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Improved Ectoine Production from Methane by Optimization of the Bio-milking Process in Engineered *Methylomicrobium alcaliphilum* 20Z

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Abstract – Methane is one of the major greenhouse gases, recently, the biotechnological conversion from methane to high-value added chemicals have emerged as an effort to reduce methane gas emission. In this study, we optimized ectoine bio-milking conditions in which cells were repeatedly used to improve intracellular and extracellular ectoine yield from methane by using *Methylomicrobium alcaliphilum* 20ZDP2. First, the cultivation and intracellular ectoine accumulation conditions were optimized with respect to the growth phase and medium salinity to achieve the highest yield of synthesis. Second, ectoine excretion was optimized by determining the ectoine secretion time (15 min) in appropriate medium salinity under hypoosmotic conditions (1% NaCl). Finally, bio-milking of ectoine was successfully repeated more than 10 times using *M. alcaliphilum* 20ZDP2, and the ectoine yield was improved up to 129.29 mg/ DCW g.

Key words: Ectoine, Bio-milking, Optimization, Methanotroph, Methane

1. Introduction

In recent years, as the amount of carbon dioxide, nitrous oxide, methane, and other greenhouse gases that are emitted into the atmosphere has increased exponentially, global warming has become a serious problem, and the solution to global warming has become a hot topic of concern worldwide. Methane, an important greenhouse gas, is second only to carbon dioxide in terms of its role in warming the atmosphere [1]. However, emission reduction using physicochemical techniques is inefficient and costly. In this context, many studies have shown that biological treatment can replace physicochemical methods by combining the conversion of diluted methane emissions with the production of high-value products as a low-cost, environmentally efficient method to mitigate climate change [2].

Recently, methane-based biotechnology has attracted attention; however, methane-conversion biocatalysis requires further research [3]. Methanotrophic bacteria are microorganisms that utilize methane or methanol as their sole carbon and energy source. Methanotrophs are used as biocatalysts to convert methane into various products, such as bioplastics, biopolymers, biofuels, and a variety of other high-value chemical compounds [4,5].

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid), first discovered in the desert in 1985 by Galinski et al. [6], is one of the most important compatible solutes produced by halophilic and halotolerant microorganisms [7]. The molecular structure of ectoine is characterized by a high degree of water solubility, and its accumulation at high intracellular concentrations can increase the intracellular osmotic pressure without affecting the normal physiological functions of biological macromolecules. Related studies have shown that ectoine is a natural protective component that is produced in the outer layer of cells to protect them from damage in extreme environments, such as high temperatures, high salinity, and exposure to high levels of UV rays, and is used as an active ingredient in skin care and sunscreen products to stabilize proteins and other cell structures, and also has wide applications in biomedicine [8,9].

Halophilic bacteria, which are considered extremophiles, are able to tolerate extreme environmental conditions and resist high extracellular osmotic pressure by accumulating osmotic pressure by compensating for solutes inside the cell to maintain cell morphology, structure, and physiological functions during high osmotic stress. *Methylomicrobium alcaliphilum* 20Z is a halophilic bacterium that genetically contains genes related to ectoine synthesis. In this study, metabolically engineered *M. alcaliphilum* 20ZDP2, constructed by deletion of *ectD* (encoding ectoine hydroxylase) and *ectR* (transcription repressor of *ectABC-ask* operon) to enhance ectoine production in our previous study, was used to optimize conditions for the bio-milking of ectoine from methane.

Most of the current industrial production of ectoine is through bio-milking, which is a method to induce the release of ectoine from cells using the concentration of salt in the culture medium, developed in 1998 by Sauer et al. [12]. In the bio-milking process, ectoine accumulates inside the cells in a high-salinity medium, and the cells are transferred to a low salinity medium. In a low-salinity medium, the cells are subjected to osmotic shock, and ectoine, which is originally inside the cell, is

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‡This paper is dedicated to the retirement of Professor Jae Wook Go of Kwangwoon University of Science and Technology.

released into the extracellular space to achieve osmotic equilibrium. This process is called bio-milking. At present, ectoine is mainly produced by bio-milking using *Halomonas* species that produce ectoine with glucose as the carbon source. This study has a great advantage in terms of economy by using methane as the sole carbon source. In this study, we determined the optimal conditions for ectoine production by bio-milking using methanotrophic *M. alcaliphilum* 20ZDP2 at the flask level.

