Thesis for the Degree of Master of Science

The signal molecule cyclic-di-GMP mediates autoflocculation in *Zymomonas mobilis*

by

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(신호전달물질 Cyclic-di-GMP 매개에 의한 *Zymomonas mobilis*의 자동 응집 유도)

Advisor: Prof. Young-Jae Jeon

by

Won-Gyeom Jung

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CONTENTS

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Abstract

Zymomonas mobilis has been studied as an important ethanologenic bacterium for bioethanol production process using lignocellulose. However, in the pretreatment step to make the plant residue more readily accessible for cellulose enzyme for lignocellulosic ethanol production process, a variety of inhibitors are generated. These inhibitors have effected on cell growth as well as overall ethanol productivity. Therefore, separation of the inhibitors or construction of resistant strains has become an important industrial mission for the process. In this respect, the cell flocculation relatively cost-efficient cell recovery characteristics, previously a cell flocculation mutant strain *Zymomonas mobilis* ZM401 has been isolated. Previous studies of transcriptomic analyses of the flocculent strain suggested that a single point mutation in the ZMO1055 (locus tag number) has been hypothesized as one of the main causatives. These proteins are predicted to regulate cell flocculation by controlling the level of cyclic di-GMP concentration. Therefore, we isolated two gene knockout mutants of ZAM1 and ZAM2 from the parental strains of *Z. mobilis* ZM4 and flocculent mutant ZM401 respectively. The results showed that the knockout mutants display a various degree of cell flocculation, decreased in the intracellular concentration of c-di-GMP and the higher robustness against such inhibitors. Consequently, this study was shown to ZMO1055 phosphodiesterase knockout was induced to cell flocculation and stable cell flocs suggested the strategies of industrial robustness and effective ethanol production for this bacterium.

1. Introduction

Carbon dioxide emissions from fossil fuels and a shortage of fossil energy cause the global environmental concerns such as climate change, and sustainable energy supply for our society. Therefore, seeking for sustainable and cleaner alternative energies over fossil fuel has been one of important urgent mission for our society worldwide [1]. Accordingly, bioethanol has emerged as a sustainable alternative over petroleum-based materials. Over severable decades, many research efforts have been paid to look for suitable feedstocks and strain development and process engineering to make more economically sensible process for biofuel production. The biofuels can be divided into three groups. First-generation biofuels include carbohydrates and starch-based materials (crops such as corn, sugarcane, and soybeans) that can be used as food, second-generation biofuels include lignocellulose-based materials (such as forest residues and special energy crops with high yields), and third-generation biofuels include carbohydrates and/or lipid produced by algae [2]. The recently identified limitations of 1st-generation biofuels produced from food source have caused greater emphasis include problems such as competition with available cultivable land used for food and ethical issues that food source used for energy production [2, 3].

Lignocellulosic biomass, in the context of enzyme-based processes for producing glucose, a pretreatment step is necessary to make the cellulose amenable to hydrolysis by cellulase. One pretreatment technology utilizing dilute H_2SO_4 also

hydrolyzes hemicellulose to sugars, producing primarily pentose sugars such xylose and arabinoase. This hydrolysate can be fermented to ethanol using a recombinant glucose/xylose-fermenting microorganism [4].

Fermentation technologies utilizing strains of *Zymomonas mobilis*, in place of the traditional yeast, have been proposed by a number of authors for lignocellulosic-based ethanol production [5]. The facultative anaerobic bacterium, *Zymomonas mobilis*, is recognized as an industrially important microorganism for the production of bioethanol and high value-added products due to its industrial robust characteristics associated with higher specific rates of sugar uptake, ethanol production, lower biomass yields as well as high tolerance against ethanol and sugars, and easier facility for genetic manipulation, when compared with other ethanologenic microorganisms [5, 6]. But, the available substrate spectrum such as glucose, sucrose, and fructose is small [7]. Because of this characteristic, recombinant *Z. mobilis* CP4 (pZB5) with 86% ethanol yield was metabolically engineered by introducing two operons encoding xylose assimilation and pentose phosphate pathway enzymes [8], and then for co-fermenting glucose, xylose and arabinose to ethanol *Z. mobilis* 8b, have been developed [9].

