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ORIGINAL PAPER

The change of the state of cell membrane can enhance the synthesis of menaquinone in *Escherichia coli*

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Abstract Menaquinone (MK) was an attractive membrane-bound intracellular chemical. To enhance its production, we tried to find the relationship between its synthesis and the state of cell membrane in producing strain. Due to non-ionic surfactant-polyoxyethylene oleyl ether (POE) and plant oil-cedar wood oil (CWO) can typically increase extracellular secretion and intracellular synthesis of MK respectively, the effect of these two substances on cell morphology, physical properties of cell membrane was investigated. Finally, two engineering strains were constructed to verify whether the state of cell membrane can enhance MK synthesis. The result showed that the edge of cells was broken when POE added in the medium. Other physical properties such as total fatty acid content decreased by 40.7% and the ratio of saturated fatty acids to unsaturated fatty acids decreased from 1.58 ± 0.05 to 1.31 ± 0.04. Meanwhile, cell membrane leakage was enhanced from 7.14 to 64.31%. Different from POE group, cell membrane was intact in CWO group. Moreover, the ratio of saturated fatty acids to unsaturated fatty acids increased from 1.58 ± 0.05 to 1.78 ± 0.04 and the average lipid length decreased from 16.05 ± 0.08 to 15.99 ± 0.10. Two constructed strains, especially *Escherichia coli* DH5α FatB, exhibited strong MK secretion ability and the extracellular MK reached 10.71 ± 0.19 mg/L. An understanding of these functionary mechanisms could not only provide a new idea for the synthesis of MK, but also provide a reference to increase the yield of intracellular membrane-bound metabolites.

Keywords Menaquinone · Cell membranes · Membrane fluidity · Leakage

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Introduction

For the important medical value in promoting bone metabolism and blood coagulation (Ishida 2008), reducing cardiovascular calcification (Scheiber et al. 2015), and curing amyotrophic lateral sclerosis (Bhalerao and Clandinin 2012; Nakagawa et al. 2010), the synthesis of menaquinone (MK) had received increasing interest. *Bacillus subtilis* natto and *Flavobacterium meningosepticum* were two major producing bacteria for MK synthesis. To promote the synthesis ability of producing strains, some methods such as random mutagenesis and gene manipulation were adopted (Hiratsuka et al. 2008; Suvarna et al. 1998; Kong and Lee 2011). Although there were indeed some improvements in the MK production, there was still a large lifting space for tapping compared with the production of ubiquinone (CoQ), which has similar structure and function to the MK.

CoQ and MK were composed of a different polar head group (benzoquinones and naphthoquinones, respectively) and a same hydrophobic side chain (isoprenoid side chains of various lengths) (Fig. 1). Most of CoQ and

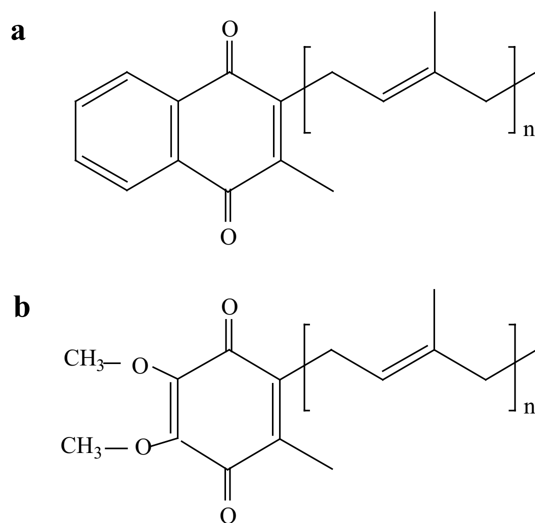

Fig. 1 Chemical structure of MK (**a**), CoQ (**b**). n represents the number of isoprenoid

MK molecules had the same function as mobile carriers of electrons in membranes. Therefore, the researches on CoQ provided a certain reference on MK. Previous study found that there was a remarkable developed multiple-layer structure of inner membrane in the cells which contained higher content of CoQ (Yoshida et al. 1998). Another study also reported that the envelope structure of cell membrane was required when MK was selective released (Hisataka et al. 1995). Besides, our recent study further confirmed that there was a certain relationship between membrane potential and MK content (Liu et al. 2016). Therefore, the change of the state of cell membrane may change the yield of these membrane-bound molecules. However, to the best of our knowledge, there has been no detailed report on which parameters of cell membrane were changed when different contents of MK was synthesized.

The cell membrane was selectively permeable. It was able to regulate what entering and exiting in the cell. Moreover, it was also responsible for internal and external material exchange and transportation in cells. These three features of cell membrane were especially important for intracellular metabolisms. The reason maybe that there were at least two major limits for the production of intracellular metabolism. First was that the formation of intracellular metabolism in the fermentation process was inhibited by intermediate metabolisms with higher concentration and intracellular enzymes. Take MK synthesis for example, the production of intracellular MK was limited in bacteria, since 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, the first regulatory enzyme in the shikimate pathway, feedback-regulating chorismate and MK-4 (Taguchi et al. 1991). Second was that the cell structure should be broken in order to extract the intracellular metabolism, so it was impossible to utilize the cells repeatedly. Sometimes the cells were still productive but didn't yield the goal metabolism only because they reached saturated state in cells. It caused the abundant waste of raw material. Therefore, it had very important significance if the cell membrane selective transfered the goal metabolism extracellularly on the premise of maintaining other nutrient substance normally exchange. Altogether, regulation of cell membrane had vital significance on the production of intracellular metabolism.

