Daejung, Korea

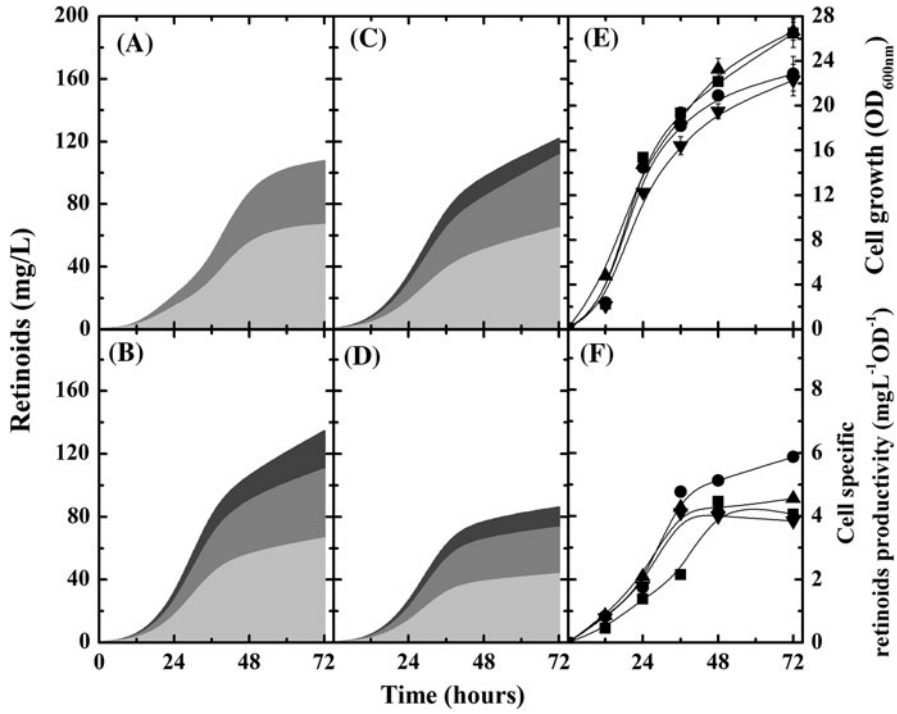


Fig. 2 Retinoid production from two-phase culture using *n*-alkanes with various carbon numbers. Two-phase culture was performed using **a** *n*-octane, **b** *n*-decane, **c** *n*-dodecane, and **d** *n*-tetradecane as extraction phases. Produced retinal, retinol, and retinyl acetate are indicated with *light gray*, *gray*, and *dark gray*, respectively. **e** *Squares*, *circles*, *triangles*, and *reversed triangles* indicate cell growth from two-phase culture using *n*-octane, *n*-decane, *n*-dodecane, and *n*-tetradecane, respectively. **f** *Squares*, *circles*, *triangles*, and *reversed triangles* indicate cell specific retinoid productivity from two-phase culture using *n*-octane, *n*-decane, *n*-dodecane, and *n*-tetradecane, respectively

not toxic to *E. coli*. The ratio of extracted retinoids was about 49–53 % retinal, 33–38 % retinol, and 9–18 % retinyl acetate at 72 h, except for *n*-octane. In the case of *n*-octane, retinyl acetate was not extracted into the extraction phase at all; however, when the intracellular amount of retinoids was measured, retinyl acetate was present at 2.06 mg/l at most (data not shown). Therefore, the production of retinyl acetate is not inhibited by *n*-octane, but its extraction is for unknown reasons.

Retinoid production from two-phase culture using mineral oils

Mineral oils are composed of a mixture of saturated hydrocarbons including various branched-chain alkanes and cyclic alkanes. They are refined from petroleum. Mineral oils are classified by their viscosity as light mineral oil with 15–25 carbon numbers and heavy mineral oil with 25–50 carbon numbers. Highly refined white mineral oil has been approved by the US FDA as a non-toxic generally regarded as safe (GRAS) ingredient and is used in medicine, cosmetics and food (Rawlings and Lombard 2012; Nash et al. 1996). *n*-Alkanes, however, can cause irritation to the skin which makes them unsuitable as raw materials for cosmetics. Another major advantage of mineral oils is their low cost, especially heavy mineral oil which is cheaper than light mineral oil. The physicochemical properties of light mineral oil and heavy mineral oil used in this study are presented in Table 1. The recombinant *E. coli* was grown for retinoid production in 2YT medium containing 0.2 % (w/v) arabinose and 2 % (w/v) glycerol for 96 h at 29 °C, and 5 ml light mineral oil as the extraction phase was layered over 5 ml of culture broth in test tubes (Fig. 3). Cell growth was relatively slow with light mineral oil and 51 mg retinoid/l was produced. This production is significantly lower than 136 mg/l with dodecane as the extraction phase. However, cell specific retinoid productivity was not significantly different at 5 and 5.2 mg/l/OD for light mineral oil and dodecane, respectively. Therefore, a lower total retinoid production with light mineral oil is possibly due to growth retardation. Light mineral oil has a viscosity of 14–17 cSt, which is higher than that of *n*-alkanes at 0.77–2.86 cSt. When 5 ml light mineral oil was overlaid as the extraction phase, it did not mix well with the culture medium due to its higher viscosity than *n*-alkanes, leading to both lower cell growth and retinoid production.

