Full Paper

The influence of medium composition on the microbial secretory production of hydroxyalkanoate oligomers

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With the aid of a chain transfer (CT) reaction, hydroxyalkanoate (HA) oligomers can be secreted by recombinant *Escherichia coli* carrying the gene encoding a lactate-polymerizing enzyme (PhaC1_{ps}STQK) in Luria-Bertani (LB) medium supplemented with a carbon source and CT agent. In this study, HA oligomers were produced through microbial secretion using a mineral-based medium instead of LB medium, and the impact of medium composition on HA oligomer secretion was investigated. The focused targets were medium composition and NaCl concentration related to osmotic conditions. It was observed that 4.21 g/L HA oligomer was secreted by recombinant *E. coli* in LB medium, but the amount secreted in the mineral-based modified R (MR) medium was negligible. However, when the MR medium was supplemented with 5 g/L yeast extract, 3.75 g/L HA oligomer was secreted. This can be accounted for by the enhanced expression and activity of PhaC1_{ps}STQK upon supplementation with growth-activated nutrients as supplementation with yeast extract also promoted cell growth and intracellular growth-associated polymer accumulation. Furthermore, upon adding 10 g/L NaCl to the yeast extract-supplemented MR medium, HA oligomer secretion increased to 6.86 g/L, implying that NaCl-induced osmotic pressure promotes HA oligomer secretion. These findings may facilitate the secretory production of HA oligomers using an inexpensive medium.

Key Words: chain transfer reaction; CT agent; osmotic pressure; PHA synthase; polyhydroxyalkanoate; yeast extract

Introduction

Many microorganisms synthesize polyhydroxyalkanoates (PHAs) in cells as intracellular granules for carbon and energy storage (Tsuge, 2002). Recently, PHAs are being used commercially as biodegradable plastics that can be synthesized from renewable biomass resources. *Ralstonia eutropha* is a well-studied bacterium that can synthesize a large amount of (R)-3-hydroxybutyrate (3HB) homopolymer [P(3HB)], the most common PHA. Meanwhile, *Pseudomonas* sp. strain 61-3, a PHA-producing bacterium, possesses a unique PHA synthase (PhaC1_{ps}) that exhibits a wide range of substrate specificity (Taguchi et al., 2012). As a pioneering study, a lactate-polymerizing enzyme mutant, PhaC1_{ps}STQK, was developed through evolutionary engineering research (Taguchi et al., 2008). This newly created mutant enzyme can incorporate not only the 3HB unit, but also the lactic acid (LA) unit into the polymeric backbone, providing a new polymer category P(LA-co-3HB) (Taguchi et al., 2008).

The molecular weight of the polymer is a crucial factor affecting the various properties as well as monomeric composition of the copolymer (Taguchi et al., 2012; Tsuge, 2016). *Escherichia coli* does not naturally synthesize PHA, but can be used as a host for PHA production with the

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help of genetic engineering techniques. Usually, PHA synthesized by recombinant *E. coli* has a higher molecular weight than that synthesized by natural PHA-producing bacteria (Agus et al., 2006). Since *E. coli* lacks a PHA depolymerase, the molecular weight of the PHA biosynthesized by *E. coli* can be maintained without undergoing a hydrolytic reaction (Tsuge, 2016). The involvement of PHA depolymerase is a significant factor in reducing the molecular weight of PHA biosynthesized by natural PHA producers (Arikawa et al., 2016; Tsuge, 2016).

On the contrary, a chain transfer (CT) reaction is also critical for determining the molecular weight of biosynthesized PHA (Tsuge, 2016). In this reaction, the growing PHA polymer chain attached to the cysteine at the active site of PhaC is transferred to a CT agent, resulting in the covalent binding of the CT agent to the carboxy terminal of the PHA (Tsuge, 2016). Thus, the CT reaction generates a low-molecular-weight PHA. This reaction can be induced by CT agents, such as hydroxy compounds like ethanol, glycerol, and ethylene glycol (Hiroe et al., 2013; Miyahara et al., 2019; Tomizawa et al., 2010; Tsuge et al., 2013).