Previously studies showed that plant oil-cedar wood oil (CWO) and non-ionic surfactant-polyoxyethylene oleyl ether (POE) had great promoting effect on the production of MK (Yoshiki and Hisataka 1988, 1989). Moreover, our lab found that POE supplementation could enhance the extracellular production of MK metabolites in shake flask cultures (Liu et al. 2014) and CWO was beneficial to the intracellular MK production (the data was shown in this paper). So, we took these two easy methods as examples to study the relationship between MK production and the state of cell membrane.

In our study, the different effects of CWO and POE on the MK synthesis and cell morphology was observed. Furthermore, we first confirmed and quantified the potentially damaging effects of CWO and POE on the cell membrane, the possible functionary mechanism of MK synthesis in *Escherichia coli* An understanding of these functionary mechanisms could not only provide a new idea for enhancing MK synthesis, but also provide a reference to increase the yield of intracellular membrane-bound metabolites.

Materials and methods

Strains and growth conditions

E. coli strain FM3-1709 was obtained as a 1-hydroxy-2-naphthoate-resistant mutant and further mutated by nitrogen ion beam irradiation as previous described (Liu et al. 2015). It was maintained on beef extract-peptone agar slants, which were made up of the following components (in g/L): beef extract 3, peptone 10, NaCl 5 and agar 20. The pH value was initially adjusted to 7.0. The slants were incubated at 37 °C for 96 h and then stored at 4 °C. The seed and basal fermentation medium contained (in g/L): glycerol 10, peptone 10, yeast extract 1.5, K_2HPO_4 4.5, NaCl 3, $MgSO_4 \cdot 7H_2O$ 0.3. The initial pH value was adjusted to 7.0. The flask culture experiments were performed in 500-mL flasks containing 100 mL of the basal medium inoculated with 10% (by volume) of the seed culture. Each culture was incubated at 37 °C on a rotary shaker incubator at 200 rpm (4.44 × g) for 120 h.

E. coli DH5α was obtained from Sangon (Shanghai, China). *E. coli* DH5α FatB and *E. coli* DH5α FatA were constructed by using *E. coli* DH5α as the plasmid hosts for heterologous expression of the FatB type acyl-ACP thioesterase from *Umbellularia californica* (BTE) and the FatA type thioesterase from Sunflower (*Helianthus annuus L.*). The bacteria were grown in LB medium which contained (in g/L): Bacto tryptone 10, Bacto yeast extract 5, NaCl 10, pH 7.0 and the liquid cultures were shaken vigorously at 37 °C.

Determination of membrane fluidity

Membrane fluidity could be measured as a fluorescence polarization or anisotropy value, which corresponded to how a fluorescent probe inside the membrane reacted to polarized light (Royce et al. 2013). Briefly, the samples were washed twice in PBS, pH = 7.0, resuspended (1×10^8 cells/mL), and incubated at 37 °C for 30 min with 6-diphenyl-1, 3, 5-hexatriene at a concentration of 0.2 µM (0.2 mM stock solution in tetrahydrofuran). Fluorescence polarization values were determined by using a Synergy 2 Multi-Mode microplate reader from BioTek and using sterile black-bottom Nunclon delta surface 96-well plates. The filters were 360/40 nm fluorescence excitation and 460/40 nm fluorescence emission filters from BioTek. The excitation polarized filter was set in the vertical position. The emission polarized filter was set either in the vertical (IVV) or horizontal (IVH) position. The polarization value was calculated by the following formula:

$$P = \frac{IVV - IVHG}{IVV + IVHG} \tag{1}$$

where G was the grating factor, assumed to be 1. The cells were treated with octanoic acid at pH 7.0 just before measurements.

Membrane leakage

The method adopted was described by previous study (Grac et al. 2002) with some modifications. Cells cultivated in control, POE or CWO addition medium and harvested at the end of the exponential growth phase (OD600 of approximately 0.6) by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0). Samples were diluted to give a final number of approximately 10^7 cells/mL. PI, as a positively charged fluorescent nucleic acid dye, was used to stain cells with compromised membranes. Stock solutions of 1.0 mg of PI (Molecular Probes) per mL were prepared in distilled water, stored in the refrigerator, and kept in the dark. 1 mL cell suspensions added with 5 µL PI was incubated at 30 °C for 10 min. The mixture was washed by potassium phosphate buffer and then analyzed by flow cytometry.

FACS was carried out on a FACS can low cytometer (Becton–Dickinson, Mountain View, USA) using the following settings: forward scatter = E00, side scatter = 420, FL-3 = 640 nm. Cells were excited with an air-cooled argon ion laser (488 nm), and FL-3 was used to detect PI fluorescence.

Membrane lipid composition

FM3-1709 cells were harvested and resuspended in 25 mL medium with 0–30 mM phosphate buffer (pH 7.0), and incubated for 3 h. The cells were washed twice by cold sterile water and split into four tubes. Then, 50 µL of 0.4 mg/mL C13/C19 (tridecanoic acid/nonadecanoic acid) in chloroform was added as internal standards. The Bligh and Dyer method was adapted for membrane lipid extraction (Bligh and Dyer 1959). Briefly, the cells were resuspended in 1.4 mL methanol, sonicated for three 20 s bursts, and incubated at 70 °C for 15 min. The cells were then centrifuged at 5,000×*g* for 10 min and the supernatant was collected. The cell pellet was further treated with 750 µL chloroform and incubated at 37 °C for 5 min shaking in a horizontal shaker at 150 rpm. A methanol/water/chloroform (1.9:1.9:1) ternary mixture was used to phase-separate the extracted lipids in the chloroform layer. The free fatty acids were concentrated with a N-Evap nitrogen tree evaporator (Organomation Associates). The lipids were methylated into fatty acid methyl esters (FAMEs) at 80 °C for 30 min by adding 2 mL 1 N hydrochloric acid in methanol to samples concentrated under nitrogen, then 1 mL 0.9% sodium chloride solution was added. The FAMEs were extracted with hexane, filtered with a 0.2 µm polytetrafluoroethylene (PTFE) filter, and analyzed by gas chromatograph-flame ionization detector/mass spectrometer (GC-FID/MS) using the following instruments: an Agilent 7890 gas chromatography, an Agilent 5975 mass spectroscopy, and a Agilent 190,915–43,330 m × 0.25 mm × 0.25 mm column (Agilent Technologies). The initial temperature was set at 50 °C, holding for 1 min, with the following temperature ramp: 20 °C/min to 140 °C, 4 °C/min to 220 °C, and 5 °C/min to 280 °C with 1 mL/min helium carrier gas. The relative retention factor of C19 was used to adjust and analyze the relative amounts of the individual fatty acids (Tvrzická et al. 2002).