However, in this ethanol fermentation step using lignocellulosic materials, the inhibitor products such as hydroxymethylfurfural, furfural, acetic acid from pretreatment of lignocellulose are directly exposed to the ethanol fermentation strain [10, 11] and leading to a decrease in the growth rate and the ethanol

production rate. This phenomenon leads to a decrease in the industrial efficiency of ethanol production using lignocellulose. Therefore, it is essential to remove the inhibitory substance in the culture medium or secure the resistance of the strain to the fermentation process. In that respect, many strains resistant to the inhibitor have been studied using *Zymomonas mobilis* as a promising ethanologenic strain. The recombinant *Z.mobilis* ZM4/AcR (pZB5) [12] and *Z. mobilis* 8b [4] strains showed the acetic acid resistance up to 16 g l^{-1}. Following chemical mutagenesis, early studies by our group resulted in the isolation of the flocculent strain *Z. mobilis* ZM401 [13, 14]. The stable cell flocculation has been traditionally used in brewing production as an easy and off-cost for cell-broth separation process [15] and possible to repeated batch or continuous cell recycle processes [14]. This is an advantage in fermentation industry by simplifying the process and reducing the time and cost as compared with batch-culture. In addition, cell flocculation has been reported to provide high robustness from various inhibitors effect in a variety of strains such as *Saccharomyces cerevisiae* and *Bacillus* [16, 17]. Interestingly, the flocculation related genes from ZM401 have been elucidated by previous transcriptomic analysis that one of many mutations occurred in a putative phosphodiesterase gene of ZMO1055, an enzyme that degrades a signal molecule c-di-GMP [14]. The ZMO1055 gene contains the putative diguanylate cyclase and phosphodiesterase domain, which synthesize and degrade c-di-GMP respectively,

and the functions of these two domains have been proven through many previous studies.

In this study, the gene encoding the phosphodiesterase domain from ZM1055 was knocked out to investigate gene function of ZMO1055, phenotypic change, and the intracellular c-di-GMP concentration. Finally, we confirmed morphological changes and resistance increase by flocculation of knockout strains, and it is intended to help development of industrially useful strains.

2. Materials and methods

2.1. Bacterial strains, growth conditions and culture storage.

The bacterial strains and plasmid used in this study are listed in Table 2.1 and 2.2. The strains of ZAM1 and ZAM2 are the Phosphodiesterase gene knockout strains of ZM4 and ZM401, respectively. The strains of *Z. mobilis* was cultivated at 30℃ on rich medium (RM medium) under the static culture conditions. The composition RM medium was 20g l^{-1} glucose, 10g l^{-1} yeast extract, and 2g l^{-1} KH_2PO_4, 1g l^{-1} $(NH_4)_2SO_4$ and 1g l^{-1} $MgSO_4$ for solid media with an addition of 1.5% agar [18]. This medium was separately autoclaved with carbon and other sources. The chloramphenicol concentration was maintained at 100ug ml^{-1} in the medium for ZAM1 and ZAM2 culture. For the growth of *E.coli* DH5α, LB medium [19] was used. Its medium composition was as followed; 10g l^{-1} tryptone, 5g l^{-1} yeast extract, 10g l^{-1} NaCl. The ampicillin concentration was maintained at 50ug ml^{-1} in the medium for the recombinant strains of *E. coli*. For a long-term storage, bacterial cultures were suspended with 80% (v/v) glycerol and stored at -70℃ in cryogenic vials.

Table 2.1. The list of strains used in this study

Strains	Characteristics	Reference
E.coli		
DH5α	hsdR17(r-m+)recA1,*end*A1	Real Biotech corporation (RBC, Taiwan)
DH5α pPBC::*pde*	ΔPDE	This study
DH5α pPBC::*pde*::*cat*	ΔPDE::cm^R	This study
Z. mobilis		
ATCC 31821	A wild-type strain	(Goodman, Rogers et al. 1982)
ATCC 31822	A flocculant strain of ZM4	(Lee, Skotnicki et al. 1982)
ZAM1	ΔPDE::cm^R	This study
ZAM2	ΔPDE::cm^R	This study

Table 2.2. The list of plasmids used in this study

Plasmid	Characteristics	Reference
pRBC::*pde*	ΔPDE	This study
pRBC:: *cat*	cm^R	This study
pRBC::*pde*::*cat*	ΔPDE::cm^R	This study
pLysS	cm^R	(Invitrogen, USA)

2.2 Isolation, amplification, and modification of DNA

To isolate genomic DNA and recombinant plasmids, a HiGene™ Genomic DNA Prep Kit (BIOFACT, Korea) and a plasmid DNA extraction mini kit (FAVORGEN, Taiwan) were used respectively. The purification of DNA fragments from PCR products and agarose gels were achieved by FavorPrep GEL/PCR purification Mini Kit (FAVORGEN, Korea) according to the Manufacture's instructions.