Recently, it has been reported that PHA-producing *E. coli* secretes hydroxyalkanoate (HA) oligomers when an extremely high concentration (such as 50 g/L) of diethylene glycol (DEG) was added as a CT agent to the culture medium (Utsunomia et al., 2017b, c). The secretory production of HA oligomers has been demonstrated using Luria-Bertani (LB) medium supplemented with sugars as a carbon source, with DEG as the CT agent. LB medium is a nutrient-rich medium composed of yeast extract, polypeptone, and NaCl. In contrast, mineral media is generally inexpensive and can be easily prepared in large amounts. The modified R (MR) medium are commonly used for high cell density cultures of *E. coli* (Kahar et al., 2005; Lee and Chang, 1993; Wang and Lee, 1997). However, in our preliminary culture test, HA oligomers were not secreted when recombinant *E. coli* was cultured in the MR medium.

HA oligomers are physiologically active in insects and bacteria (Kato et al., 1992; Ogita et al., 2006; Schulz and Toft, 1993). Recently, terminally methyl-esterified HA oligomers have been reported to be effective scavengers for hydroxyl radicals (Ma et al., 2019). Alternatively, the HA oligomers can be used as building blocks to be assembled into poly(ester-urethane) via a polyaddition reaction with diisocyanate (Utsunomia et al., 2017d). Thus, HA oligomers have various applications as functional biomolecules and chemical platforms.

This study investigated the secretory production of HA oligomers using a mineral-based medium instead of LB medium and the influence of medium composition on HA oligomer secretion. The effects of adding yeast extract and NaCl, the constituents of LB medium, to the MR medium were also examined. It was observed that HA oligomers were effectively secreted by supplementing the MR medium with 5 g/L yeast extract and 10 g/L NaCl.

Materials and Methods

Bacterial strains and plasmids used. *E. coli* BW25113Δ*adhE* (JW1228) was used as the host strain for

Fig. 1. The biosynthetic pathway and enzymes involved in the synthesis of diethylene glycol (DEG)-capped hydroxyalkanoate (HA) oligomers.

PhaA_{Re}, *R. eutropha* β-ketothiolase; PhaB_{Re}, *R. eutropha* acetoacetyl-CoA reductase; PhaC_{1ps}STQK, Ser325Thr/Gln481Lys (STQK)-mutated *Pseudomonas* sp. 61-3 PHA synthase 1; Pct, *M. elsdenii* propionyl-CoA transferase.

HA oligomer production in this study. This strain, which lacks the alcohol dehydrogenase gene (*adhE*), was kindly provided by the National BioResource Project (NBRP), Japan, from its Keio collection (Baba et al., 2006). The plasmid pTV118N*pctCIAB*(STQK) (Taguchi et al., 2008) carrying Ser325Thr/Gln481Lys (STQK)-mutated PHA synthase 1 gene (*phaC*_{1ps}STQK) from *Pseudomonas* sp. 61-3, propionyl-CoA transferase gene (*pct*) from *Megasphaera elsdenii*, and 3HB monomer supplier genes (*phaAB*_{Re}) from *R. eutropha* H16 were used for HA oligomer and PHA polymer production. The biosynthetic pathway and enzymes involved in HA oligomer synthesis are shown in Fig. 1.

