J. Microbiol. Biotechnol. (2019), 29(3), 382–391
https://doi.org/10.4014/jmb.1811.11028

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# Discarded Egg Yolk as an Alternate Source of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) S

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*Received: November 19, 2018*
*Revised: January 6, 2019*
*Accepted: January 8, 2019*

First published online
January 11, 2019

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

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Many poultry eggs are discarded worldwide because of infection (*i.e.*, avian flu) or presence of high levels of pesticides. The possibility of adopting egg yolk as a source material to produce polyhydroxyalkanoate (PHA) biopolymer was examined in this study. *Cupriavidus necator* Re2133/pCB81 was used for the production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) or poly(3HHx), a polymer that would normally require long-chain fatty acids as carbon feedstocks for the incorporation of 3HHx monomers. The optimal medium contained 5% egg yolk oil and ammonium nitrate as a nitrogen source, with a carbon/nitrogen (C/N) ratio of 20. Time course monitoring using the optimized medium was conducted for 5 days. Biomass production was 13.1 g/l, with 43.7% co-polymer content. Comparison with other studies using plant oils and the current study using egg yolk oil revealed similar polymer yields. Thus, discarded egg yolks could be a potential source of PHA.

**Keywords:** Egg yolk oil, *Cupriavidus necator*, medium optimization, feedstock, co-polymer

## Introduction

Chicken farms in South Korea and Europe have suffered severely from exposure to residual pesticides used to protect hens from red mite [1, 2]. Billions of chicken eggs exposed to the toxic chemicals were discarded for safety reasons because they were unusable as food. Even though the quantity of pesticide detected in these eggs is not an immediate threat to human health, disposal was inevitable considering the possible effects of long-term consumption of even low concentrations of pesticides [3]. Finding other uses for eggs slated for disposal would be useful in lessening the environmental burden arising from periodic disposal of large quantities of unconsumable poultry eggs.

Eggs are an affordable food with high nutrition content. Egg yolk contains 43.8% solids, consisting of protein, lipid, minerals, and carbohydrates. The specific nutritional properties can vary depending on the type of chicken feed [4, 5]. Egg yolk lipid is composed mainly of triglycerides (65%), phospholipids (28.3%), and cholesterol (5.2%) [6]. Besides these major structural components, egg yolk contains yellowish pigments, such as lutein and zeaxanthin. In addition to these carotenoid compounds, egg yolk oil also contains fat-soluble vitamins A, D, and E [7].

One way to utilize lipids from discarded eggs is as a feedstock for microbial cultures of industrial importance. Production of biodegradable plastic can be achieved by the cultivation of a variety of species of microorganisms as biocatalysts. Polyhydroxyalkanoate (PHA) granules accumulate in many bacterial strains under nutritionally unfavorable circumstances, such as limitation of nitrogen, phosphorus, or magnesium. Various wild-type or engineered bacterial species and strains that have been studied include *Pseudomonas* sp., *Bacillus* sp., *Cupriavidus* sp., and *Escherichia*

*coli* [8−11]. More than 150 monomers can be incorporated in PHA polymers produced by microorganisms. Among them, 3-hydroxybutyrate (3HB) in the homopolymer polyhydroxybutyrate (PHB) has been extensively studied [12−16]. However, the PHB polymer is brittle, breakable, and inflexible, which limits its use as a thermoplastic [9, 10, 17]. Attempts to overcome these limitations of PHB have led to incorporation of additional hydroxyalkanoate (HA) monomers, which include 3-hydroxyvalerate, 4-hydroxyvalerate, and 3-hydroxyhexanoate. The resulting P(3HB-*co*-3HV), P(3HB-*co*-4HV), and P(3HB-*co*-3HHx) copolymers, respectively, have better thermal and mechanical properties than PHB [18−21].

Many PHA-producing organisms can attain a high cell density culture that is attainable using a defined medium and refined carbon source. However, to reduce the price of the process, and the final product, PHA synthesis using crude and waste materials has been investigated [13, 22−27]. Industrial waste materials, such as crude whey and glycerol lipid phase remaining from biodiesel production, have been utilized as feedstocks to produce PHA at a more reasonable price [20, 25, 28]. Also, oils extracted from waste soybean, palm, spent coffee, and other sources are another potential target substrate for production of PHA by microorganisms [27, 29, 30]. Metabolic engineering has enabled the accumulation of high concentrations of medium chain length PHA (mcl-PHA) by different organisms [17, 24]. One key benefit of using oils and fats as carbon sources is that their breakdown provides medium chain length precursors required to synthesize mcl-PHA, especially for the production of P(3HB-*co*-3HHx) [31].