The saturated/unsaturated fatty acids ratio (S/U) and weighted-average lipid length were calculated as follows:

$$S/U = \frac{C12{:}0 + C14{:}0 + C16{:}0 + C18{:}0}{C12{:}1 + C14{:}1 + C16{:}1 + C17cyc + C18{:}1 + C19cyc} \tag{2}$$

$$L = \frac{12 \times (C12{:}0 + C12{:}1) + 14 \times (C14{:}0 + C14{:}1) + 16 \times (C16{:}0 + C16{:}1 + C17cyc) + 18 \times (C18{:}0 + C18{:}1 + C19cyc)}{C12{:}0 + C12{:}1 + C14{:}0 + C14{:}1 + C16{:}0 + C16{:}1 + C17cyc + C18{:}0 + C18{:}1 + C19cyc} \tag{3}$$

Scanning electron microscopy (SEM)

Cells cultivated in control, POE or CWO addition medium were harvested by centrifugation. Wash twice by distilled water and fixed at 4 °C with 2.5% (v/v) glutaralde-hyde in 0.1 M phosphate buffer (pH 7.2). After three buffer washes, samples were dehydrated in a graded series of increasing ethanol concentrations (30, 50, 70, 90, 100, and 100% (v/v), 15 min each step). After air drying, the fixed material was sputter-coated with gold for 180 s. The mate-rial was examined and photographed with a field emission scanning electron microscope (Sirion200, FEI, Hillsboro, OR, USA).

Transmission electron microscopy (TEM)

To get a dense bacterial suspension, 1 L of sample was concentrated to about 4 mL with the hollow-fiber system and fixed with formaldehyde (2% v/v). For calibration, latex spheres with a density of 1.055 g/cm^3 and diameters of 0.093, 0.282, and 0.415 mm (Molecular Probes, Eugene, Oreg) were sprayed in microdroplets onto grids and air dried. For transmission electron microscopy (TEM), copper slotgrids (Gropl, Tulln, Austria) supported with Formvar film and coated with 0.5% bovine serum albumin (Sigma, Vienna, Austria) were used. Drops of cultures were added directly onto the grids. After 1 min, the drops were carefully drained off with filter paper, and examined and photographed by Transmission electron microscopy (Zeiss, Oberkochen, Germany).

Menaquinone analysis by HPLC

Cells and culture fluid were separated by centrifuging the culture broth at 8000×*g* for 5 min. The cells collected were freeze-dried and dissolved with 3 mL methanol by shaking at 55–60 °C for 10 min. In each run, the mixture was stirred well and then centrifuged at 999×*g* for 10 min to separate the organic and aqueous layers. The amounts of intracellular and extracellular MK in the upper organic phase were determined by high performance liquid chromatography (HPLC) (Waters 600, Waters, USA) equipped with a Phenomenex C18 column with 250×4.60 mm, 4 µm at 35 °C. Methanol-dichloromethane (8:2, v/v) was used as mobile phase with a flow rate of 1.0 mL/min. A wavelength of 248 nm was selected for calibration and analysis. Commercially available MK (Sigma–Aldrich, St. Louis, Mo.), processed in parallel with the samples, was used as a standard.

Statistical analysis

The p values were obtained using one-way ANOVA and Tukey–Kramer pair analysis with the JMP v/8.02 statistical program (SAS Institute, Cary, NC, USA). The statistical significance of the linear regression estimates of the weight-average lipid length was performed in JMP using the t-test on the intercept and the slope (concentration).

Results

Effect of cedar wood oil (CWO) and polyoxyethylene oleyl ether (POE) on MK synthesis

In order to explore enhancing mechanism of CWO and POE on MK synthesis metabolism, the exact effects of two factors on intracellular and extracellular MK yield were compared by varying different concentrations of CWO and POE (Fig. 2).

A rapid increase in intracellular MK yield was observed when the concentration of CWO ranged from 0.2 to 0.4 g/L, and decreased slowly with a further

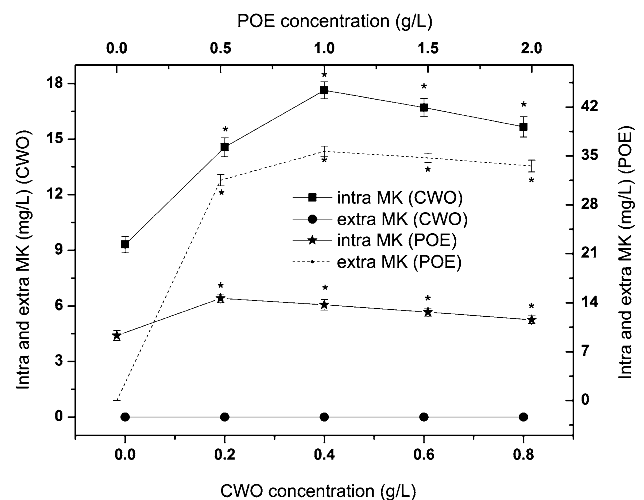

Fig. 2 The intracelluar and extracellular MK production of FM3-1709 modeled as a function of the concentration of CWO and POE. MK amount was measured at 120 h of cultivation. Intra MK–intracellular MK, extra MK–extracellular MK. All data represents the average of at least three biological replicates, with error bars indicating standard deviation values. An *asterisk* indicates significantly different values from the control ($p < 0.05$)

increase of CWO concentration. Extracellular MK was hardly detected in CWO addition medium. Intracellular MK increased slowly and kept stable when the concentration of POE ranged between 0.5 and 2.0 g/L, the tendency also similar in the extracellular MK.