All PCR reactions were performed in a 2720 PCR System (Thermo, USA) and using a TaKaRa Ex Taq polymerase (TaKaRa, Japan) for DNA amplification. The composition of PCR was set up with 0.25ul Taq-polymerase (5unit/ul), 4ul dNTP (2.5mM), 1ul/1ul forward/reverse primers (10pmol), 5ul buffer (20mM Tris-HCl, pH 8.0), 100mM KCl, 0.1mM EDTA, 1mM Dithiothreitol, 0.5% Tween 20, 0.5% NP-40, 50% glycerol), 1ul Template(<500ug) and the rest was filled with D.W up to 50ul. All PCR products and DNA fragments were separated on 1% agarose gels in TAE buffer (2M Tris-acetate, 50mM EDTA) and the size of DNA was compared with a 10kb DNA ladder (SMOBIO, Taiwan). All PCRs were performed at pre-denaturation at 94℃ for 5min, denaturation at 94℃ for 20s, annealing at 55℃ for 20s, extension at 72℃ for 1.5min. The number of PCR cycles was thirty and final extension was achieved at 72℃ for 7min. DNA sequence analyses were performed using Sanger methods [20].

Restriction enzyme NsiI used in this study was supplied from New England Biolab (UK). Digestion of DNA fragments with the restriction enzymes was performed at

37℃ for 2hr using 1ul endonuclease (10,000units/ul), 2ul NEBuffer 3.1, 17ul DNA and the enzyme was inactivated at 65℃ for 20min.

The ligation of DNA fragments was achieved using a Rapid Ligation Kit (RBC, Taiwan). The ligation mixture was set up with 1ul T4 ligase(3U/ul), 2ul the cloning vector, 1ul ligation buffer A, 1ul ligation buffer B and 5ul PCR product, and the rest was filled with D.W up to 20ul distilled water.

2.3 Construction of pRBC::*pde*::*cat* in *E. coli*.

To construct phosphodiesterase gene knockout strains of *Z mobilis,* the gene coding for phosphodiesterase (locus No: ZMO1055) was targeted for homologous recombination event. The PCR and sequencing primers used for suicide vectors construction and genetic confirmation are the list in Table 2.3. To construct pRBC::*pde,* the gene ZMO1055 was amplified from the genomic DNAs of ZM4 and ZM401 respectively, using primers, ZMO1055F2 and ZMO1055DW. The 2.9kb PCR products were cloned into a TA cloning vector using the RBC TA cloning kit (RBC, Taiwan) via an overnight ligation reaction at 4℃. The ligation mixture was transferred into DH5α competent cells (RBC, Taiwan). The objective recombinant plasmids were screened by blue-white selections and the putative recombinant strains of E. coli (pRBC::*pde*) were further confirmed by PCR analysis using M13F and M13R.

To construct pRBC::*pde*::*cat,* the chloramphenicol resistant gene (cat), was amplified from pLysS using primers catFNsiI and CatRNsiI. The 1kb PCR product was subcloned into TA cloning vector. The ligation mixture was transformed into DH5α competent cells. The transformants were isolated by blue-white selection, a cm^R and the putative strains of E. coli (pRBC::*cat*) were further screened and confirmed by PCR analysis using M13F and M13R.

Finally, to construct of pPBC::*pde*::*cat*, the 5.6kb-NsiI-fragment of pRBC::*pde* and 1kb- NsiI-fragment of the cat were isolated respectively from 1% agarose gels. The DNAs were recovered by the DNA purification kit (FAVORGEN, Korea). The ligation mixture was subsequently transformed into DH5α competent cells. The transformed cells were isolated on LB medium containing 34ug/ml of chloramphenicol. Genetic confirmation of pRBC::*pde*::*cat*, were further confirmed by PCR using M13F and M13R.

Table 2.3 Oligonucleotide primers used in this study

Primer	Sequence(5'→3')	Reference
ZMO1055F2	CATGACCATAGCCCTTATCGG	This study
ZMO1055DW	GATTGCCAGCGAAGTCTTG	
ZMO1054netF	CGGTCGTGGTTTTATTACCCAG	This study
ZMO1056netR	GATCGCGACGAAGCGACAGTC	
CatFNsil	ATGCATTTGCTTTCGAATTTCTGCCATTC	This study
CatRNsiI	CCATGCATTGGCGGCGGAATTTCTGCCATTC ATCC	This study
M13F	TTTCCCAGTCACGACGTTGTAA	RBC
M13R	TCACACAGGAAACAGCTATGAC	RBC

2.4 The construction of *pde* gene knockout mutants of *Z. mobilis*

To knockout phosphodiesterase gene in *Z. mobilis*, the electro-competent cell of *Z. mobilis* was prepared using the method previously reported by [21]. All competent cell preparation was processed on ice. For the competent cell preparation, a single colony was transferred into a 50ml conical tube (Falcon, USA) containing 10ml RM broth. This inoculum was transferred and cultured in a 250ml medium bottle (SAMWOO, Korea) containing 90ml RM broth at 30℃. When the cell concentration was reached up to 0.8 to 1.0 (OD_{660nm}), the culture was incubated on ice for 20min and centrifuged at 5000xg at 4℃ for 5min. The harvested cells were