2. Materials and Methods

2-1. Bacterial strain and medium

An engineered strain of *M. alcaliphilum* 20ZDP2 was used, as constructed in a previous study. In the engineered strain, *ectD*, and *ectR*, which are related to ectoine production, were deleted from the native plasmid. *M. alcaliphilum* 20ZDP2 was cultivated at 30 ℃ and 230 rpm in a 500 ml baffled flask with 50 ml of *Methylomicrobium* medium consisting of NaCl, 30.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2 \cdot 2H_2O$, 0.02 g/L; KNO_3, 1.0 g/L; and 1 mL trace elements (EDTA, 5 g/L; $CuCl_2 \cdot 5H_2O$, 0.1 g/L; $FeSO_4 \cdot 7H_2O$, 2 g/L; $ZnSO_4 \cdot 7H_2O$, 0.1 g/L; $NiCl_2 \cdot 6H_2O$, 0.02 g/L; $CoCl_2 \cdot 6H_2O$ 0.2 g/L; Na_2MoO_4, 0.03 g/L; $MnCl_2 \cdot 4H_2O$, 0.03 g/L; H_3BO_3, 0.03 g/L) and supplemented with 20 mL phosphate buffer (KH_2PO_4, 14.0 g/L; $Na_2HPO_4 \cdot 12H_2O$, 30 g/L), 50 mL of 1 M $NaHCO_3$, 5 mL of 1 M Na_2CO_3.

2-2. Methane fermentation

Prior to fermentation, M. *alcaliphilum* 20ZDP2 was cultivated in a 500 mL baffled flask containing 50 mL of *Methylomicrobium* medium supplemented with 1% methanol at 30 ℃ and 230 rpm for 48 h. The pre-culture was inoculated (final OD_{600} = 0.2) into a 250 mL baffled flask with a screw cap seal containing 50 mL of *Methylomicrobium* medium with a headspace medium ratio of 4:1, and a supplemental air and methane mixture was used as a carbon and energy source. The medium was refreshed every 12 h with 300 SCCM (300 S cm^3/min) at a mass ratio of 7:3 using a mass flow controller (Alicat Mass Flow Controller, Alicat Scientific Inc., AZ, USA). To examine the salt tolerance and ectoine accumulation of the strain, *M. alcaliphilum* 20ZDP2 was cultivated at four different salinities of *Methylomicrobium* medium (3% NaCl, 6% NaCl, and 9% NaCl) using methane as a carbon source. Tungsten (0.05 μM) was also added to the *Methylomicrobium* medium as needed. Cells were harvested every 24 h, and the optical density and presence of any metabolites were analyzed. All experiments were performed in triplicate. Raw data were within ± 5% of the average.

2-3. Optimization of the ectoine bio-milking

To optimize the bio-milking conditions, the accumulation of intracellular ectoine was measured in the cell growth phase. *M. alcaliphilum* 20ZDP2 was cultivated in *Methylomicrobium* medium containing 6% NaCl, using methane as a carbon source. Cells were harvested at appropriate time intervals and intracellular ectoine was measured. In order to determine the appropriate time to maximize the accumulation of intracellular ectoine, *M. alcaliphilum* 20ZDP2 was cultivated in *Methylomicrobium* medium containing 3% NaCl until the OD_{600} of the cell reached 5; then, all of the cells were collected by centrifugation (Combi 514R, Hanil Scientific Inc. KOREA) at 4 ℃ and 13000 rpm for 10 min, which allowed for removal of the medium. The harvested cells were transferred to hyperosmotic conditions (6% NaCl-containing medium), and cell growth and metabolites were examined at 3, 6, 12, and 24 h.