In this study, we extracted oil from discarded egg yolk by liquid-liquid extraction with hexane/methanol mixed solvent. To produce P(3HB-*co*-3HHx) from the extracted oil, engineered *Cupriavidus necator* strain Re2133/pCB81(Δ*phaB1*, Δ*phaB2*, Δ*phaB3*, Δ*phaC1* and containing pBBR1 MCS-2 harboring a synthetic PHA operon: *phaC2*$_{Ra}$-*phaA-phaJ1*$_{Pa}$), designed to produce P(3HB-*co*-3HHx) co-polymer using precursors synthesized by fatty acid degradation intermediates, was selected as a PHA co-polymer producing biocatalyst. The strain was cultured in a minimal medium optimized for PHA production [22, 32, 33]. We selected proper oil concentrations in the medium and the type of nitrogen source to increase productivity. Furthermore, an optimal concentration ratio between the carbon and nitrogen sources, which can govern the PHA content in microorganisms, was determined. Biopolymer accumulation in the optimized medium was monitored with time. We evaluated the possibility of egg yolk oil as a carbon feedstock for P(3HB-*co*-3HHx) production by comparison with previous data using plant oils.

## Materials and Methods

**Bacterial Strains and Culture Conditions**

The aforementioned *C. necator* strain Re2133/pCB81 was used as a PHA co-polymer producing biocatalyst [32, 33]. The strain was cultured in tryptic soy broth (TSB) supplemented with 10 μg/ml gentamicin and 50 μg/ml kanamycin for 24 h at 30°C and was stored in 15% glycerol at −80°C. A single colony from a tryptic soy agar plate was precultured in 5 ml TSB. Cells from the culture were harvested and washed twice with sterile de-ionized water. The washed cells were re-suspended in minimal medium lacking a carbon source with the same initial volume and inoculated into 20 ml of PHA production minimal medium supplemented with proper concentrations of gentamicin and kanamycin, as indicated above, in a 100-ml Erlenmeyer flask. The optimized PHA production medium used in this study consisted of 1.5 g/l $KH_2PO_4$, 3.6 g/l $Na_2HPO_4$, 1.25 g/l $NH_4NO_3$, 0.2g/l $MgSO_4 \cdot 7H_2O$, 0.2%(w/v) Tween-80, and 1 ml/l trace element solution. The trace element solution contained 10.00 g/l $FeSO_4 \cdot 7H_2O$, 2.25 g/l $ZnSO_4 \cdot 7H_2O$, 0.64 g/l $CuSO_4$, 0.56 g/l $MnSO_4 \cdot H_2O$, 0.4039 g/l $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, and 10 ml/l of 35% (v/v) HCl. Autoclaved egg yolk oil was supplemented as a carbon source ranging from 0.5% to 10% (w/v).

**Egg Yolk Oil Extraction and Analysis of Composition**

Oil extraction was conducted with a previously described nonpolar/polar solvent extraction method [34] with some modifications. Disrupted egg yolk was mixed with two volumes of organic solvents, which were composed of nonpolar solvent (hexane) and polar solvent (alcohols) at a ratio of 7:3, respectively. To compare the extraction efficacy using different chain length alcohols as polar solvents, methanol, ethanol, and isopropanol were used. The egg yolk and solvent mixture were vigorously mixed at room temperature (25°C) and then centrifuged to separate the nonpolar solvent layer and precipitated proteins. The organic layer was collected and heated at 80°C for 16 h. The egg yolk oil was autoclaved and stored at room temperature. The collected oil was added as a carbon source for the growth and PHA synthesis experiments.

To determine fatty acid content, oils were diluted with chloroform to obtain a 10 μg/ml solution. Oils in the spent culture broth were collected by adding chloroform to the medium, followed by centrifugation and condensation using an $N_2$ evaporator. Fatty acid methyl ester (FAME) derivatization was performed according to the methanolysis method described below. After reaction, the samples were injected into a gas chromatography-mass spectrometry chromatograph (Perkin Elmer Calrus 500) and composition analysis was performed under conditions reported previously [35]. Each peak was assigned structures based on the MS-fragmentation data. Online libraries by

Wiley (http://www.palisade.com) and NIST (http://www.nist.goc) were utilized for confirmation. Methyl-heneicosanoate (10 mg/ml) was used as an internal standard to quantify the fatty acid.