washed with 1% ice-cold glycerol, this was repeated five times to remove all metal ions and cell debris. The cell pellets were resuspended with 120ul of 10% glycerol. If the cells were not used immediately, it was stored in -70℃ for further use. The 40ul of the electro-competent cell was transferred into a Gene Pulser® cuvette (0.1cm electrode gap, BioRad, USA) and various DNA concentrations were added into the electroporation cuvettes. The electroporation conditions used in this study were set up at 200 Ω, 25 μF, 1.6 kV cm -1. After the electric shock for 3s, a 1ml of RM broth was added immediately into the electroporated cells and the electroporation mixture was recovered for 1.5hr at 30℃. A 100ul of the recovered cell was spread on to RM agar plates containing 100ug ml^{-1} Chloramphenicol and then incubated at 30℃ for 5 days. The putative transformants of *pde* gene knockout mutants conferring cm^R were subjected for genetic confirmation. The genetic confirmations were carried out by PCR using the primer set ZMO1055F2, ZMO1055DW, ZMO1054F and ZMO1056R with various recombinations.

2.5 Intracellular cyclic-di-GMP quantification from *Z. mobilis* strains

Intracellular c-di-GMP quantification was carried out using the method previously reported by [22]. Various strains such as *Z. mobilis* ZM4 and ZAM1, ZM401 and ZAM2 were cultured up to the exponential phase an 0.8 to 1.0 (OD_{660nm}), and their supernatants were removed by centrifugation at 6000xg for 15 min. The cell pellet was suspended in 1 ml lysis buffer (10mM Tris, 10mM MgCl2, 10mM

dithiothreitol, pH 7.6) and the pellet was transferred to a zirconium glass bead tube (0.5mm diameter, Benchmark, USA). This tube was placed in a bead homogenizer (benchmark, USA) and homogenized at 2800rpm for 30s followed by incubation on ice for 30s. The cell lysis step was repeated ten times. Finally, the whole lysate was centrifuged at 15,000xg for 10 min and the only soluble fraction of cell lysates was collected and used to measure c-di-GMP quantification. The 100ul of the soluble crude extract was used for total protein assay and the rest supernatant was filtered through a 0.2um filter (Polypropylene membrane, 13mm, Millipore, USA). For the quantitative analysis of c-di-GMP, total protein assay was performed using a Pierce ™ 660nm Protein Assay Kit (Thermo Fisher, U.S.A). To quantify the total protein concentration, bovine serum albumin was used as a standard. Quantitative analysis of c-di-GMP was carried out by HPLC Alience e2695 (waters, UK). For quantification, a calibration curve was prepared using an analytical grade of c-di-GMP (98%, Sigma-Aldrich, USA). Two mobile phases were prepared as followed; 10 mM ammonium acetate in water (A) and in methanol (B) respectively. The solvents were filtered through a 0.45um membrane filter (Hydrophilic polyethersulfone, 47mm, Pall Corporation, USA) and subsequently degassed for 2 hours. As a typical analysis condition, UV detector (Waters, UK) was set up at 253nm, 1.2 nm resolution. The chemical was separated by SunFire C^{18} reverse-phase column (5um, waters, UK). The mobile phase gradient mode was set up to the first mode for 0 to 28 min with 1% of B solvent and 99% of A solvent and

second mode for 28 to 38 min with 15% of B solvent and 85% of A solvent and the third mode for 38 to 48 min with 25% of B solvent and 75% of A solvent and the fourth mode for 48 to 62 min with 90% of B solvent and 10% of A solvent and the final mode for 62 to 72 min with 1% of B solvent and 99% of A solvent. Injection volume was 20ul and the flow rate was maintained at 2min ml^{-1}. Oven temperature was set up at 35℃.

2.6 Cell motility assay of *Z. moilis* strains

To measure the cell motility of ZM4, ZAM1, ZM401, and ZAM2, semisolid agar plates were prepared using 0.2% agar with RM broth. A single colony was inoculated in a 15ml conical tube (Falcon, USA) containing 10ml RM broth at 30℃ for overnight. A 10ul inoculum was dropped onto the middle of semisolid agar plates and incubated at 30℃ for 24hr. To measure the cell motility, the increased cell culture diameter was measured by subtraction of initial cell culture diameter.

2.7 Bacterial cellulose quantity assay of *Z. moilis* strains

For the analysis of bacterial cellulose quantity using the previously reported by [23], four strains were incubated in a 50ml conical tube containing 20ml RM broth at 30℃ for overnight. The 5ml of inoculate was transferred into 45ml RM broth in a 50ml conical tube and the inoculums were cultivated up to a middle log phase(OD_{600nm} 0.8 to 1.0). The 20 ml culture was collected and centrifuged at

4000xg for 15 min followed by the removal of the supernatant. The harvested cells were washed using D.W and then further centrifuged at 5000xg for 10 min. The recovered cell pellet was stained with 1% (v/v) crystal violet for 15 min and the cell suspension was centrifuged at 10,000xg for 20min to remove the crystal violet. The pellet was washed again with D.W and then destained with a 20 ml 90% ethanol.