These results showed that POE could enhance extracellular MK production and CWO could promote intracellular MK synthesis in *E. coli* FM3-1709. In order to unravel the intrinsic differences among different cultivation conditions, the cell morphology and membrane integrity in MK synthesis were analyzed.

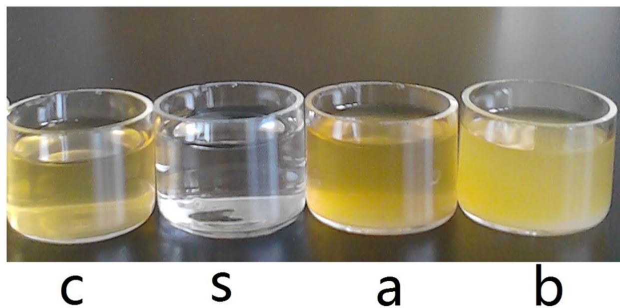

Fig. 3 Appearance of different medium for strain cultivation. *a* basal fermentation medium; *b* basal fermentation medium supplemented with 0.2 g/L CWO; *c* basal fermentation medium supplemented with 0.5 g/L POE; *s* sterile water

Effect of CWO and POE on cell morphology

The effect of CWO and POE on culture medium (Fig. 3) and cell morphology (Fig. 4) was then investigated. Among them, CWO addition made the medium more turbid than the control (Fig. 3b). On the other hand, medium added with POE was clearer and more transparent (Fig. 3c). The medium turbidity decreased in the following order: medium added with CWO > Control > medium added with POE > Sterile water. Given that the decrease of surface tension was beneficial to dissolve the components of medium and decrease fermentation broth viscosity, POE addition was more conducive to the mass transfer and substance exchange between cells and fermentation broth.

Cells harvested from different medium were microscopically visible in Fig. 4. *E. coli* FM3-1709 was a straight rod by scanning electron microscope (SEM) observation(Fig. 4a). There was a large amount of snowflake shaped substrates covering the surface of the cells in CWO group (Fig. 4b), while a large amount of granular substances covering the surface of the cells in POE group (Fig. 4c). Therefore, CWO and POE had obvious effect on the cell surface. Snowflake shaped substrates may prevent the internal and external substrates exchanging. Due to POE has the similar polarity to phospholipid of membrane, POE may dissolve part of phospholipid molecules and form

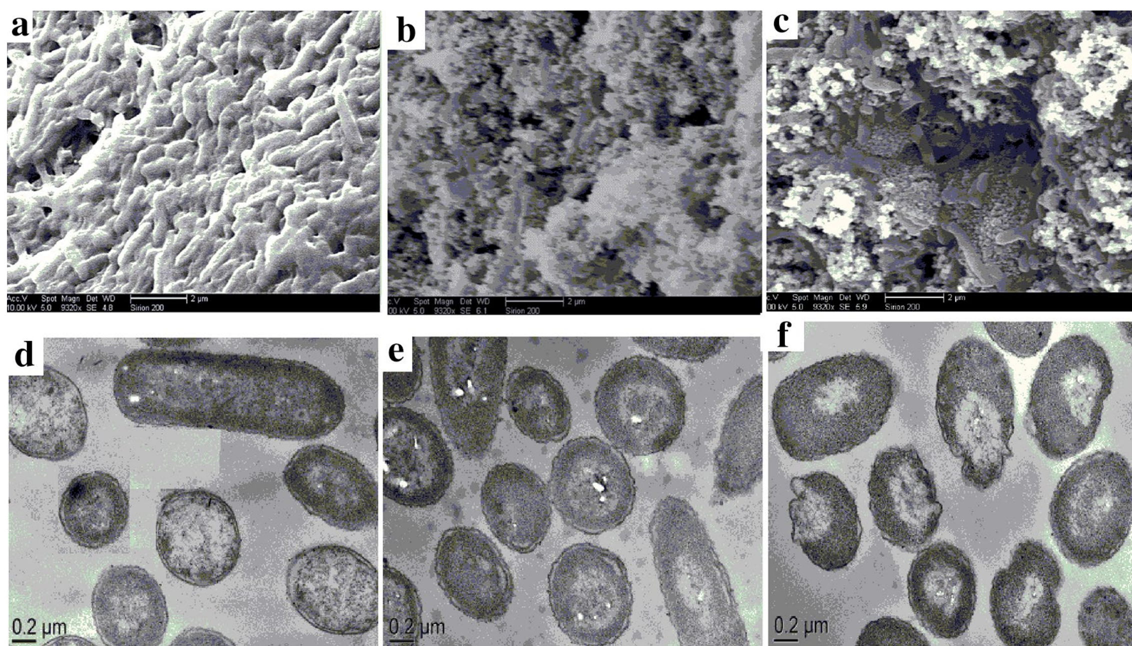

Fig. 4 Morphology of cells harvested from different medium by SEM (**a**–**c**) and TEM (**d**–**f**) observation. *Bars* represent 2 µm (SEM) and 0.2 µm (TEM). **a** and **d** represent morphology of cells cultivated in basal fermentation medium; **b** and **e** represent morphology of cells cultivated in basal fermentation medium supplemented with 0.2 g/L CWO; **c** and **f** represent morphology of cells cultivated in basal fermentation medium supplemented with 0.5 g/L POE

granular substances on the surface of cells, then cell membrane structure was changed and MK was secreted.