**PHA Analysis and Extraction Method**

For quantification and composition analysis of the PHA produced in this work, a previously described method was used with slight modification [36]. Culture broth was centrifuged and washed twice with de-ionized water. The washed cells were transferred to a glass vial for lyophilization and measurement of dry cell weight. Equal volumes of chloroform and 15% (v/v) $H_2SO_4$/85% methanol solution (2 ml total volume) were added to the glass vial. Methanolysis was then conducted for 2 h at 100°C, after which the glass vial was cooled to room temperature. A 1-ml aliquot of de-ionized water was added to the methyl ester solution, and it was vortexed for 5 s. The chloroform layer was drawn off the glass vial to a microtube containing crystalline $Na_2SO_4$ to remove residual water content. Filtered 1 µl aliquots of samples were injected with split mode (1/10) into a model 6500 gas chromatograph (Young-lin, Seoul, Korea) equipped with a fused silica capillary column (HP-FFAP, 30 m × 0.32 mm, i.d. 0.25 um film; Agilent, USA) and flame ionization detector (FID). The inlet temperature was 210°C, and helium was supplied as a carrier gas at 3 ml/min. Oven temperature was controlled by the following gradient program: 0-5 min at 80°C then 12-17 min at 220°C. FID temperature was maintained at 230°C during operation. For comprehensive data interpretation, the quantified results were processed using the following equations:

$$\text{PHA content \%} = \frac{\text{PHA mass (g/l)}}{\text{Biomass (g/l)}} \times 100\%$$

$$\text{3HHx mole fraction \%} = \frac{\text{3HHx concentration (mol/l)}}{\text{PHA concentration (mol/l)}} \times 100\%$$

**Co-Polymer Extraction from Biomass**

P(3HB-*co*-3HHx) co-polymer extraction from cells was conducted using methyl ethyl ketone (MEK) and a previously described protocol [37]. Cultures were harvested and washed with de-ionized water and hexane to remove medium components and residual oil. The washed cells underwent lyophilization and the dried cells were submerged in MEK (1 ml solvent/mg cell dry mass). Extraction was performed with gentle stirring at 60°C for 16 h. MEK solvent containing co-polymer was collected by centrifugation, followed by filtering in a Minisart RC25 apparatus (0.2 um, Sartorius AG, Germany). A refined co-polymer solution was heated at 80°C for 1 h to evaporate solvent, and the polymer was completely dried at room temperature until a plastic film was formed. Small samples of the P(3HB-*co*-HHx) were cut from the film when required for further analysis.

**Thermal Analysis and NMR**

Differential scanning calorimetry (DSC) and nuclear magnetic resonance spectroscopy (NMR) analyses were performed at the Korea Polymer Testing & Research Institute. DSC analysis was conducted with a DSC213 Polyma system (NETSCH, Germany) at a temperature ranging from −60°C to 200°C. The heating rate was 10°C/min in an $N_2$ atmosphere. Samples were dissolved in $CDCl_3$ (0.02 g/0.7 ml) for a final concentration of 2.8%, with tetramethylsilane as a reference. The $^1$H-NMR analysis was performed using a JNM-LA 400 instrument (JEOL, Japan) with a low frequency generator.

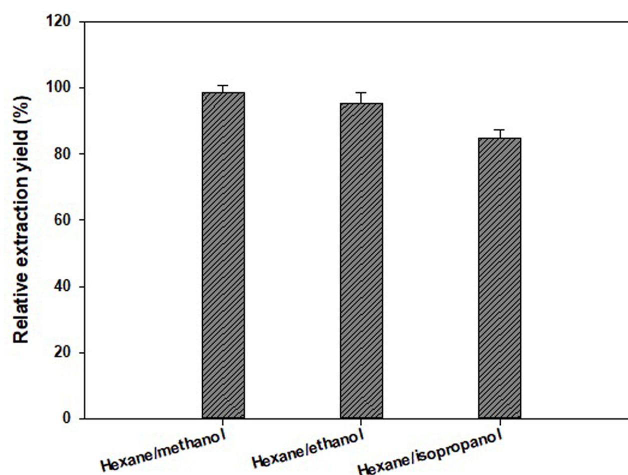


**Fig. 1.** Egg yolk extraction yield depending on carbon length of alcohols.

The ratio of non-polar solvent (hexane) to polar solvent (alcohols) was fixed at 7:3. Methanol, ethanol, and isopropanol were used as polar alcohols for the separation of lipid from lipoprotein.

## Results

**Preparation of Lipids from Egg Yolk**

Based on a previously published extraction method that used hexane/isopropanol as extraction solvent for liquid egg yolk [34], the extraction yield of short-chain alcohols, such as methanol and ethanol, were compared with isopropanol (IPA), which is a relatively expensive solvent (Fig. 1). Extraction efficiency was decreased as the carbon chain length of the alcohol increased. Based on extraction yield when methanol was used, relative extraction yield was decreased to 95.3% for ethanol and 84.6% for IPA.