The optical density at 590 nm was measured using a spectrophotometer. The biofilm forming abilities of all *Z. mobilis* strains were compared based on the intensity of crystal violet staining.

2.8 Cell robustness test for *Z. mobilis* strains

The toxic chemicals including furfural, hydroxymethylfurfural, ethanol and acetic acid derived from cellulosic ethanol production process were main subjects to test cell robustness of *Z. mobilis* ZM4, ZAM1, ZM401, and ZAM2. All tested toxic chemicals were an analytical grade furfural (≥99%) purchased from Sigma-Aldrich, USA, hydroxymethylfurfural (≥99%,) purchased from Sigma-Aldrich, USA, ethanol (≥99.5%) purchased from SAMCHUN, Korea and acetic acid (99.9%) purchased from DUKSAN, Korea. Various concentrations of all tested chemicals were added into a 50ml conical tube containing 10ml cell culture prepared at mid-log phase (OD_{660nm} 0.8 ~ 1.0). The cell cultures were incubated for 24h, and a part of the culture was serially diluted with RM broth and 10ul of each dilution was

dropped onto RM plate with the triplicates. The method was used previously reported by [24]. Appeared colonies on the plates were counted for cell robustness against such toxicities.

3. Results

3.1 The genetic confirmation of the phosphodiesterase gene knockout mutants of *Z. mobilis*

To construct the *pde* (ZMO1055) knockout strains of *Z mobilis,* the chloramphenicol gene was inserted into the NsiI recognition sites of pRBC::*pde*. The resultant recombinant plasmid pRBC::*pde*::*cat,* a suicide vector was transferred in *Z. mobilis* ZM4 and ZM401 by electroporation respectively. The putative transformants showing chloramphenicol resistance were isolated. The strain isolated from the parental strains of ZM4 and ZM401 were named as ZAM1 and ZAM2 respectively. The confirmation of the gene knockouts of *Z. mobilis* was carried out via PCR using various primer sets. The results are shown in Fig 2. According to the previous genome analysis data for ZM4 and ZM401 [25], [26] both parental strains have similar DNA sequences around the targeted homologous recombination regions. Therefore to confirm the successful homologous recombination on both genomes, the chromosomal DNA of the parental strain ZM4 was used as a control reagent for this purpose. As can be seen in Fig2, as the primer set CatFNsiI and ZMO1055F2 were used, 2.9kb PCR products were generated from both ZAM1 and ZAM2 strains (lane 6 and 10). However, the control strain ZM4 did not generate any PCR products under the same PCR conditions using the same primers as shown in the lanes 2 and 3. In addition, as the primer set, CatRNsiI and ZMO1055DW were used against the genomic DNAs of ZAM1 and ZAM, 2.

2kb PCR fragments were amplified respectively shown in Lane 7 and 11. The 3.9kb PCR products as ZMO1055F2 and ZMO1055DW were used, and the 3.6kb PCR products as ZMO1054F and ZMO1056R were used, were respectively generated from both mutant strains (Lane 5,8 and 9,12), whereas the PCR products generated from the control strain using the same primer set under the same PCR conditions were 2.9 and 2.6 kb respectively (Lane 9 and 13). The appearance of increased in DNA fragment size suggested that the occurrence of double homologous recombination in the chromosome from both strains. According to the above results, the gene (*cat*, chloramphenicol acetyltransferase) coding chloramphenicol resistance was inserted into the middle of phosphodiesterase domain in ZMO1055, and its direction was reverse direction of *pde* gene expression. The PCR fragments derived from both mutant strains were further sequenced and confirmed by sequence analysis using BLAST (National Center for Biotechnology Information)(data not shown).