In order to find the structural changes of cells, cells harvested from different medium were observed using transmission electron microscope (TEM). It can be seen from the cut surface of cells, there was an intact cell membrane around the cells in the control and CWO group (Fig. 4d, e). While in the POE group, the edge of cells was swelled, ruptured, and intracellular contents secreted outside (Fig. 4f). In addition, compared with control, the color of cells was deeper in CWO group, the contents of cells increased in CWO group may inhibit TEM light passing through.

These results demonstrated that there were great differences in cell morphology and membrane integrity between different groups. Identification of these changes can provide insight into the mechanism of MK synthesis.

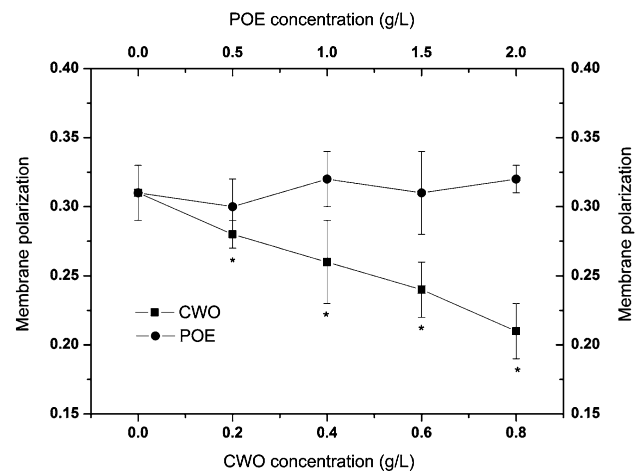

Fig. 6 Fluorescence polarization of FM3-1709 cultivated in CWO and POE addition medium after 120 h cultivation. The polarization values are unitless. Eight technical replicates were averaged. An *asterisk* indicates significantly different values from the control

Membrane integrity and fluidity

Previous studies had speculated that some metabolites secretion was imposed by membrane damage (Desbois and Smith 2010; Lennen et al. 2011). The damage in terms of membrane integrity and membrane fluidity was quantified. Measurements of the membrane integrity and membrane fluidity confirmed that POE and CWO really damaged cell membrane (Figs. 5, 6; Table 1). One indication of a damaged membrane was the membrane porosity, as reflected by % gated, which represented the ratio of PI staining cells to all cells. When the cell membrane permeability increased, there would be more PI enter into cells and bind with DNA. From Table 1, POE addition resulted in the 64.31% PI staining, which was higher than the control sample (7.14%) and the CWO group (6.78%). On the contrary, CWO addition resulted in the 6.78% PI staining, which was even lower than the control. Therefore, cell membrane leakage was obviously enhanced when POE added to the medium.

Another indication of a damaged membrane was the membrane fluidity. As CWO concentration increased, the

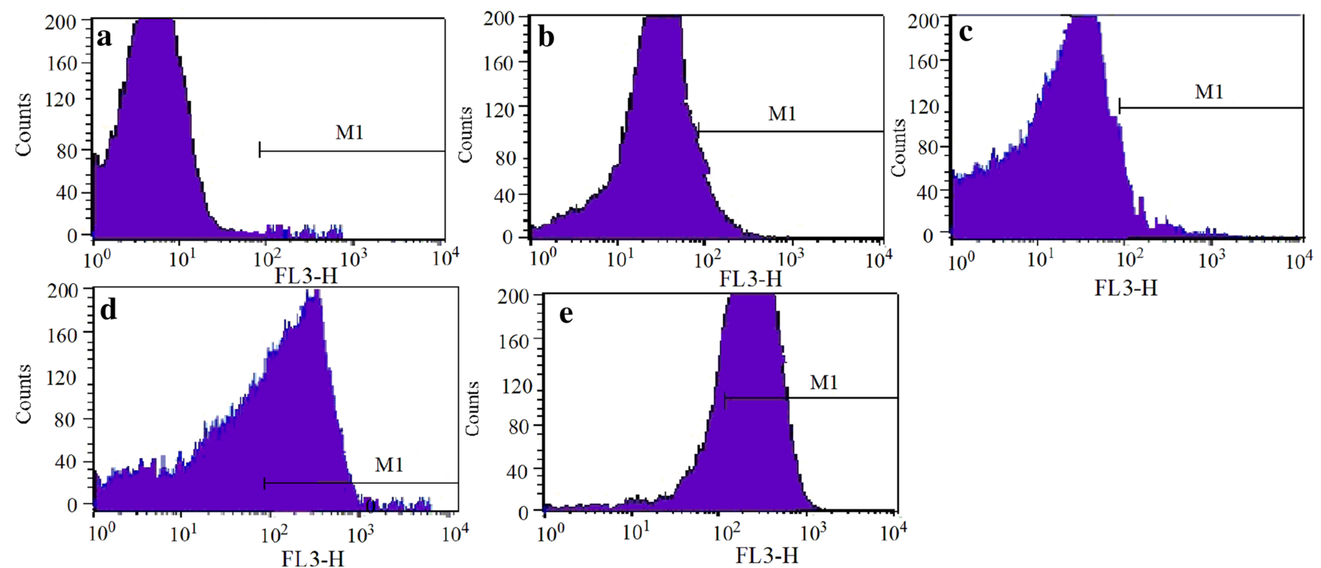

Fig. 5 Cell membrane permeability measured by flow cytometry–PI staining (**a** negative control, that means cells cultivated in basal fermentation medium not stained by PI; **b** cells cultivated in basal fermentation medium; **c** cells cultivated in basal fermentation medium supplemented with 0.2 g/L CWO; **d** cells cultivated in basal fermentation medium supplemented with 0.5 g/L POE; **e** positive control that means cells cultivated in basal fermentation medium, destroyed by ultrasonic waves at a frequency of 20 kHz for 5 min and stained by PI)