The fatty acid composition of lipids extracted using our modified hexane/methanol solvent was determined by GC-MS analysis of FAME products from the extracted egg yolk oil (Table 1). Egg yolk oil used as a carbon feedstock in this study consisted of tetradecanoic acid (C14:0, 0.34%), 7-hexadecenoic acid (C16:1, 0.81%), palmitoleic acid (C16:1, 3.41%), palmitic acid (C16:0, 25.6%), linoleic acid (C18:2, 11.7%), oleic acid (C18:1, 53.25), stearic acid (C18:0, 5.91%),

**Table 1.** Comparison of fatty acid composition (%) before and after fermentation with plant oils and egg yolk oil.

| Fatty acid | | Fatty acid profile | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Olive oil | | Soybean oil | | Corn oil | | Egg yolk oil | |
| | | Before | After | Before | After | Before | After | Before | After |
| C14:0 | Tetradecanoic acid | - | - | - | - | - | - | 0.335 | 0.255 |
| C16:1 | 7-Hexadecenoic acid | - | - | - | - | - | - | 0.807 | 0.790 |
| C16:1 | Palmitoleic acid | 0.385 | 0.036 | - | - | 0.049 | 0.142 | 3.41 | 2.81 |
| C16:0 | Palmitic acid | 7.76 | 7.45 | 12.1 | 11.4 | 12.0 | 10.7 | 25.6 | 23.7 |
| C18:2 | Linoleic acid | 16.9 | 15.0 | 50.7 | 48.6 | 50.9 | 43.0 | 11.7 | 11.1 |
| C18:1 | Oleic acid | 71.2 | 72.4 | 31.9 | 33.3 | 34.4 | 32.8 | 53.2 | 53.1 |
| C18:0 | Stearic acid | 2.61 | 3.57 | 4.35 | 5.35 | 1.94 | 2.67 | 5.91 | 7.70 |
| C20:1 | Gondoic acid | 0.616 | 0.747 | 0.259 | 0.300 | 0.233 | 0.874 | 0.163 | 0.578 |
| C20:0 | Eicosanoic acid | 0.472 | 0.733 | 0.346 | 0.533 | 0.350 | 0.534 | - | - |
| C21:0 | Docosanoic acid | - | - | 0.422 | 0.557 | 0.133 | 0.392 | - | - |

and eicosanoic acid (C20:0, 0.53%). The egg yolk oil extracted with hexane/methanol had a similar fatty acid composition as oil extracted by previous methods [34].

**Optimization of PHA Production Medium**

Autoclaved oil was supplemented as a carbon source for *C. necator* cultures in PHA production minimal medium. The effects of oil content in the medium were studied using concentrations from 0.5% to 10% (w/v). To minimize any inhibition to oxygen transfer by the oil layer, 0.25% (w/v) Tween 80 was used [27]. Similar biomass was obtained after 3 days of culture using an oil content of 0.5% to 10%. A relatively high PHA content was observed in cells grown in cultures containing 5% and 10% oil than in cultures with lower oil concentrations. The highest PHA content was 42.8% from cultures containing 5% egg yolk oil in the production medium (Fig. 2A). Monomer composition of the resulting PHA was monitored as described in the Materials and Methods. Although the carbon to nitrogen (C/N) ratio was altered by increasing oil concentration, the overall monomer composition in intracellular PHA was not affected. A 3HB mole fraction of 79.6% and a 3HHx mole fraction of 20.4% was obtained in cells from cultures of all oil concentrations tested (Fig. 2B). Even though other medium components were not yet optimized, 5% oil concentration was considered an optimal concentration for further experiments, as a high PHA content was obtained. Furthermore, 5% egg yolk oil was a sufficient carbon source when biomass concentrations increased following optimization of the growth medium.

Cell growth and PHA accumulation can be affected by the nitrogen source used [38]. Equivalent molar nitrogen concentrations were used to test the effects of different nitrogen sources on the growth of *C. necator*. Cell dry mass was not appreciably changed using different nitrogen sources. Ammonium nitrate was the best nitrogen source in terms of cell growth (8.79 g cell dry mass/L) and PHA content (44.9%) (Fig. 2C). The 3HB/3HHx ratio from cells grown with the different nitrogen sources was similar, except for ammonium sulfate. The mole fraction of 3HHx monomer was 19.5%, 19.1%, 20.2%, 26.8%, and 19.9% for urea, ammonium nitrate, ammonium sulfate, ammonium chloride, and diammonium phosphate, respectively (Fig. 2D).