To investigate the appropriate time to excrete accumulated ectoine under hypo-osmotic conditions, all cells cultivated under hyperosmotic conditions (6% DSMZ medium) were collected and transferred to hypo-osmotic conditions (3% DSMZ medium). The cells were sampled every 15 min for 1 h, and the growth and metabolites were analyzed. To determine the optimal concentration to perform osmotic shock on the cells, the secretion levels of ectoine were determined in four culture media with different salt concentrations (0%, 1%, 2%, and 3% NaCl). Cells were cultivated under hyperosmotic conditions (6% NaCl containing medium) for 24 h and then transferred to a hypo-osmotic medium. The cells were exposed to the hypo-osmotic condition for 15 min. Optical density and intracellular and extracellular levels of ectoine were then measured.

2-4. Bio-milking of ectoine under optimized conditions

M. alcaliphilum 20ZDP2 was cultivated in *Methylomicrobium* medium containing 3% NaCl until the OD_{600} of the cell reached 5. Then, all the cells were collected by centrifugation (Combi 514R, Hanil Scientific Inc. KOREA) at 4 ℃ and 13000 rpm for 10 min. The harvested cells were transferred to a 6% NaCl-containing medium and cultivated for 24 h. Ectoine-accumulated cells were harvested and transferred to a 1% NaCl-containing medium and exposed for 15 min. The cells were then transferred to a hyperosmotic medium (6% NaCl-containing medium). The process described above was repeated 10 times, and the extracellular ectoine and intracellular ectoine produced in each process were measured.

2-5. Analytic procedures

Cell growth was determined using a UV-VIS spectrophotometer (Biochrom WPA Lightwave II, Biochrom Ltd., Cambridge, UK) at 600 nm. The culture broth was diluted appropriately to maintain the linear range of the calibration curve for optical density (OD_{600}). The unit value of OD600 corresponded to 0.198 g dry cell weight [13].

Ectoine extraction was performed according to a previously reported method [14]. To extract intracellular ectoine, the cultivated broth was centrifuged (Combi 514R, Hanil Scientific Inc. Gimpo, Korea) at 4 ℃ and 13000 rpm for 10 min. The cell pellet was freeze-dried for more than 48 h using a freeze dryer (TFD8503, IlShin BioBase, Korea), and 10 mg of cell mass was extracted using 570 μL of ectoine extraction solution (methanol/chloroform/water, 10:5:4, v/v) by vigorous shaking for 5 min, followed by the addition of 170 μL of chloroform and water. The mixture was shaken again for 10 min, and

the phase separation was enhanced by centrifugation. The extracted ectoine was derivatized with acetonitrile and measured using high-performance liquid chromatography (HPLC). Extracellular ectoine was determined by collecting the supernatant of the culture medium, and subsequent derivatization and HPLC analysis were performed in the same manner as stated for the extracted ectoine.

Ectoine was measured by HPLC (DGU-20A degassing unit, LC-20AD pump, SIL-20A automatic injector, RID-20A refractive index, SPD-20A UV-Vis detector, and CTO-20A column oven, Shimadzu, Kyoto, Japan) equipped with a prominence UV/VIS detector and ZORBAX NH_2 column (4.6 × 250 mm, 5-Micron, Agilent, USA) under the following conditions: sample volume, 1 mL; mobile phase, 70% acetonitrile; flow rate, 1 mL/min; column temperature, 40 ℃; detection time, 25 min; pressure limits, 0 and 25 MPa [15].

3. Results and Discussion

3-1. Effect of medium salinity on cell growth and ectoine production

Since various parameters of the culture process can affect cell growth and production of ectoine in *M. alcaliphilum* 20ZDP2, optimal culture parameters for ectoine production, such as culture temperature, pH, and agitation speed, were investigated. The optimal conditions for cell growth and ectoine production were determined to be cultured in a medium (pH 9~9.5) at 30 ℃ shaking 230 rpm for *M. alcaliphilum* 20ZDP2 (data not shown). *M. alcaliphilum* 20ZDP2 could not grow below pH 6.8 (data not shown).