Table 1 Fluorescence distribution of the different regions of cells measured by FCM

Sample ID	Marker	Events	% Gated	% Total	Mean
a	All	96,500	100.00	80.76	7.38
	M1	50	0.05	0.04	287.11
b	All	96,500	100	63.48	66.57
	M1	6892	7.14	4.53	135.97
c	All	96,500	100.00	62.29	67.01
	M1	6550	6.78	4.22	150.81
d	All	96,500	100.00	42.34	174.12
	M1	62,059	64.31	27.23	241.70
e	All	96,500	100.00	52.21	371.59
	M1	85,066	88.15	46.02	412.94

PI staining (M1 represents the outside area of cell autofluorescence; a: negative control, that means cells cultivated in basal fermentation medium not stained by PI; b: cells cultivated in basal fermentation medium; c: cells cultivated in basal fermentation medium supplemented with 0.2 g/L CWO; d: cells cultivated in basal fermentation medium supplemented with 0.5 g/L POE; e: positive control that means cells cultivated in basal fermentation medium, destroyed by ultrasonic waves at a frequency of 20 kHz for 5 min and stained by PI). Marker: all or gate events which was marked by PI; M1: gate events which was marked by PI; % Gated: all or M1 events/all events × 100%; % Total: all or M1 events/(all + M1) events × 100%; Mean: the fluorescence intensity of each part of events in average

fluorescence polarization was significantly decreased in *E. coli* FM3-1709, corresponding to increased fluidity (Fig. 6). However, fluorescence polarization had no significant change when POE concentration ranged from 0.5 to 2.0 g/L.

These results confirmed that POE has a significant impact on integrity of *E. coli* FM3-1709 while CWO has a significant impact on membrane fluidity. So, maybe membrane leakage related to MK secretion and membrane fluidity related to MK intracellular synthesis. Due to the close relationship between membrane composition and membrane fluidity and integrity, the fatty acid composition under these conditions was further tested.

Membrane lipids

In order to better understand the changes in the membrane fluidity and integrity that occur during CWO or POE addition and lead to maintenance of fluidity, the membrane lipid content and composition of *E. coli* cells grown in the control, CWO and POE medium were first quantified (Table 2). Data were average values of at least three determinations. As previously observed from bacteria, the membrane lipids would change upon environmental perturbations (Di et al. 2007).

Table 2 gave a complete overview of these parameters. As expected, there was a substantial difference between

Table 2 Membrane lipid composition, mol ratio of saturated to unsaturated fatty acids and the weight-average lipid length of FM3-1709 cells cultivated in different medium after 120 h cultivation

Group	Fatty acids	Mol% membrane lipids								S:U ratio	Average liquid length
	μg/mg dry wt	C12:0	C14:0	C16:1	C16:0	C17cyc	C18:1	C18:0	C19cyc		
Control	18.9 ± 0.5	1.1 ± 0.2	6.5 ± 0.5	19.8 ± 1.1	52.5 ± 1.4	8.7 ± 0.8	9.1 ± 0.7	1.1 ± 0.1	1.2 ± 0.3	1.58 ± 0.05	16.05 ± 0.08
CWO	19.1 ± 0.8	2.0 ± 0.2	7.2 ± 0.5	17.5 ± 1.2	53.9 ± 1.4	8.9 ± 0.7	8.5 ± 0.5	0.9 ± 0.2	1.1 ± 0.3	1.78 ± 0.04	15.99 ± 0.10
POE	11.2 ± 0.7	0.9 ± 0.2	4.7 ± 0.4	21.4 ± 1.3	50.3 ± 1.2	8.4 ± 0.6	12.2 ± 0.6	0.8 ± 0.2	1.3 ± 0.2	1.31 ± 0.04	16.16 ± 0.06

C12:0–lauric acid, C14:0–myristic acid, C16:1–palmitoleic acid, C16:0–palmitic acid, C17cyc–cyclopropane C17:0, C18:1–vaccenic acid, C18:0–stearic acid, C19cyc–linolenic acid. Mol ratio of saturated to unsaturated fatty acids and the weight-average lipid length after adaptation

control, CWO and POE groups. The total amount of liquids, represented by the fatty acids, decreased by 40.7% in POE group (11.2 ± 0.7 μg/mg dry wt) and increased by 1.0% in CWO group (19.1 ± 0.8 μg/mg dry wt) compared to the control (18.9 ± 0.5 μg/mg dry wt); also, S/U was definitely changed in POE and CWO group. The analysis of individual fatty acids revealed both a relative increase in C16:0 [(53.9 ± 1.4)%] and a relative decrease in C16:1 [(17.5 ± 1.2)%] compared to the control. While C16:0 and C16:1 were responsible for the change in S/U upon addition of CWO. However, upon addition of POE, the effect was reversed to the CWO group. Interestingly, the shorter lipids (C12:0 and C14:0) decreased whereas the longer lipids (C18:1) increased upon addition of CWO. The shorter lipids (C12:0 and C14:0) decreased whereas the longer lipids (C18:1 and C18:0) increased upon addition of POE. The change is apparent in S/U and the weight-averaged lipid length (Table 2). The S/U significantly increased and the lipid length slightly decreased upon CWO addition. These combined changes of total fatty acids, S/U and the lipid length may be the mechanism behind the maintenance of the membrane fluidity and integrity.