Culture conditions. Recombinant *E. coli* BW25113Δ*adhE* harboring pTV118N*pctCIAB*(STQK) was cultured in 100 mL LB and MR media supplemented with 40 g/L xylose and 50 g/L DEG in a 500 mL flask shaken at 130 rpm, at 30°C or 37°C for 48 h. LB medium contained 10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract dissolved in water, while MR medium (pH 7.0) contained 13.5 g/L KH₂PO₄, 4 g/L (NH₄)₂HPO₄, 1.4 g/L MgSO₄·7H₂O, and 1.7 g/L citric acid dissolved in water with an additional 10 mL/L trace metal solution (Kahar et al., 2005; Wang and Lee, 1997). The trace metal solution contained 10 g/L FeSO₄·7H₂O, 2 g/L CaCl₂, 2.2 g/L ZnSO₄·7H₂O, 0.5 g/L MnSO₄·4H₂O, 1 g/L CuSO₄·5H₂O, 0.1 g/L (NH₄)₆Mo₇O₂₄·4H₂O, and 0.02 g/L Na₂B₄O₇·10H₂O dissolved in 0.1 M HCl. The plasmid was maintained in the cell through antibiotic selection by adding 100 mg/L ampicillin to each medium. After cultivation, the pelleted cells were washed twice with water to remove the remaining culture medium components and were then lyophilized.

Assay to determine the extracellular HA oligomer concentration. The culture supernatant was analyzed before and after acid hydrolysis to determine the extracellular

Table 1. The effect of culture medium composition and temperature on the production of HA oligomer from xylose in the presence of DEG.^a

Medium	Temp. (°C)	Dry cell wt. (g/L)	Intracellular PHA (g/L) ^b			Extracellular HA oligomer (g/L) ^c			Extracellular HA monomer (g/L) ^c		
			LA	3HB	Total	LA	3HB	Total	LA	3HB	Total
LB	30	8.13 ± 0.30	0.65 ± 0.07	1.43 ± 0.16	2.08 ± 0.22	3.49 ± 0.05	0.72 ± 0.05	4.21 ± 0.02	0.22 ± 0.05	0.20 ± 0.03	0.42 ± 0.07
	37	1.95 ± 0.03	n.d.	n.d.	n.d.	0.04 ± 0.04	0.02 ± 0.02	0.06 ± 0.04	0.06 ± 0.00	0.62 ± 0.04	0.68 ± 0.04
MR	30	3.80 ± 0.10	n.d.	0.03 ± 0.01	0.03 ± 0.01	n.d.	0.02 ± 0.01	0.02 ± 0.01	0.31 ± 0.03	0.03 ± 0.00	0.35 ± 0.03
	37	2.41 ± 0.03	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.01	0.01 ± 0.01	0.16 ± 0.01	0.01 ± 0.00	0.17 ± 0.01

HA, hydroxyalkanoate; DEG, diethylene glycol; PHA, polyhydroxyalkanoate; LA, lactic acid; 3HB, 3-hydroxybutyrate; LB, Luria-Bertani; MR, modified R.

^a Cells were cultured in 100 mL medium supplemented with 40 g/L xylose and 50 g/L DEG for 48 h. Results shown are the mean of results of three individual experiments.

^b PHA composition and content in the cells were determined by gas chromatography.

^c Extracellular secretion products in the culture supernatants were determined by an enzymatic assay.

Table 2. Effect of yeast extract in MR medium on HA oligomer production from xylose in the presence of DEG.^a

Yeast extract (g/L)	Dry cell wt. (g/L)	Intracellular PHA (g/L) ^b			Extracellular HA oligomer (g/L) ^c			Extracellular HA monomer (g/L) ^c		
		LA	3HB	Total	LA	3HB	Total	LA	3HB	Total
0	3.80 ± 0.10	n.d.	0.03 ± 0.01	0.03 ± 0.01	n.d.	0.02 ± 0.01	0.02 ± 0.01	0.31 ± 0.03	0.03 ± 0.00	0.35 ± 0.03
1	5.53 ± 0.02	0.06 ± 0.01	0.89 ± 0.10	0.95 ± 0.11	0.52 ± 0.13	0.52 ± 0.07	1.04 ± 0.20	0.31 ± 0.04	0.26 ± 0.01	0.57 ± 0.04
5	6.31 ± 0.26	0.58 ± 0.03	2.21 ± 0.11	2.79 ± 0.14	3.08 ± 0.17	0.67 ± 0.02	3.75 ± 0.18	2.17 ± 0.09	0.21 ± 0.02	2.38 ± 0.11
10	7.08 ± 0.41	0.24 ± 0.03	0.99 ± 0.04	1.23 ± 0.07	1.17 ± 0.49	2.07 ± 0.14	3.24 ± 0.61	1.13 ± 0.15	0.30 ± 0.00	1.43 ± 0.15