To examine the salinity adaptation range and optimal concentration for the accumulation of ectoine in *M. alcaliphilum* 20ZDP2 strain, the cells were cultured in three different media containing 3%, 6%, and 9% of NaCl, respectively, for 120 h. It was confirmed that cell growth was the fastest in the medium containing 3% NaCl, and the growth rate decreased as the NaCl concentration increased. In particular, *M. alcaliphilum* 20ZDP2 showed insubstantial growth in the medium containing 9% NaCl (Fig. 1a). Ectoine production in medium containing 6% NaCl was much higher (142.32 mg/L) than that in medium containing 3% NaCl (71.08 mg/L) (Fig. 1b). Although cell growth was slower in medium containing 6% NaCl than in medium containing 3% NaCl, the amount of ectoine accumulated was significantly increased. Therefore, the culture containing 6% NaCl provides the optimal environment for the accumulation of ectoine.

3-2. Comparison of the ectoine production according to growth phase

As shown in Fig. 1, ectoine increased with an increase in cell growth, and showed a tendency to decrease rapidly when cells entered the late exponential phase.

To determine the best cell growth phase for ectoine accumulation, the cells were cultured in medium containing 6% NaCl and it was sampled each time the OD600 value increased (OD 1–9). As shown in Fig. 2, the accumulation of ectoine increased with cell growth, and when the cell optical density was between 5 and 6, the cells could

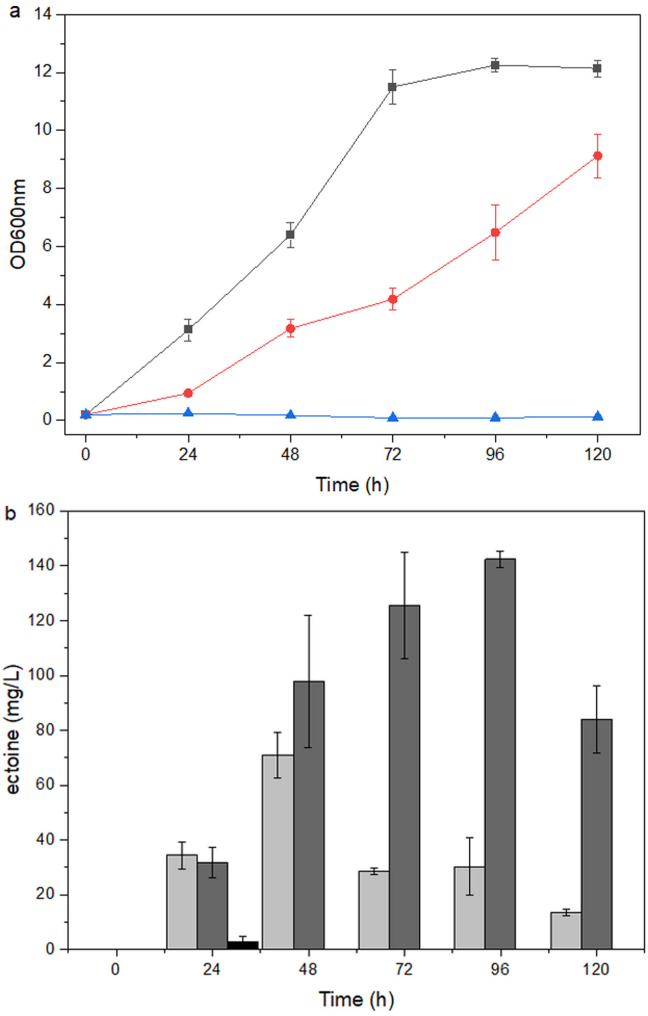

Fig. 1. The effect of medium salinity on cell growth and ectoine production with methane as the carbon source. Time course of the cell growth and ectoine production by *M. alcaliphilum* 20ZDP2 at different medium salinities. (a) The symbols represent cell growth in 3% NaCl-containing medium (■), 6% NaCl-containing medium (●), and 9% NaCl-containing medium (▲). (b) Ectoine production is represented by the light gray bar (3% NaCl), dark gray bar (6% NaCl), and black bar (9% NaCl).

metabolize the most ectoine up to 138.4 ± 9.934 mg/L. After reaching the highest ectoine production, it decreased dramatically with cell growth. This indicates that when the amount of intracellular ectoine accumulates to a certain level, synthesis is inhibited, that is, ectoine synthesis is limited by the intracellular ectoine concentration.