From the results above, it could be predicted that membrane leakage was the main reason for MK extracellular secretion, while the decrease in total fatty acid and decrease in S/U could promote the membrane leakage. On the other hand, membrane fluidity maybe the reason for intracellular MK synthesis. The difference between membrane leakage and fluidity was mainly due to different S/U and total fatty acid concentration, which could lead to the change of the physical properties of the phospholipid bilayer, such as selective permeability. It could be speculated that when total fatty acid decreased and S/U decreased, the cells could be induced to excrete excessive MK extracellularly and the intracellular MK concentration kept below the level to feedback the inhibition of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, then the improvement in the total MK concentration was realized.

Change of membrane composition alters MK synthesis

To investigate whether total fatty acid and S/U were the cause of the increase of intracellular and extracellular MK in *E. coli* FM3-1709, two genome engineering strain *E. coli* DH5α FatB and *E. coli* DH5α FatA were constructed, which harbored FatB type acyl-ACP thioesterase from *U. californica* (BTE) and the FatA type thioesterase from Sunflower (*H. annuus L.*), respectively. The result of quantitative western blot analysis showed that there was no significant difference between the protein expression level of FatB type acyl-ACP thioesterase and FatA type thioesterase in the cells (data not shown). Their fatty acid composition and ability to produce MK in different *E. coli*

Table 3 Membrane lipid composition, mol ratio of saturated to unsaturated fatty acids, the weight-average lipid length and MK production of *E. coli* DH5α cells after 120 h cultivation

Group	Fatty acids	Mol% membrane lipids								S:U ratio	Average liquid length	Intra MK	Extra MK
	μg/mg dry wt	C12:0	C12:1	C14:0	C14:1	C16:1	C16:0	C17cyc	C18:1			mg/L	mg/L
DH5α	14.5 ± 0.2	12.1 ± 0.2	8.7 ± 0.2	1.8 ± 0.5	0.9 ± 0.2	9.8 ± 0.4	33.9 ± 1.4	0.7 ± 0.2	32.1 ± 0.7	0.92 ± 0.03	15.76 ± 0.08	3.21 ± 0.21	0 ± 0.03
DH5α FatA	15.1 ± 0.3	14.1 ± 0.4	6.2 ± 0.2	7.4 ± 0.3	1.4 ± 0.1	0 ± 0.1	39.1 ± 1.4	1.4 ± 0.3	30.4 ± 0.5	1.54 ± 0.04	15.62 ± 0.07	4.75 ± 0.19	0 ± 0.03
DH5α FatB	13.2 ± 0.3	10.3 ± 0.3	9.5 ± 0.2	1.5 ± 0.4	1.3 ± 0.2	7.8 ± 0.3	34.3 ± 1.2	1.0 ± 0.2	34.3 ± 0.4	0.85 ± 0.04	15.90 ± 0.06	4.36 ± 0.17	10.71 ± 0.19

C12:1–cis-5-dodecenoic acid, C14:1–cis-7-tetradecenoic acid

DH5α mutants were tested (Table 3). When *E. coli* DH5α expressed the FatA type thioesterase from *H. annuus*, it predominantly cleaved unsaturated C16 and increased S/U by approximately 12.7%. On the other hand, *E. coli* DH5α FatB resulted in greatly elevated levels of unsaturated phospholipids, which was consistent with previous study (Voelker and Davies 1994). Most interestingly, although there was a slightly decreased in total fatty acids in *E. coli* DH5α FatB group (13.2 ± 0.3 μg/mg dry wt) compared to the control (14.5 ± 0.2 μg/mg dry wt), extracellular MK increased significantly compare to the control. After 120 h cultivation, the extracellular MK reached 10.71 ± 0.19 mg/L in *E. coli* DH5α FatB group, while it can't be detected in *E. coli* DH5α group and *E. coli* DH5α FatA group. Altogether, total fatty acids decrease and unsaturated liquids increase were beneficial to MK secretion, while otherwise could only slightly increase intracellular MK production.

Discussion

Genetic engineering which consisted of gene knockout and overexpression based on transposons, plasmid overexpression, gene shuffling, and other methods of mutagenic manipulation, have been widely used for the reconstruction of microbial strains. As for MK- production strains, previous study focused on the strains by overexpressing or knocking out genes of key enzymes in the pathway of MK synthesis (Kong and Lee 2011). However, metabolic network was a system of mutual restraint interrelated. Changing one or one series of enzymes may not necessarily affect entire metabolic flux. So, it should find another way to enhance MK production for its bright application prospect.

As we know, the change of the state of cell membrane may have obviously effect on promoting membrane-bound molecules. Many of the functional responses were directly caused by the change of composition and structure of membrane lipid, which conversely influence either bulk lipid fluidity or specific lipid domains. The conformation or quaternary structures of certain transporters, receptors, and enzymes were probably sensitive to changes in the structure of their lipid microenvironment, leading to the changes in activity. Furthermore, several studies had shown that the modifications of membrane fatty acid composition was extensive to alter membrane fluidity and affect a number of cellular functions, including stimulation of glucosyltransferase secretion (Jacques et al. 1985), improvement of free fatty acid tolerance in *E. coli* (Lennen and Pfleger 2013) and full transportation of lactose permease (Vitrac et al. 2013). In our study, it could be found that the change of the state of cell membrane can enhance the synthesis of menaquinone in *E. coli*. It not only provided a new idea for the synthesis of MK, but also provided a reference to enhance the yield of intracellular membrane-bound metabolites.