MR, modified R; HA, hydroxyalkanoate; DEG, diethylene glycol; PHA, polyhydroxyalkanoate; LA, lactic acid; 3HB, 3-hydroxybutyrate; LB, Luria-Bertani.

^a Cells were cultured in 100 mL MR medium supplemented with 40 g/L xylose and 50 g/L DEG at 30°C for 48 h. Results are shown as the mean of results of three individual experiments.

^b PHA composition and content in the cells were determined by gas chromatography.

^c Extracellular secretion products in the culture supernatants were determined by an enzymatic assay.

Table 3. Effect of NaCl concentration in MR(Y5) medium on HA oligomer production from xylose in the presence of DEG.^a

NaCl (g/L)	Dry cell wt. (g/L)	Intracellular PHA (g/L) ^b			Extracellular HA oligomer (g/L) ^c			Extracellular HA monomer (g/L) ^c		
		LA	3HB	Total	LA	3HB	Total	LA	3HB	Total
0	6.31 ± 0.26	0.58 ± 0.03	2.21 ± 0.11	2.79 ± 0.14	3.08 ± 0.17	0.67 ± 0.02	3.75 ± 0.18	2.17 ± 0.09	0.21 ± 0.02	2.38 ± 0.11
5	6.39 ± 0.08	0.67 ± 0.04	2.37 ± 0.08	3.04 ± 0.12	3.72 ± 0.48	0.90 ± 0.12	4.62 ± 0.60	1.59 ± 0.04	0.08 ± 0.01	1.67 ± 0.04
10	8.49 ± 0.39	0.83 ± 0.05	3.53 ± 0.17	4.36 ± 0.21	5.05 ± 0.34	1.81 ± 0.16	6.86 ± 0.50	0.98 ± 0.07	0.06 ± 0.01	1.04 ± 0.07
20	8.98 ± 0.24	0.70 ± 0.02	3.99 ± 0.13	4.69 ± 0.15	3.55 ± 0.08	1.86 ± 0.14	5.41 ± 0.21	0.47 ± 0.03	0.08 ± 0.00	0.55 ± 0.03

MR(Y5), 5 g/L yeast extract-supplemented modified R; HA, hydroxyalkanoate; DEG, diethylene glycol; PHA, polyhydroxyalkanoate; LA, lactic acid; 3HB, 3-hydroxybutyrate; LB, Luria-Bertani.

^a Cells were cultured in 100 mL MR(Y5) medium supplemented with 40g/L xylose and 50 g/L DEG at 30°C for 48 h. Results are shown as the mean of results of three individual experiments.

^b PHA composition and content in the cells were determined by gas chromatography.

^c Extracellular secretion products in the culture supernatants were determined by an enzymatic assay.

Fig. 2. Culture results with different NaCl concentration in 5 g/L yeast extract-supplemented modified R [MR(Y5)] medium.

A. Intracellular polyhydroxyalkanoate (PHA), B. extracellular HA oligomer, and C. extracellular HA monomer.

HA oligomer concentration in the supernatant (Utsunomia et al., 2017c). HCl was added to the supernatant at a final concentration of 2 M and incubated at 100°C for 2 h to hydrolyze the HA oligomer, after which the reaction mixture was neutralized with NaOH. The amount of LA and 3HB in the culture supernatant was determined using an enzyme assay with D-lactate/L-lactate and D-3HB enzyme assay kits (J.K. International; Tokyo, Japan).