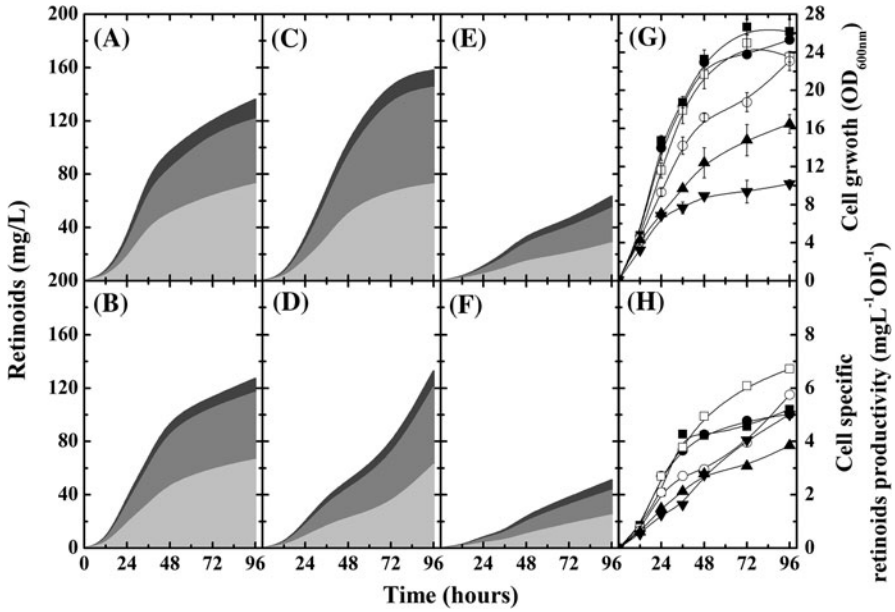


Fig. 3 Retinoid production from two-phase culture using light mineral oil. Two-phase culture was performed using **a** 5 ml dodecane, and **b** 1 ml, **c** 2 ml, **d** 3 ml, **e** 4 ml, and **f** 5 ml of light mineral oil as extraction phases. Produced retinal, retinol, and retinyl acetate are indicated with *light gray*, *gray*, and *dark gray*, respectively. For **g** cell growth and **h** cell specific retinoid productivity, the dodecane and the overlaid light mineral oil volumes are indicated with the following symbols: 5 ml dodecane *closed squares*, 1 ml *closed circles*, 2 ml *open squares*, 3 ml *open circles*, 4 ml *closed triangles*, 5 ml *closed reversed triangles*

Since the excessive addition of light mineral oil caused growth retardation, various volumes of light mineral (1–5 ml) were used during the culture (Fig. 3). As the volume of light mineral oil increased, retinoid production decreased due to slower cell growth. The highest retinoid production of 158 mg/l was obtained after 96 h with 2 ml light mineral oil, which was ~1.2-fold higher than that with 5 ml dodecane (136 mg/l). Cell specific retinoid productivity was also the highest with 2 ml light mineral oil, at 6.7 mg/l per OD unit. Since 2 ml mineral oil produced larger amounts of retinoid than 5 ml dodecane, it can be concluded that light mineral has a higher retinoid extracting efficiency.

Heavy mineral oil was also used as the extraction phase to compare retinoid production. The recombinant *E. coli* was grown in 2YT medium containing 0.2 % (w/v) arabinose and 2 % (w/v) glycerol for 96 h at 29 °C, and 2 ml heavy mineral oil was overlaid on 5 ml culture broth (Fig. 4). With 2 ml heavy mineral oil, the total retinoid production after 96 h was 88 mg/l, which is lower than that with light mineral oil at 158 mg/l. Just as with light mineral oil, retinoid production probably decreased due to growth retardation. The viscosity of heavy mineral oil at 63–70 cSt is 3.7–5 times higher than that of light mineral oil (~14–17 cSt). Therefore, it was expected that the total retinoid production with heavy mineral oil would increase if the extraction phase and the culture medium were mixed well enough as to not slow down cell growth. The recombinant *E. coli* was grown with heavy mineral oil in the same conditions as before, but with the test tube being slanted to facilitate mixing of the two phases (Fig. 4). Cell growth did not change significantly but the efficiency of retinoid production increased. Furthermore, retinoid production after 96 h was 174 mg/l and the cell specific retinoid productivity was 7.6 mg/l per OD unit, higher than 6.7 mg/l per OD unit with 2 ml light mineral oil. Since cell growth did not change much, the observed increase in the efficiency of retinoid production was due to a better mixing of heavy mineral oil and culture medium. When 2 ml light mineral oil was used as the extraction phase, the ratio of retinoids was 46 % retinal, 46 % retinol and 8 % retinyl acetate, whereas when 2 ml heavy mineral oil was used, the ratio was 58 % retinal,