MK was a membrane bound compounds. In molecular structure, MKs consisted of a polar head group and a hydrophobic side chain. The latter part provided the molecules with a lipid-soluble character to allow them to perform vital functions in membrane lipid bilayer, while the former group enables interaction with membrane proteins. Therefore, the synthesis of MK related to the state of cell membrane. Our study also confirmed this hypothesis. When different methods were used to promote the yield of MK, cell membrane changed through cell morphology observation, membrane integrity, fluidity and lipids content detection (Figs. 2, 3, 4, 5, 6; Tables 1, 2). The result was consistent with that of ubiquinone-10, which had a similar structure and function to that of MK. There was a remarkably developed multiple-layer structure of inner membrane in the cell when different content of ubiquinone-10 was obtained (Yoshida et al. 1998).

Membrane leakage indicated the cellular membrane porosity. Due to its important functions such as selectively exclude harmful compounds and retain valuable metabolites, membrane leakage was an important index for the synthesis of fermentation product. Given that the mechanisms of MK synthesis in *E. coli* was due to the integrity of cell membrane, the cell morphology and cell membrane leakage were compared when CWO and POE added to the medium. From cell morphology observation, POE obviously destroyed the cell membrane, the edge of cells was swelled, ruptured, the contents secreted outside (Fig. 4f) and membrane porosity was damaged.

However, the cell was still intact and only the color was deeper than the control in CWO group (Fig. 4e). FCM detection confirmed that membrane leakage was affected by POE but not by CWO (Fig. 5). It indicated that membrane leakage was beneficial for MK secretion.

Membrane fluidity as an important physiological element which was used to quantify membrane damage and study membrane dynamics was detected. Addition of CWO decreased fluorescence polarization, which indicated an increase in fluidity (Fig. 6). While POE didn't affect Membrane fluidity. Therefore, part of CWO may flow laterally and transiently within the lipid bilayer and reduce hydrophobic interactions of the fatty acid tails thus increasing fluidity.

It was known that when the environment condition changed, the membrane composition in bacteria also made corresponding changes (Epand and Epand 2009), which in turn affect the membrane leakage and fluidity. Biological membranes are composed of a thin film of phospholipids and proteins. Total amount of fatty acids and S/U were important component of phospholipids, which was seemed to play a crucial role in intracellular metabolism secretion

in many studies (Hartmann et al. 2015). *E. coli* can use total fatty acids, the S/U and the average lipid length to retain the optimum membrane fluidity and leakage. In our study, *E.coli* FM3-1709 changed the membrane lipid composition when cultivated in CWO and POE medium (Table 2), which possibly allowed the cell to resist CWO and POE invasion (Fig. 6). The change in membrane lipid composition in CWO group was characterized by increase in total fatty acids, increase in the S/U and a slight decrease in the average lipid length (Table 2). Therefore, CWO altered the content of total fatty acids and S/U in cells. In POE group, the total amount of fatty acids and S/U decreased sharply, and the average lipid length increase compared to the control. The lipid profile was dominated by C16:0, which accounted for ~50% of the total lipids. Cyclopropane lipids were often included in S/U ratio calculations as it was believed to act similarly to unsaturated lipids. Sharply decrease in total amount of fatty acids contributed to the decrease of the membrane lipid bilayer thickness, for average liquid length had just a slight increase. Alterations in the membrane thickness may also distort curvature and protein conformation, which may disrupt membrane protein functions (Engelman 2005; Veld et al. 1991). Decrease in membrane thickness accompanied by increase in unsaturated fatty acid, membrane leakage was definitely increased in POE group.

To verify whether fatty acids were the reason for promoting MK synthesis, *E. coli* DH5α FatA strain by using *E. coli* DH5α as the plasmid hosts for heterologous expression of the FatA type thioesterase from Sunflower (*H. annuus L.*) and *E. coli* DH5α FatB strain by using *E. coli* DH5α as the plasmid hosts for heterologous expression of the FatB type acyl-ACP thioesterase from *U. californica* (BTE) were next constructed. The result showed that the majority of unsaturated fatty acids decreased and saturate fatty acids increased in cell membrane of *E. coli* DH5α FatA compared with the control group, which was consistent with previous study (Serrano-Vega et al. 2005). As expected, the increase in total fatty acids and S/U ratio promoted the intracellular MK (Table 3). Meanwhile, unsaturated and cyclopropane phospholipids were greatly elevated in cell membrane of *E. coli* DH5α FatB compared with the control group. Excitedly, the decrease in total fatty acids and the increase in unsaturated fatty acids really stimulated the MK secretion (Table 3), even though the main component of unsaturated fatty acids was changed from C16:1 to C18:1 when the strain from *E. coli* FM3-1709 was changed to *E. coli* DH5α FatB. These results further confirmed the change of the state of cell membrane can enhance the synthesis of menaquinone in *E. coli*. Especially, the decrease of total fatty acids and increase of unsaturated fatty acids were beneficial to MK secretion.

Altogether, MK synthesis was depended on the state of cell membrane, which include the content of total fatty acids, saturated and unsaturated fatty acid. Obviously, extracellular secretion was more beneficial for the total production of MK. Therefore, future studies will design strategy to promote membrane stability and increase the extracellular MK production includes the shrink of total fatty acids and increase unsaturated fatty acids. A current challenge in strain construction is to find a fast and reliable method for rationally engineering biosynthesis for the production of target molecules at industrially relevant yields, titers, and productivities. *E. coli* as a model strain was studied in this research, further study will perform *B. subtilis* natto or *Flavobacterium meningosepticum* for instead. Our framework for studying the relationship between MK and cell membrane may be helpful in the engineering design of biosynthesis of other intracellular metabolisms. While this work is aimed at MK, it can be applicable to a broad range of potential target intracellular membrane-bound metabolisms.

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