Intracellular PHA analysis. The PHA content of the dried cells and PHA monomer composition were determined using gas chromatography (GC). The samples for GC were prepared through methanolysis using 15% v/v sulfuric acid in methanol and incubated at 100°C for 140 min to hydrolyze the PHA polymer.

Electrospray ionization time-of-flight mass spectrometry (ESI TOF-MS) of the extracellular HA oligomer. The HA oligomers secreted in the culture supernatant were extracted with chloroform for 1 h at room temperature, then washed thrice with water to remove the remaining culture medium components, and dried. To determine the molecular weight of the extracellular HA oligomers, the oligomers collected from the culture supernatant were analyzed using electrospray ionization time-of-flight mass spectrometry (ESI TOF-MS). The oligomer samples were dissolved in methanol and passed through a polytetrafluoroethylene (PTFE) filter before being injected into the ESI TOF-MS system.

PHA synthase activity assay. The activity of PHA synthase (PhaC1_{ps}STQK) was measured using the protocol described in a previous study (Hyakutake et al., 2014). Briefly, the recombinant *E. coli* BW25113Δ*adhE* strain was cultured in each medium supplemented with 40 g/L xylose and 50 g/L DEG at 30°C for 24 h. The cells were ultrasonically disrupted and centrifuged at 1,500 × *g* for 5 min at 4°C to obtain the lysates containing PHA synthase bound to the PHA granules (the precipitate was collected as the insoluble fraction). PHA synthase activity was determined using the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) assay (Harada et al., 2019; Valentin and

Steinbüchel, 1994). The assay mixture containing 50 mM sodium phosphate (pH 7.0), 0.6 mM *R*-3HB-CoA, and the soluble fraction of cell lysate was incubated at 30°C for 2 min. Then, trichloroacetic acid was added to stop the enzymatic reaction. Next, 1 mM DTNB solution was added to the assay mixture which was then incubated for 2 min at room temperature and the absorbance was measured at 412 nm. The molar absorbance coefficient ($\epsilon_{412} = 14.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) was used to determine the thiol group concentration of free CoA. The protein concentrations were determined using a Quant-iT protein assay kit (Invitrogen; CA, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. SDS-PAGE was performed according to the standard procedures using 12.5% polyacrylamide gels. Five micrograms of proteins containing PhaC1_{ps}STQK (62.3 kDa) were loaded. The gels were stained with Coomassie Brilliant Blue.

Results

HA oligomer secretion in LB and MR media

The alcohol dehydrogenase gene-deficient *E. coli* strain BW25113Δ*adhE* produces less ethanol (Hiroe et al., 2013). This strain was used as a production host for HA oligomers to yield highly DEG-capped HA oligomers by reducing the ethanol-capped end.

Using LB medium supplemented with 40 g/L xylose and 50 g/L DEG, the secretory production of HA oligomers was first performed. The results are listed in Table 1. After cultivation at 30°C for 48 h, 4.21 g/L HA oligomer was secreted, which consisted of 85 mol% LA units. A total of 2.08 g/L PHA also accumulated in the cells, comprising 65 mol% 3HB units. Interestingly, the secreted HA oligomer was predominantly composed of LA units, whereas the intracellular PHA was predominantly composed of 3HB units. A similar compositional difference between intracellular PHA and extracellular HA oligomers was also reported in a previous study (Utsunomia et al., 2017c). A total of 0.22 g/L LA and 0.20 g/L 3HB were

Fig. 3. Culture results with increased NaCl concentration in Luria-Bertani (LB) medium. A. Intracellular PHA, B. extracellular HA oligomer, and C. extracellular HA monomer. LB medium originally contains 10 g/L NaCl, which was doubled to increase the osmotic pressure.

secreted, respectively. In contrast, HA oligomers were not secreted when culturing at 37°C, which is the optimal growth temperature for *E. coli*, due to the absence of PHA accumulation in the cells.