To select the optimal C/N ratio, the effect of cultivation at a wide range of ratios ranging from 5 to 80 was examined. A fixed concentration of egg yolk oil (5%, w/v) was provided as a carbon source, and the C/N ratio was controlled by changing the ammonium nitrate concentration (w/v). PHA accumulation in cells was gradually increased as the C/N ratio increased. The maximum PHA content was 49.2% at a C/N ratio of 80. However, the overall cell dry mass (CDM) of the culture displayed a different trend as compared to that of the PHA content. Total biomass increased as the C/N ratio increased from 5 to 20. At higher ratios, cell growth decreased, in contrast to PHA content. At C/N = 20, maximum biomass (CDM/L) and total PHA/L were 8.39 g and 3.39 g, respectively. This ratio was adapted to the production medium (Fig. 2E). The 3HHx monomer mole fraction was decreased from 41.2% to 20.2% with increased C/N ratio. Maximum and minimum 3HHx mole fractions were observed at C/N = 5 and 80, respectively (Fig. 2F).

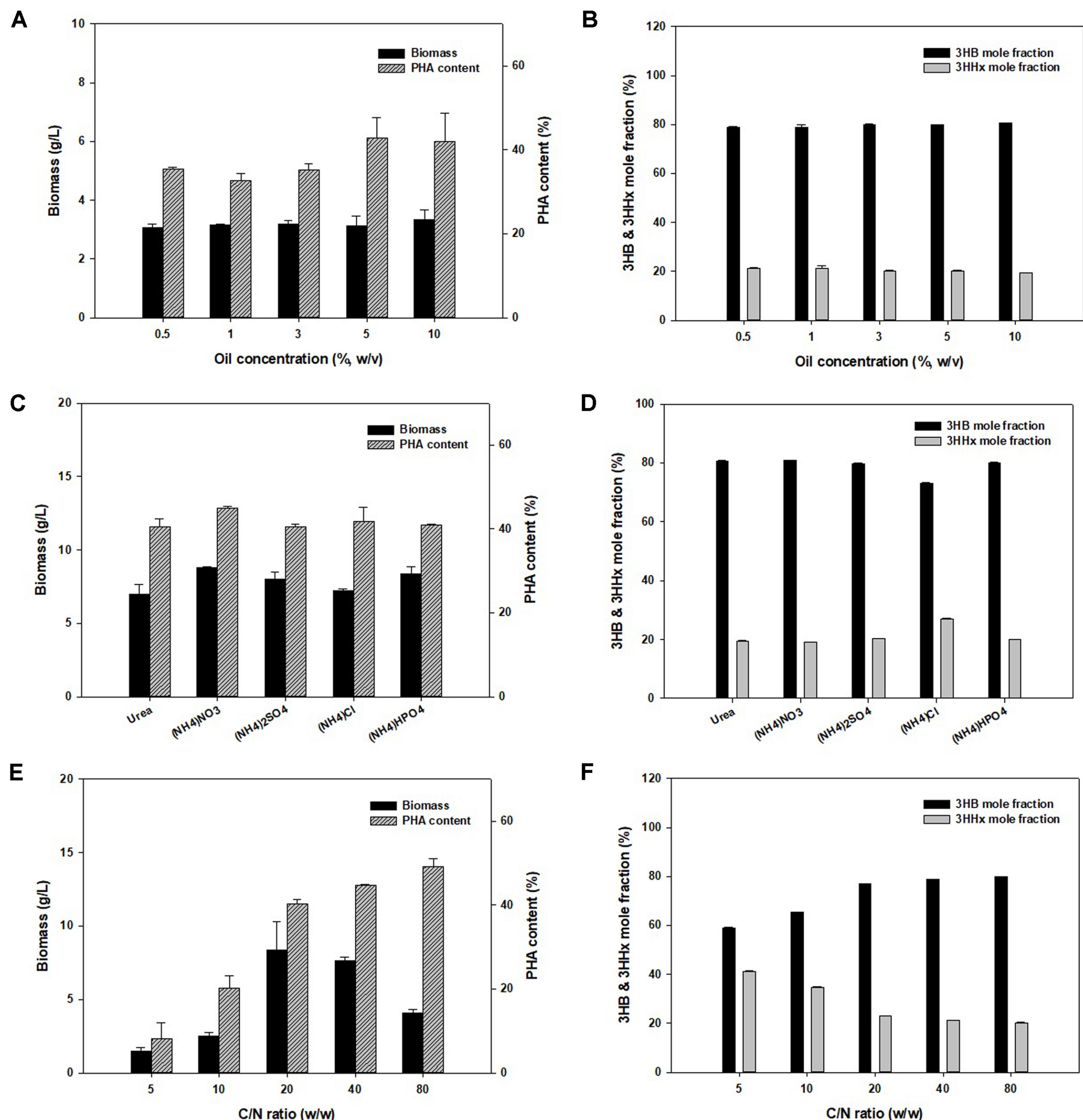


**Fig. 2.** Oil and nitrogen source optimization in PHA production medium.

(**A** and **B**) Extracted egg yolk oil was added to minimal medium in concentrations ranging from 0.5% to 10% (w/v). Accumulated dried cell weight and PHA content (**A**), and PHA monomer compositions (**B**) were examined. (**C** and **D**) Inorganic or organic nitrogen sources were used in egg yolk oil cultures for comparison. Biomass concentration and percentage of PHA produced in cells (**C**) and 3HB and 3HHx monomer mole fractions (**D**) were examined. (**E** and **F**) Oil concentration was fixed at 5.0% (w/v) and the carbon-nitrogen (C/N) ratio was controlled by changing the concentration of the nitrogen source. Accumulated biomass concentration, PHA content (**E**), and composition of PHA (**F**) were examined.