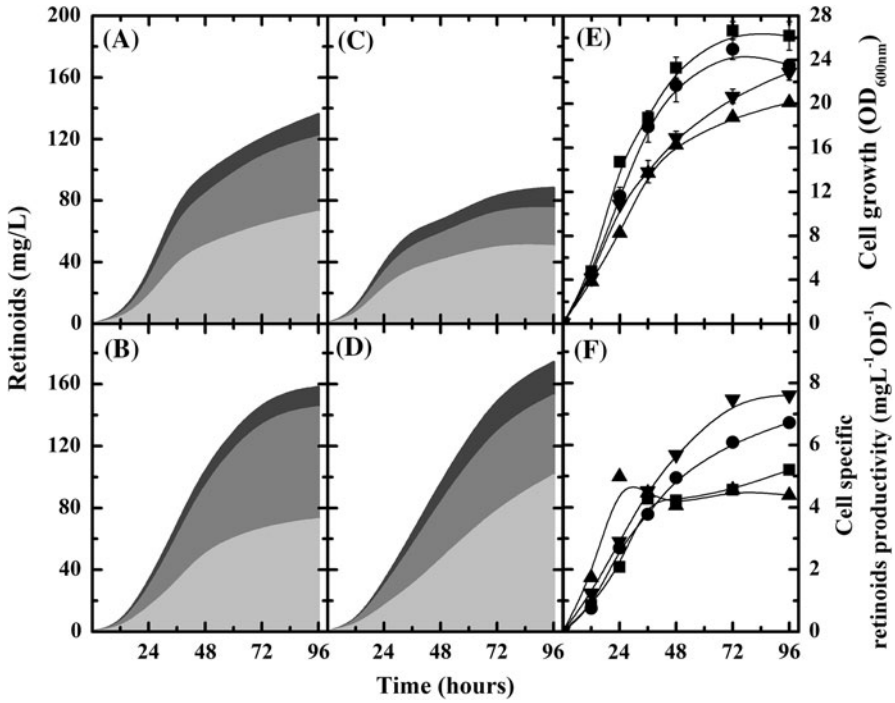


Fig. 4 Retinoid production from two-phase culture using mineral oils. Two-phase culture was performed using **a** 5 ml dodecane and **b** 2 ml light mineral oil, and **c** vertical and **d** slant position of 2 ml heavy mineral oil as extraction phases. Produced retinal, retinol, and retinyl acetate are indicated with *light gray*, *gray*, and *dark gray*, respectively. For **e** cell growth and **f** cell specific retinoid productivity, the extraction phases are indicated with the following symbols: 5 ml dodecane *squares*, 2 ml light mineral oil *circles*, vertical position of 2 ml heavy mineral oil *triangles*, slanted position of 2 ml heavy mineral oil *reversed triangles*

30 % retinol and 12 % retinyl acetate. With heavy mineral oil, the proportion of retinal was higher but the proportion of retinol was lower than with light mineral oil. Retinal was more effectively extracted using heavy mineral oil, before it was intracellularly converted to retinol. Therefore, if the extraction phase and the culture medium are well mixed, a higher yield of retinoid production could be achieved with heavy mineral oil, when compared to light mineral oil.