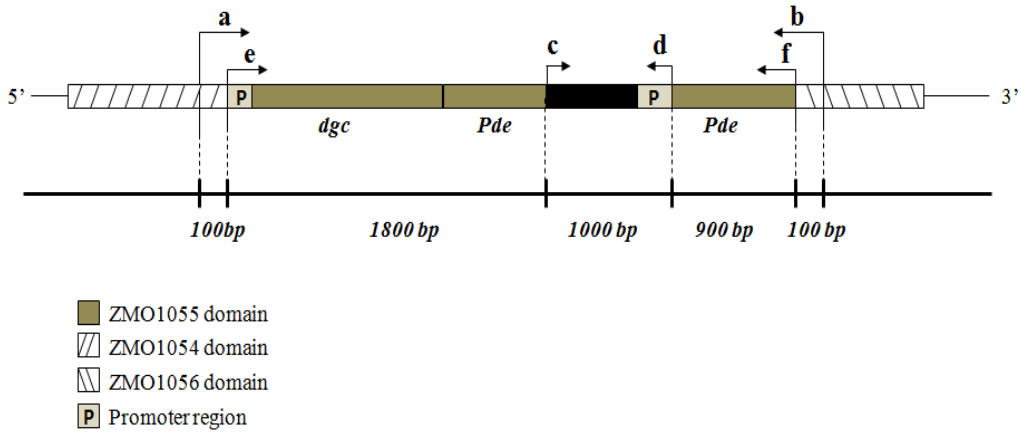


Figure 1. Schematic diagram showing the strategy to disrupt the phosphodiesterase gene via homologous recombination with left-(ZMO1054) and right-(ZMO1056) flanking regions from the ZMO1055 site. (a) ZMO1055F2 primer, (b) ZMO1055DW primer, (c) CatRNsiI primer, (d) CatFNsiI primer, (e) ZMO1054F primer, (f) ZMO1056R primer. A 1kb cat gene fragment was inserted between phosphodiesterase gene.

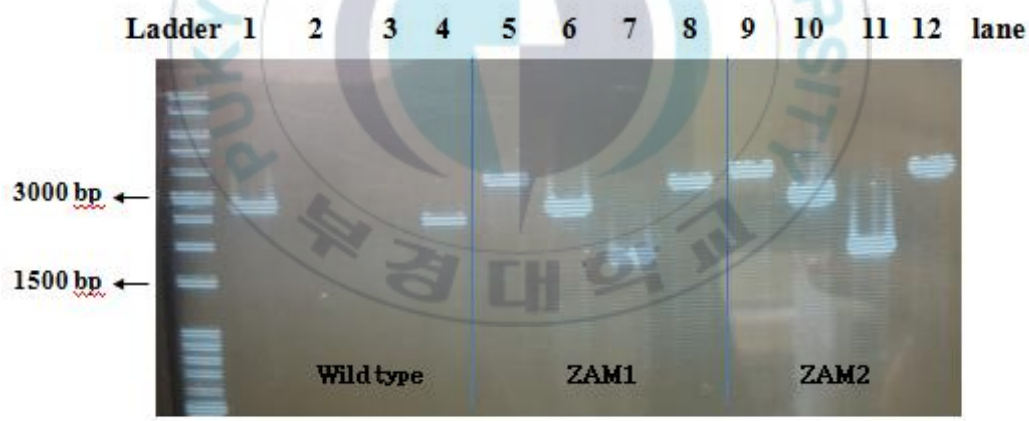


Figure 2. PCR analysis for insertional inactivation for PDE using the genomic DNAs of ZMO1055 of ZM4 and ZM401 with various primers set. Lane 1~4 was the Wild-type. Lane 5~8 was ZAM1. Lane 9~12 was ZAM2. Lane 1,5 and 9, PCR product from the *Z. mobilis* DNA amplified with ZMO1055F2 and ZMO1055DW. Lane 2,6 and 10, PCR product from the *Z. mobilis* DNA amplified with ZMO1055F2 and CatFNsiI. Lane 3,7 and 11, PCR product from the *Z. mobilis* DNA amplified with CatRNsiI and ZMO1055DW. Lane 4,8 and 12, PCR product from the *Z. mobilis* DNA amplified with ZMO1054F and ZMO1056R. The ladder was used size marker (SMOBIO, 10kb gene Ruler).

3.2 Intracellular C-di-GMP concentration of ZMO1055-phosphodiesterase-knockout strains.

The putative ZMO1055-phosphodiesterase defective strains were expected to accumulate c-di-GMP concentration *in vivo*. To determine the amount of the c-di-GMP in the total cell soluble extracts from both ZAM1 and ZAM2, a standard calibration curve was made using the various concentration of c-di-GMP (Fig 4). Based on the calibration curve, the concentration of c-di-GMP in the soluble cell extracts was measured. The result of ZAM1, ZAM2 and ZM401 strains exhibited a higher C-di-GMP contents (about 85 nmol ug^{-1}) than that of the control ZM4 (82 nmol ug^{-1}) indicating the gene knockout of ZMO1055 from both knockout strains contributed to the increase in the intracellular C-di-GMP concentration.