**Monitoring of PHA Production and Analysis of Chemical Properties**

Under the optimized conditions, cell growth and PHA accumulation were monitored for 5 days at a larger scale. *C. necator* Re2133/pCB81 was inoculated into 100 ml of optimized medium in a 500-ml non-baffled Erlenmeyer flask. Biomass concentration continuously increased throughout cultivation. For the first 48 h of cultivation, intracellular

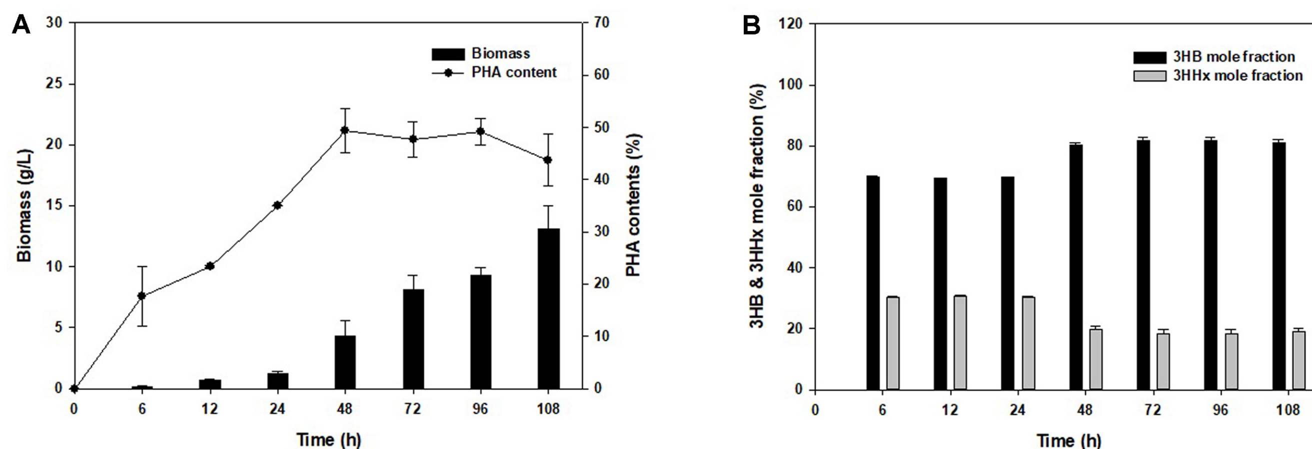


**Fig. 3.** Time course monitoring of PHA production under the optimized condition.
Cell growth and intracellular PHA accumulation of *C. necator* Re2133/pCB81 strain in optimized egg yolk oil medium were monitored for 108 h (**A**). Change in mole fraction of 3HB and 3HHx was monitored during 5 days of culture (**B**).

PHA content increased and then plateaued thereafter. The biomass and PHA content at the final 96-h culture was 13.1 g/l and 43.7%, respectively (Fig. 3A). During the initial growth phase, a relatively high 3HHx mole fraction (30.1%–30.7%) was observed for PHA. However, after 48 h, when the culture PHA concentration had plateaued, the 3HHx mole fraction decreased to 18.3% to 19.8% (Fig. 3B).

For larger scale production of P(3HB-*co*-3HHx) product, a 500-ml culture was grown in a 2-L Erlenmeyer flask. After 5 days of culture, cells were harvested, and the co-polymer was extracted using MEK as the solvent. The extracted co-polymer had a relatively high 3HHx fraction of 38.6%.