Retinoid production from two-phase culture using cosmetic raw materials

As cosmetic raw materials, isopropyl myristate, ODO (caprylic/capric triglyceride), cetyl ethylhexanoate, and phyto-squalane were used for the two-phase culture. These extraction phases are either synthetic or natural oils widely used in the cosmetic and pharmaceutical industries as a premium emollient, emulsifier, thickening agent, lubricant, parting agent, antifoaming agent, and moisturizer (Traul et al. 2000; Kuo et al. 2012; Kim and Karadeniz 2012). If such cosmetic raw materials are used as the extraction phase in retinoid production, they can be used as a cosmetic ingredient directly without further separation or refinement, which offers a huge advantage. Also, caprylic/capric triglyceride and phyto-squalane are present in coconut oil and pure olive oil, respectively, both of which are natural plant oils, highly accepted and preferred by consumers of cosmetic products. The physicochemical properties of cosmetic raw materials used in this study are presented in Table 1. The recombinant *E. coli* was grown for retinoid production in 2YT medium containing 0.2 % (w/v) arabinose and 2 % (w/v) glycerol for 96 h at 29 °C, and 2 ml cosmetic raw material was layered over 5 ml culture broth in test tubes (Fig. 5). Isopropyl myristate produced the highest amount of retinoids at 181 mg/l, and the cell specific retinoid productivity was also the highest at 8.05 mg/l/OD. Cetyl ethylhexanoate produced 142 mg/l of retinoids, which is slightly lower than that produced with 2 ml light mineral oil (158 mg/l); however, the cell specific retinoid productivity was higher with cetyl ethylhexanoate (7 mg/l per OD unit) than with light mineral oil (6.7 mg/l per OD unit). On the other hand, ODO (caprylic/capric triglyceride) and phyto-squalane produced 114 and 122 mg/l of retinoids, respectively. Their viscosities are 23 and 37.4 cSt, respectively, and are relatively higher than other cosmetic raw materials. Since the same volume was used, the higher viscosity seems to have affected cell growth, and thus, retinoid

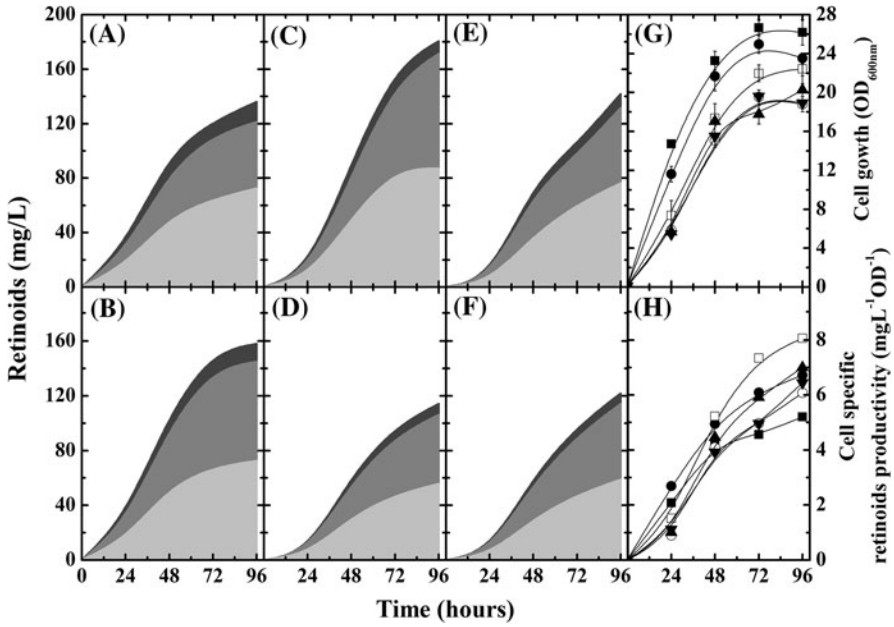


Fig. 5 Retinoid production from two-phase culture using cosmetic raw materials. Two-phase culture was performed using **a** 5 ml dodecnae, **b** 2 ml light mineral oil, **c** 2 ml isopropyl myristate, **d** 2 ml ODO, **e** 2 ml cetyl ethylhexanoate, and **f** 2 ml phyto-squalane as extraction phases. Produced retinal, retinol, and retinyl acetate are indicated with *light gray*, *gray*, and *dark gray*, respectively. For **g** cell growth and **h** cell specific retinoid productivity, the extraction phases are indicated with the following symbols: 5 ml dodecane *closed squares*, 2 ml light mineral oil *closed circles*, 2 ml isopropyl myristate *open squares*, 2 ml ODO *open circles*, 2 ml cetyl ethylhexanoate *closed triangles*, 2 ml phyto-squalane *closed reversed triangles*

extraction. When 5 ml of cosmetic raw material was used, all four types produced 61–68 % fewer retinoids than when 2 ml was used, due to the same reasons as before (data not shown).

To overcome the unstable nature of retinoids, which leads to intracellular degradation, in situ extraction is used to simultaneously produce and extract. Dodecane was previously used since it can prevent degradation but it is relatively expensive and irritates the skin making it unsuitable for use as a cosmetic ingredient. To replace dodecane, mineral oils and cosmetic raw materials were used as the extraction phase. In particular, heavy mineral oil and isopropyl myristate produced 174 and 181 mg/l of retinoids, respectively, which is about 1.3-fold that produced when using dodecane. This means that different types of oils with various properties can be applied for retinoid production to shorten the production process and reduce the production cost, thus broadening their industrial use. In the future, it will be necessary to investigate a detailed mechanism of the direct extraction of retinoids from cells, which can be used to design a highly efficient in situ extractive fermentation system.

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