Subsequently, MR medium supplemented with 40 g/L xylose and 50 g/L DEG was used for HA oligomer production, because mineral media have not been used for HA oligomer production until now. After culturing for 48 h at 30 and 37°C, cell growth was observed at both temperatures, but almost no PHA and HA oligomers were produced. Mineral media are suitable for fermentation because they are inexpensive and compositionally simple. However, HA oligomers were not secreted efficiently in media composed of mineral salts only.

The effect of yeast extract supplementation on HA oligomer secretion in MR medium

HA oligomers were not secreted in the MR medium as mentioned above. However, interestingly, HA oligomers were secreted in a mineral-based MR medium supplemented with 1–10 g/L yeast extract. The results are listed in Table 2.

The dry cell weight increased with increasing yeast extract concentration in the medium, implying that yeast extract acts as a nitrogen source for cell growth. However, the production of intracellular PHA and extracellular HA oligomers and monomers was maximum in 5 g/L yeast extract-supplemented MR [MR(Y5)] medium.

The effect of NaCl supplementation on HA oligomer secretion in MR(Y5) medium

The MR(Y5) medium was further modified for efficient HA oligomer secretion through supplementation with 5–20 g/L NaCl. The results are listed in Table 3 and illustrated in Fig. 2.

Intracellular PHA accumulation and extracellular HA oligomer secretion increased with increasing NaCl concentration in the MR(Y5) medium. Notably, HA oligomer secretion was maximum in 10 g/L NaCl-supplemented MR(Y5) medium and then decreased in 20 g/L NaCl-sup-

plemented MR(Y5) medium. In contrast, HA monomer secretion decreased with increasing NaCl concentration. Thus, the behavior of only HA monomer secretion was different between yeast extract and NaCl supplemented media. The osmotic pressure exerted by NaCl had a significant impact on HA oligomer secretion, as well as polymer accumulation.

Enhanced secretion of HA oligomers in LB medium

The osmotic pressure exerted by NaCl enhanced HA oligomer production in the MR(Y5) medium. We then attempted to reconfirm this finding using LB medium supplemented with an additional 10 g/L NaCl. A comparison of the secretion in this modified medium with that in the original LB medium is shown in Fig. 3. PHA accumulation, as well as HA oligomer secretion, were promoted by NaCl supplementation. Consequently, the HA oligomer secretion increased to 6.42 g/L. Therefore, NaCl supplementation is useful for enhancing HA oligomer secretion. However, NaCl concentrations above 30 g/L did not enhance HA oligomer secretion in LB medium. Moreover, KCl supplementation in LB medium did not promote HA oligomer secretion.

Analysis of the size of the HA oligomers

The effect of NaCl supplementation on HA oligomer size was investigated using LB and MR(Y5) media. The secreted HA oligomers were extracted from the culture broth and subjected to ESI TOF-MS analysis. The results are shown in Fig. 4. It was inferred that the HA oligomers were predominantly DEG-capped at the carboxy end, with its oligomeric size distributed over a range of m/z 200–1,000 with m/z 500–700 (5–7 mer) as the peak oligomer. Compared with that in LB medium, the HA oligomers produced in MR(Y5) medium had a slightly larger size. However, NaCl supplementation had almost no effect on the size of the HA oligomers.

Analysis of PHA synthase expression

We further checked the expression and activity of

Fig. 4. Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) analysis of HA oligomers extracted from the supernatant of different culture media at 48 h of cultivation.

Fig. 5. PHA synthase activity (soluble fraction) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the soluble and insoluble fractions of recombinant *E. coli* BW25113 Δ *adhE* cells grown under different conditions.

The cell lysates were prepared using 24 h-cultured cells, whereas the HA oligomer yields were measured after 48 h of cultivation. Arrows indicate the position of PhaC1_{ps}STQK.