DSC analysis showed that the P(3HB-*co*-38.6mol%3HHx) had a glass transition temperature ($T_g$) of –12.4°C, cold crystallization temperature ($T_c$) of 53.5°C, and melting temperature ($T_m$) of 142.3°C. The DSC scanning also determined that the change in enthalpy for crystallization ($\Delta H_c$) and melting ($\Delta H_m$) was –3.20 J/g and 5.72 J/g, respectively (Fig. S1). In contrast to glass transition, crystallization and melting were not detected during the first cooling and second heating (Table S1). The monomer content of the polymer was confirmed by $^1$H NMR (Fig. S2). The co-polymer produced from egg yolk oil had comparable thermal properties with previously produced P(3HB-*co*-3HHx) with similar 3HHx mole fractions [39].

## Potential of Egg Yolk Oil as a Carbon Source for PHA Production

PHA production using an animal-derived source was documented in the ANIMPOL project, which focused on the usage of trans-esterified oils (biodiesel) or crude glycerol [23, 25, 26]. Similarly, P(3HB-*co*-3HHx) production from waste animal fat with a step-wise production strategy showed that animal-derived carbon sources have considerable potential as a feedstock [40]. As this is the first report about PHA production from egg yolk waste, P(3HB-*co*-3HHx) production with egg yolk oil was compared to commercially available plant oils (olive, corn, and soybean oil). Different oils were added as a carbon source in the same initial quantity into the optimized production medium. After 5 days, cell growth in terms of biomass was the highest (6.88 CDM/L) in cultures where egg yolk oil was the carbon source. Although the PHA content of cells grown with egg yolk oil was relatively lower (36.5%) than those of plant oils, total PHA amount was higher in egg yolk oil cultures, compared to plant oil cultures. In these experiments, PHA content and biomass seemed to have a negative correlation. Soybean oil cultures achieved the highest PHA content (55.9%). Simultaneously, these cultures yielded the lowest biomass accumulation of 2.61 g CDM/L (Fig. 4A). PHA monomer composition (3HB and 3HHx) showed no significant differences between oil cultures, except for a slightly higher 3HHx mole fraction with egg yolk oil as carbon source than with other oils (Fig. 4B). The 3HHx mole fraction for soybean, corn, olive, and egg yolk oil cultures were 17.8%, 17.3%, 17.2%, and 18%, respectively.

The fatty acid composition of oils used for comparison in this study was analyzed and the change in fatty acid profile after culturing was determined. The type of fatty acids involved was similar among all oils tested. However, egg

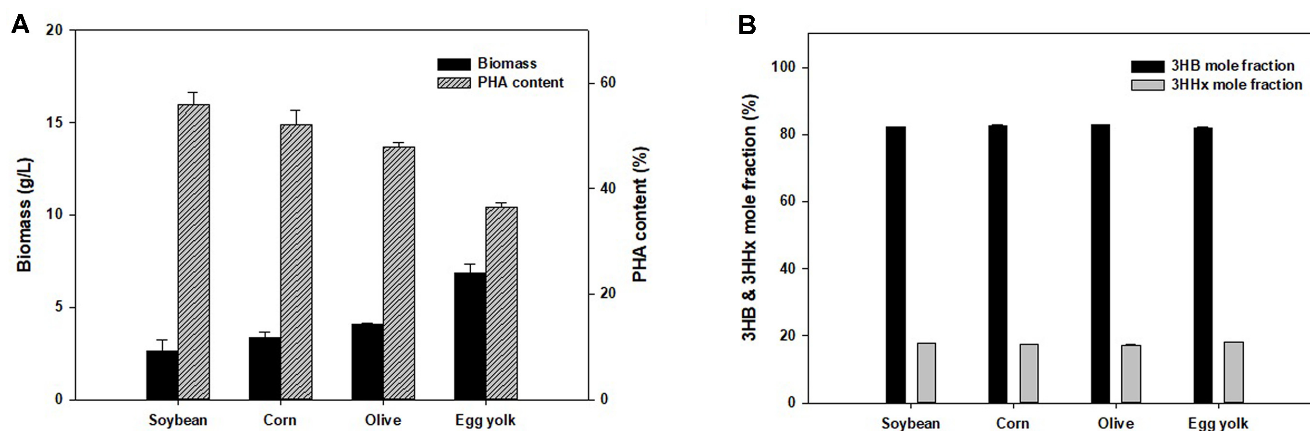


**Fig. 4.** Comparison of PHA production in different oil sources.
Viability of commercially available soybean, corn, and olive oil as carbon feedstocks was compared with that of extracted egg yolk oil. Oils were provided in PHA production medium with the same mass concentration. To estimate the viability of egg yolk lipids as carbon sources, cell growth and PHA accumulation were observed (**A**) and the composition of the produced P(3HB-*co*-3HHx) polymer was analyzed (**B**).

yolk oil tends to contain a higher percentage of shorter chain length fatty acids like palmitoleic acid (3.41%) and palmitic acid (25.6%). In addition, C14 fatty acid was detected in egg yolk oil, even though its content was low (0.34%). Generally, all four oils displayed compositional changes following cultivation involving the decreased content of shorter chain fatty acid (Table 1).