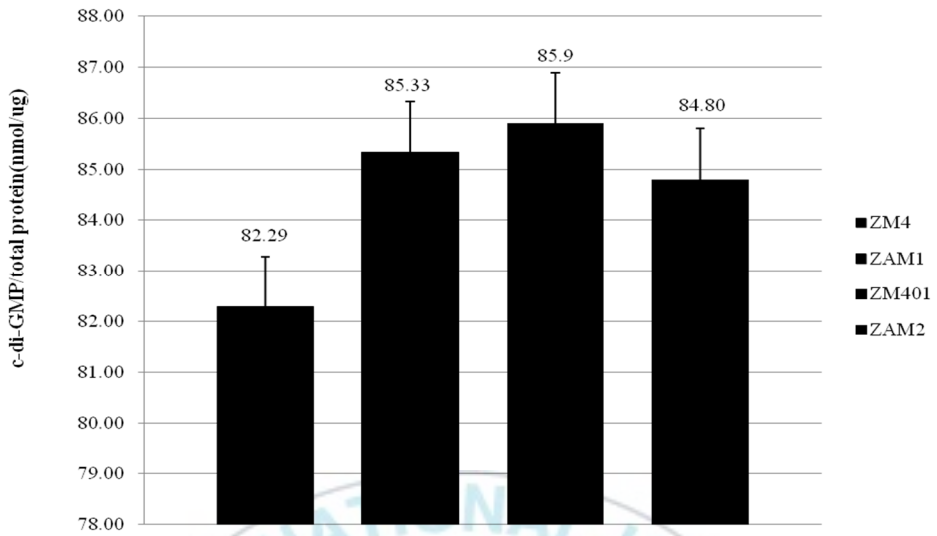


Figure 3. The total c-di-GMP concentration of soluble cell extracts from various *Z. mobilis* strains. Bars present were intracellular c-di-GMP concentration with a standard deviation of the mean. Error bars were calculated using Excel.

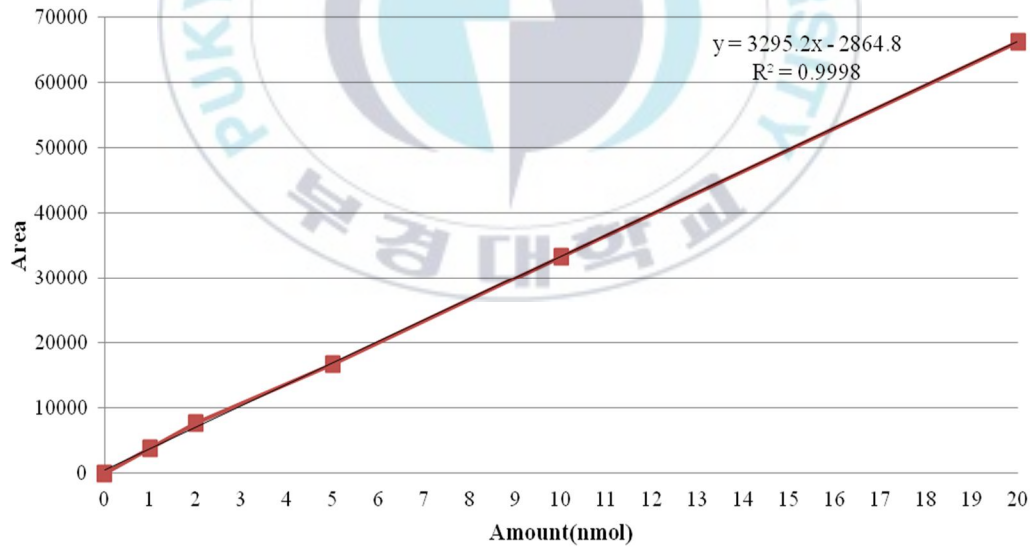


Figure 4. C-di-GMP HPLC standard curve. The standard curve was generated by plotting the peak areas obtained following the separation of 10 µl aliquots of c-di-GMP standards (here: 0, 2, 5, 10, and 20 nmol/µl) versus the total c-di-GMP amounts in nmol.

3.3 Phenotypic characterizations of ZMO1055-phosphodiesterase-knockout strains

3.3.1 Auto-flocculation capacities of both mutant strains

Since the ZMO1055-phosphodiesterase gene knockout mutants showed the accumulation of the signal molecule in the cell, the next tasks were to investigate whether both strains may show any phenotypic changes such as an increase in cell flocculation and decreases cell motility [27, 28]. To observe cell flocculation of ZAM1 and ZAM2, ZM4 and ZM401 were served as negative and positive controls respectively. As shown Fig 5, as expected, but ZM4, all mutant strains showed auto-flocculation in RM liquid broth. However, the degree of cell flocculation phenomena from the both knockout mutants was different. The degree of cell flocculation was most severe from ZAM2, which was fairly similar to ZM401 flocs, whereas ZAM1 cell flocs were not as strong as ZM401 flocs. The cell flocs of ZAM1 were easily resuspended in the medium when an additional force such as shaking was provided. The phenotypic change of both strains ZAM1 and ZAM2 was further investigated through the optical microscopy as shown in Fig. 6. The cell aggregation from ZAM1 and ZAM2 was confirmed, but not found in the wild-type ZM4. Especially, ZAM2 showed the largest flocculation. The morphology and size of cell flocs of ZAM1 and ZAM2 under the microscope were similar to those of the previously studied ZM401.