PhaC1_{ps}STQK, a key enzyme that can generate HA oligomers, under various culture conditions. The PhaC1_{ps}STQK protein sample was recovered as PHA granule-associated soluble fraction and subjected to SDS-

PAGE analysis and enzymatic activity measurement. The expression patterns of PhaC1_{ps}STQK are shown in Fig. 5. When yeast extract was added to the MR medium, the expression of PhaC1_{ps}STQK was enhanced, feasibly cor-

relating with the increased PhaC1_{ps}STQK activity and HA oligomer generation. On the contrary, NaCl supplementation only enhanced HA oligomer generation in yeast extract-supplemented MR(Y5) medium, and it did not affect PhaC1_{ps}STQK expression and activity. In 10 g/L NaCl-supplemented LB medium, PhaC1_{ps}STQK expression and activity, and HA oligomer generation were all increased. Moreover, a good relationship was observed between the band intensity and enzymatic activity of PhaC1_{ps}STQK secreted in LB and MR(Y5), but not in MR, when the soluble PhaC1_{ps}STQK fraction was analyzed.

Discussion

HA oligomers are considered to be produced by increasing the frequency of CT reactions during PHA polymerization. The HA oligomer generated within the cell can be secreted outside through several probable routes (Utsunomia et al., 2017a). The secreted HA oligomer and intracellular PHA are predominantly composed of LA and 3HB, respectively (Fig. 2). This compositional difference in polymerized products is attributed to the difference in hydrophilicity of LA and 3HB. LA is more hydrophilic than 3HB and the hydrophilic oligomers are more easily secreted into the extracellular environment. Therefore, LA-rich oligomers are more easily secreted than 3HB-rich oligomers.

The MR medium has been used for high-cell density cultures of recombinant *E. coli* to produce intracellular PHA. For instance, the efficient production of P(3HB) by *E. coli* XL1-Blue has been demonstrated using MR medium without yeast extract supplementation (Kahar et al., 2005; Wang and Lee, 1997; Woo et al., 2000), as well as P(LA-co-3HB) production by *E. coli* MG1655 (Hori et al., 2019). In contrast, yeast extract-supplemented mineral media has been reported to support PHA synthesis in other *E. coli* strains (Fadzil et al., 2018; Hiroe et al., 2016).

In this study, we found that supplementing MR medium with yeast extract and NaCl increased the HA oligomer secretion by recombinant *E. coli* BW25113Δ*adhE*, and yeast extract supplementation increased both PhaC1_{ps}STQK expression level and PHA synthase activity (Fig. 5). Since yeast extract is an important source of amino acids, it may upregulate the PhaC1_{ps}STQK expression level simply due to the increased amount of amino acids available for protein synthesis. Meanwhile, the increase in PHA synthase activity may occur due to the activation of intracellular chaperons in host cells. Generally, chaperons play an important role in the proper folding of newly synthesized proteins, including PHA synthases, as demonstrated in our previous study (Thomson et al., 2013). Additionally, it has been reported that a chaperon-mediated protein disaggregation in *E. coli* is drastically activated by the existence of compatible solutes (osmolytes) and NaCl stress (De Marco et al., 2005; Diamant et al., 2003). If yeast extract functions as an osmolyte source, it is reasonable to assume that the chaperons were activated in response to the media components, resulting in enhanced PHA synthase activity. The same effect may have also occurred in LB medium with increased NaCl concentration (Fig. 5).

In conclusion, this study demonstrates that HA oligomers can be effectively secreted in 5 g/L yeast extract and 10 g/L NaCl supplemented MR medium. Additionally, NaCl supplementation was useful for enhancing HA oligomer secretion even in LB medium. The findings of this study may be beneficial for developing an efficient method to secrete HA oligomers using inexpensive mineral-based media. Moreover, supplementation with osmotic regulators, such as NaCl, may also be a novel approach for improving intracellular PHA productivity as well as extracellular HA oligomer productivity.

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