## Discussion

Large quantities of eggs are discarded when it is judged they are unsafe for human consumption. In most cases, the reason for disposal is the overuse of pesticides to kill red mites, which can be a host for avian influenza [41]. The residual level of pesticides in the eggs is not damaging to human health immediately. However, recycling these nutritionally valuable materials as a biotechnological feedstock is limited because of concern of the possible detrimental effects on human health effect of consuming low levels of pesticides for a long time [3, 42]. The annual cost to control red mite and production losses is reportedly €130 million in Europe and other countries [1]. Although methods for reducing damage from *Dermanyssus gallinae* (poultry red mite) have been actively studied, some chicken farms continue to use pesticides, such as bifenthrin and fipronil, in combination with other approaches to control red mites because of the effectiveness and convenience of the approach [1, 43, 44]. Urban agricultural societies in some third world countries have been exposed to the overuse of toxic pesticides including chlorinated pesticides [45]. As red mite can be a vector for the influenza virus, use of pesticides to eradicate red mites is indispensable in poultry farms globally. Thus, it is important to figure out a solution to minimize huge economic loss and negative environmental effects that come from the occasional massive disposal of poultry eggs.

Concerning bioplastic production, disposed eggs can be an inexpensive and accessible starting material for PHA production after the oil extraction process. Especially for the production of copolymers like P(3HB-*co*-3HHx), PHA producers require additional carbon substrates like butyrate, hexanoate, octanoate, or fats [18]. The high content of lipid in egg yolk can support cell growth and PHA accumulation by *C. necator* when oils are used as a substrate [17]. Although the triglyceride content in egg yolk oil is relatively higher than that of noble or waste oils from plants used to induce the growth of microorganisms for PHA production, *C. necator* excretes lipases that can fully convert triglycerides to free fatty acids and glycerol to utilize egg yolk oil as a feedstock [46]. In addition, fat-soluble vitamins and antioxidative carotenoid compounds in egg yolk oil can aid the growth of bacterial cells and the accumulation of PHA [47].

Egg yolk oil can be easily extracted from eggs by one of several well established methods [6, 34, 48–52]. These include the use of diethyl ether or chloroform for oil extraction [53] and polar/nonpolar mixed solvents to detach lipid from lipoproteins [50, 52]. A similar solvent extraction method replaces heat evaporation and bleaching at high temperature with vacuum evaporation under

moderate temperature, which results in the decreased decomposition of poly-unsaturated $C_{20}$ and $C_{22}$ fatty acids [6]. Comprehensive extraction of oil from liquid egg yolk with hexane/IPA or chloroform/ethanol has been detailed [34]. The latter study achieved a higher extraction yield with hexane/methanol, rather than with longer alcohols like ethanol and propanol, which likely reflected the higher relative polarity of methanol (0.762) compared to that of ethanol (0.654) and isopropanol (0.546). Phospholipids consisting of the total lipid content of eggs (28.3%) might be extracted more by alcohols with higher polarity [6, 54]. Furthermore, liquid-liquid extraction with polar/nonpolar organic solvent can add more value to egg yolk oil as a feedstock for PHA as well as for biodiesel production, as the extraction method can exclude the phospholipid content in egg yolk oil, which can inhibit lipases like *Candida antarctica* B-lipase and *Thermomyces lanuginose* lipase (TTL) [34, 55−57].

In conclusion, egg yolk oil can be a suitable extra substrate for P(3HB-*co*-3HHx) co-polymer production. Sustained growth of *C. necator* in and comparison of PHA production from egg yolk oil relative to that in plant oil cultures demonstrates the potential of egg yolk oil as a feedstock for biopolymer production. Although the utilization of egg yolk oil is well established for the isolation of β-carotene, oil-soluble vitamins, or lecithin, our approach promises a potential alternative way to utilize egg yolk from large quantities of discarded, unconsumable eggs to produce bulk quantities of eco-friendly plastics.

## Acknowledgments

This study was supported by the Research Program to Solve Social Issues of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2017M3A9E4077234), NRF-2015M1A5A1037196, and the Polar Academic Program (PE18900). Consulting service from the Microbial Carbohydrate Resource Bank (Korea) is kindly appreciated.

## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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