3-3. Optimization of ectoine bio-milking under hyperosmotic and hypo-osmotic conditions

M. alcaliphilum 20ZDP2 showed the best ectoine accumulation in the medium containing 6% NaCl, but the cell growth rate was significantly lower than that in the medium containing 3% NaCl. To overcome this problem, the cells were rapidly grown to OD 5–6 in medium containing 3% NaCl.

The cells were then transferred to a medium containing 6% NaCl

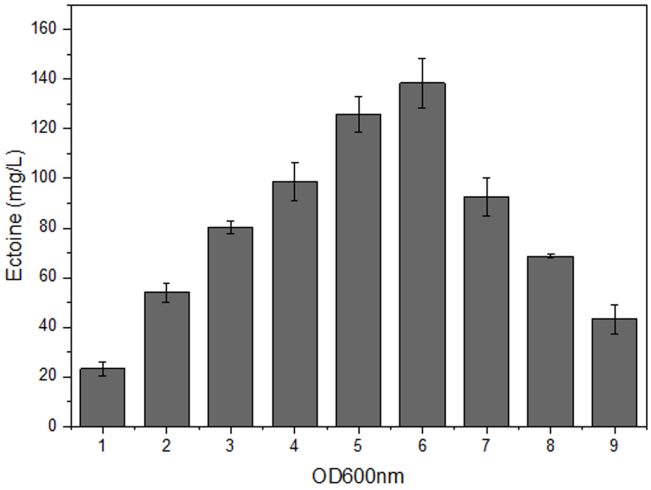

Fig. 2. Comparison of ectoine production and cell growth. *M. alcaliphilum* 20ZDP2 was cultivated in 6% NaCl and W added medium supplied with methane. Cells were harvested (OD 1-9) to measure intracellular ectoine.

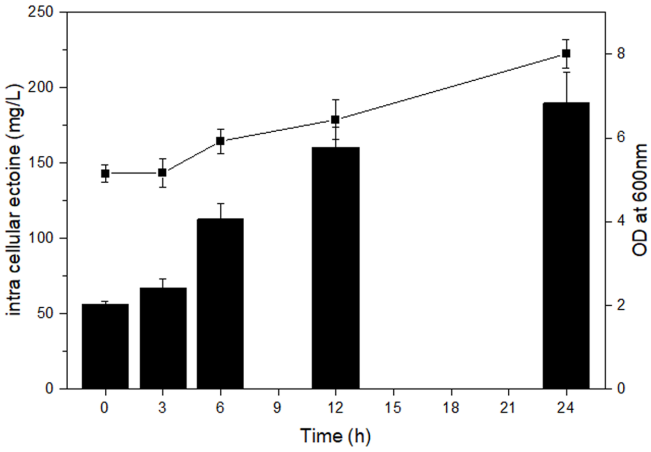

Fig. 3. Ectoine accumulation by *M. alcaliphilum* 20ZDP2 under hyperosmotic conditions. *M. alcaliphilum* 20ZDP2 was cultivated in medium containing 3% NaCl until the OD_{600} reached 5. The cells were then transferred into fresh medium containing 6% NaCl. Ectoine was measured by sampling the culture medium at the appropriate time intervals. The symbols represent cell growth under hyperosmotic conditions (■) and black bars represent the amount of intracellular ectoine.

to induce the accumulation of ectoine. Thus, we explored the appropriate time required for ectoine accumulation to reach the highest level in a medium containing 6% NaCl. The amount of intracellular ectoine was measured at 3, 6, 12, and 24 h. As a result, the amount of ectoine accumulation increased gradually with time, and the highest yield was obtained at 24 h (Fig. 3). It was confirmed that the amount of ectoine accumulated after 24 h was similar to that at 24 h (data not shown). Therefore, the optimal conditions for ectoine accumulation during the bio-milking process were a medium containing 6% NaCl and a period of 24 h.

To investigate the optimal conditions for ectoine secretion that accumulated intracellular ectoine in a medium containing 6% NaCl,

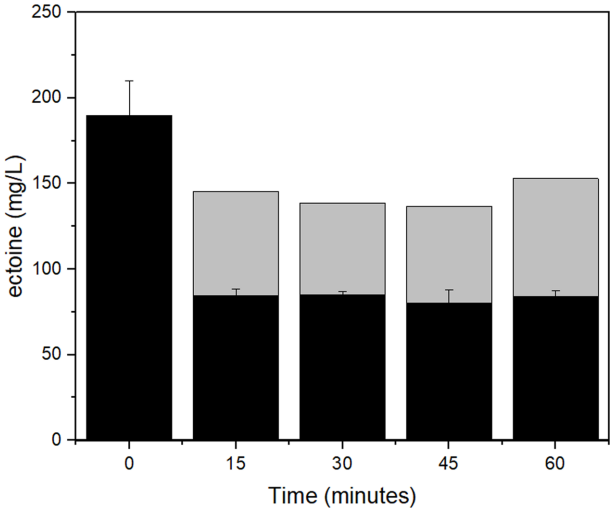

Fig. 4. Ectoine secretion by *M. alcaliphilum* 20ZDP2 under hypoosmotic conditions. Ectoine was accumulated in medium containing 6% NaCl by *M. alcaliphilum* 20ZDP2 for 24 hours. The cells were then transferred into fresh medium containing 3% NaCl. Extracellular and intracellular ectoine was measured by sampling the culture medium at the appropriate time intervals. Black bars represent the accumulated intracellular ectoine and gray bars represent the excreted extracellular ectoine.

the cells in the medium containing 6% NaCl were transferred to a medium containing 3% NaCl, and the amount of intracellular ectoine and the amount of ectoine excreted and were measured every 15 min for 1 h. Adequate excretion of intracellular ectoine was observed in the culture medium containing 3% NaCl within 15 min (Fig. 4), and approximately the same amount of ectoine was also obtained to the outside of the cells after 15 min. Therefore, the optimal time for ectoine excretion in cells was determined to be 15 min.

3-4. Optimization of ectoine secretion at different salinities under hypo-osmotic conditions

In order to determine the salinity that can result in maximum extracellular ectoine excretion with minimal cell destruction, which causes osmotic shock, we measured the secreted ectoine at different salinities in hypo-osmotic medium containing 0%, 1%, 2%, and 3% NaCl. Ectoine was synthesized in *M. alcaliphilum* 20ZDP2 under hyperosmotic stress for 24 hours (139.13 ± 12.56 mg/L). In the medium containing 0% NaCl, it was confirmed that more than 98% of the intracellular ectoine was excreted out of the cells, but the cell growth did not recover during the subsequent ectoine accumulation process, indicating that the cells were destroyed (Fig. 5). In addition, it was observed that the excretion of ectoine increased as the salinity decreased (1%, 2%, and 3% NaCl). When a medium containing 1% NaCl was used, not only 82% of intracellular ectoine was released, but there was also no significant effect on cell growth after hypo-osmotic shock. Therefore, we decided to use a medium containing 1% NaCl for the optimal hypo-osmotic shock to excrete intracellular ectoine.

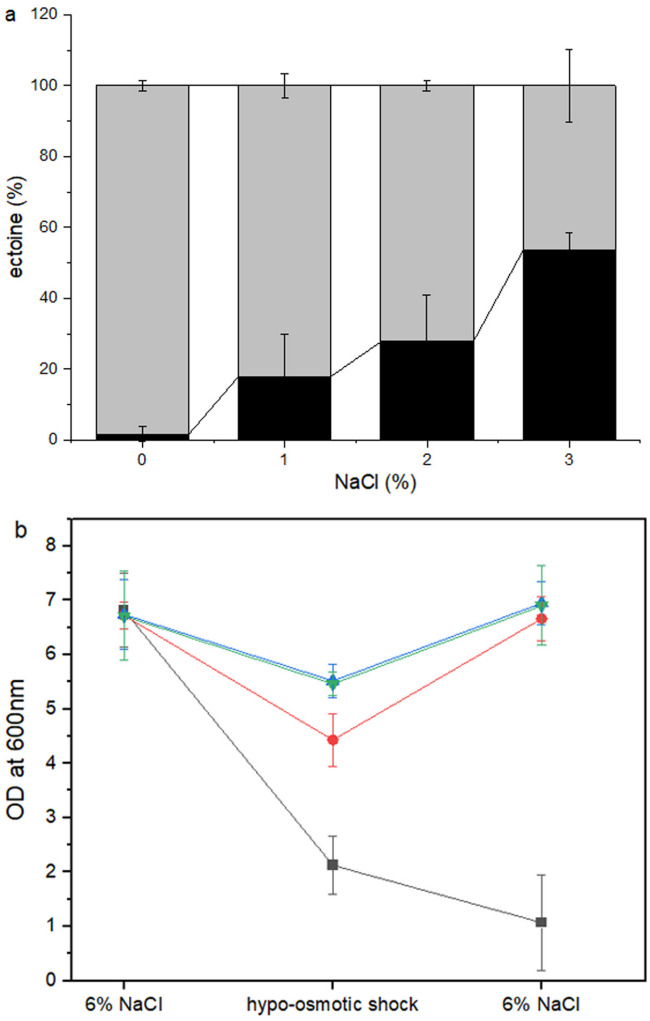

Fig. 5. Ectoine secretion at different salinities by *M. alcaliphilum* 20ZDP2. Ectoine was accumulated in medium containing 6% NaCl by *M. alcaliphilum* 20ZDP2 for 24 hours. The cells were then transferred to medium containing 0%, 1%, 2%, and 3% NaCl. (a) Extracellular and intracellular ectoine were measured by sampling each culture medium after 15 min of exposure to hypoosmotic conditions. (b) Comparison of cell growth after exposure to hypoosmotic conditions. The symbols represent the hypoosmotic conditions of 0% NaCl (■), 1% NaCl (●), 2% NaCl (▲), and 3% NaCl (▼).

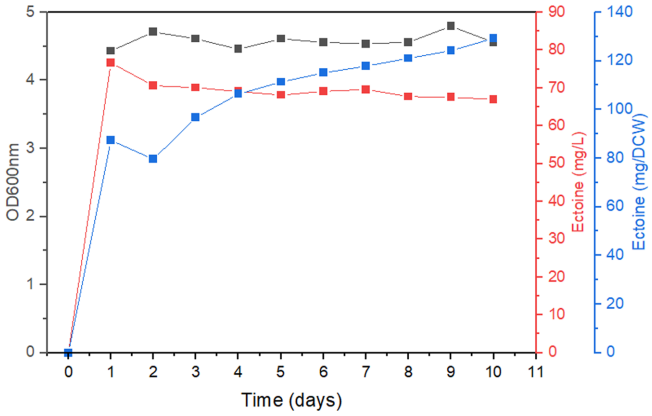

Fig. 6. Time course of the extracellular ectoine production by optimization of the ectoine bio-milking process using *M. alcaliphilum* 20ZDP2. The symbols represent the cell growth (■) and concentration of extracellular ectoine (▲, mg/L; ●, mg/DCW g).

3-5. Bio-milking of ectoine under optimal conditions using *M. alcaliphilum* 20ZDP2

We optimized the bio-milking of ectoine using *M. alcaliphilum* 20ZDP2. By applying these optimized conditions, bio-milking of ectoine was repeated more than 10 times using *M. alcaliphilum* 20ZDP2. As shown in Fig. 6, the concentration of ectoine excreted from the cell remained constant (67 mg/L) even when repeated bio-milking was performed more than 10 times, and it was observed that ectoine yield increased as bio-milking proceeded (129.29 mg/DCW g).

Until now, the ectoine production was focused on sugar fermentation using *Halomonas* species, but recently, ectoine production process from methane using halophilic methanotrophs such as *M. alcaliphilum* 20Z has been actively conducted. In the Table 1, the ectoine production by bio-milking system and continuous batch fermentation was compared according to cultivation type from previous reports and this study. Sauer and Galinski [12] obtained the highest ectoine production (155 mg/DCW g) from bioreactor scale with bacterial milking by *Halomonas elongata* DSM 142^T used glucose as carbon source. The Cantera group obtained 66.9 ± 4.2 mg/DCW g of ectoine from methane in fed-batch fermentation [18,19] and 70.4 ± 14.3 mg/DCW g of ectoine

Table 1. Comparison of ectoine production by cultivation type

Host bacteria	Carbon source	Cultivation type	Culture scale	Ectoine titer (g/L)	Ectoine yield (mg/DCW g)	References
Halomonas elongata DSM 142^T	Glucose	Bacterial milking	Bioreactor	-	155	[12]
Methylomicrobium alcaliphilum 20Z	Methane	Continuous (13-day tests)	Batch gas-tight reactor	0.005	40.7	[19]
Methylomicrobium alcaliphilum 20Z	Methane	Continuous (50 days)	Continuous stirred tank reactor	-	37.4	[21]
Methylomicrobium alcaliphilum 20Z	Methane	Continuous Bio-milking	Two sequential continuous stirred tank reactor	0.043	70.4	[20]
Methylomicrobium alcaliphilum 20Z mixed haloalkaliphilic consortium	Biogas (CH_4, O_2, CO_2, He)	Bioconversion	Cylindrical glass bubble column bioreactor	-	108.7	[22]
Methylomicrobium alcaliphilum 20ZDP2	Methane	Bio-milking	Flask	0.143	129.3	This study

in bio-milking process [18,20]. So far, it has been reported that the production of ectoine from methane by using methanotrophs was relatively lower than that of sugar based ectoine production. However, biological processes that produce high value-added products using methane are attracting attention in industrial biotechnology.

In this study, the ectoine was produced up to 126.3 ± 5.965 mg/ DCW g in flask cultivation by optimization for ectoine bio-milking. In particular, this study suggests that a constant level of ectoine could be excreted even cells were reused more than 10 times during bio-milking process, and thus the ectoine yield (mg ectoine/g DCW) was gradually increased. The bio-milking system of this study is continuously producing ectoine by recycling the same cells. This system is different from the other research group such as Sauer and Galinski group and Cantera group [12,20]. Applying this optimized bio-milking system would facilitate the mass production of ectoine commercially in the industrial biological process.

4. Conclusion

In this study, we optimized the bio-milking of ectoine by engineered methanotroph strain, *M. alcaliphilum* 20ZDP2, which uses methane as the sole carbon source. First, the culture condition according to the cell growth phase (OD600 5-6) and the salinity of medium (6% NaCl) was optimized for maximize the accumulation of ectoine (yield up to 138.4 ± 9.934 mg/L). Second, the bio-milking conditions for effective osmotic shock were optimized. The time for optimal ectoine accumulation under hyper-osmotic cultivation conditions was 24 hours. Also, the time for optimal ectoine excretion under hypo-osmotic cultivation condition was 15 minutes with appropriate salinity medium (1% NaCl). By applying these optimized conditions, the bio-milking of ectoine was successfully repeated more than 10 times using *M. alcaliphilum* 20ZDP2. Finally, the concentration of ectoine excreted out of the cell was constant (67 mg/L) even when bio-milking was performed more than 10 times, and the ectoine yield was increased up to 129.29 mg/ DCW g. The results of this study suggest that this optimized bio-milking system would applied in the industrial ectoine process.

Acknowledgments

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