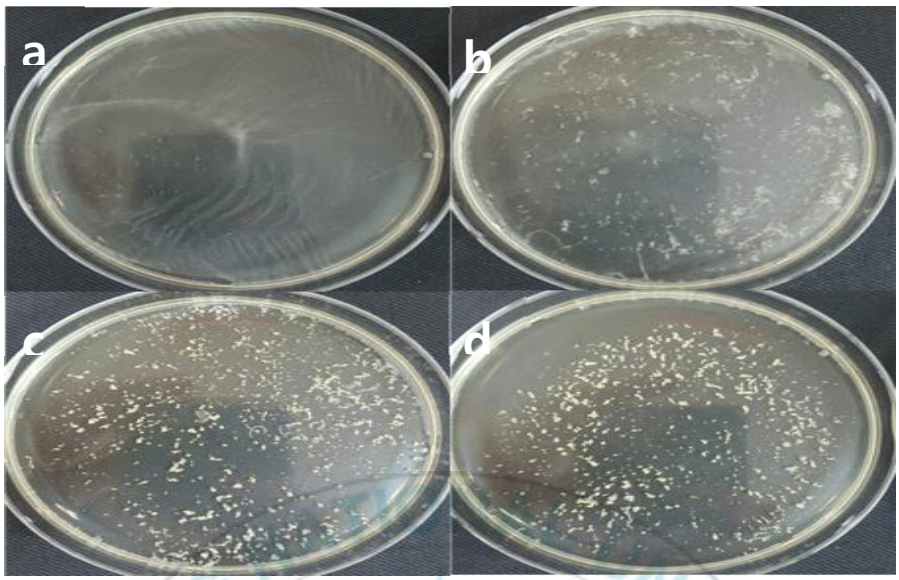


Figure 5. Cell flocculation behaviors from (a) the wild-type ZM4 (negative control), (b) ZAM1, (c)ZM401 (positive control), (d) ZAM2. The number and degree of flocculation from two mutant strains ZAM1 and ZAM2 were higher than the wild type.ZM401 and ZAM2 were similar forms of cell flocs.

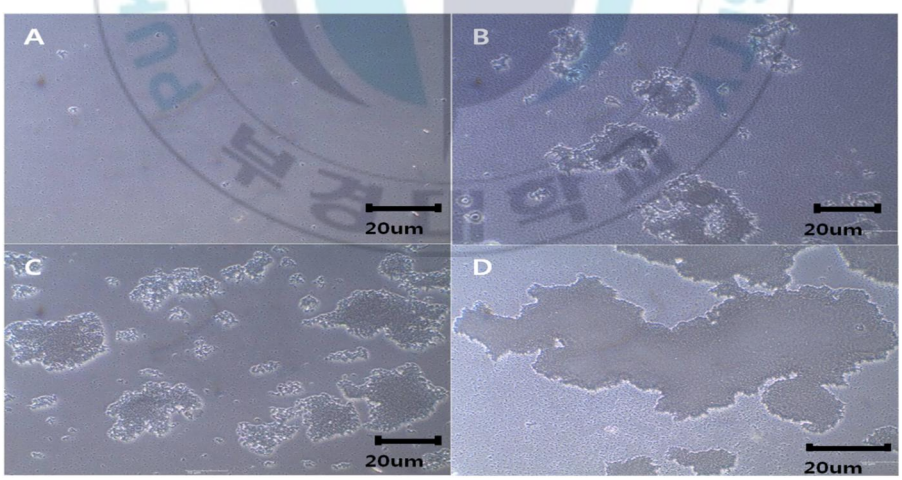


Figure 6. Cell flocculation observed by optical microscopy at 1000x Magnification. The (a) wild-type ZM4, (b) ZAM1, (c) ZM401, (d) ZAM2. The wild-type ZM4(a) was confirmed no sight the flocculation more than flocculant mutants that ZAM1, ZAM2, and ZM401. Flocs appeared to variously size, even ZAM2 was shown the flocs of about more than 100um.

3.3.2 Cell motility of both knockouts strains

It has been reported that *Z. mobilis* has a single flagellum providing cell motility as wells as chemotaxis. Also has been reported that the cell motility of several flagella harboring bacteria was governed by the intracellular c-di-GMP concentration regulating bacterial cell motility through the inhibition of the flagella synthesis [28, 29]. Therefore the cell motility assay was performed to investigate such phenomenal changes from the strains according to the methods described in the earlier section. The results are shown in Fig. 7. The diameter of culture extension zone from ZAM1 was decreased to 1.1cm as compared to that of ZM4 (1.4 cm) indicating the cell motility of ZAM was decreased. These results support the degradation of c-di-GMP was inhibited by phosphodiesterase gene inactivation and this would lead to a decrease in cell motility. However, the motility of ZAM1 was not comparable to ZM401 which has a point mutation of phosphodiesterase gene (ZMO1055), whereas the motility of ZAM2 was similar to that of ZM401.

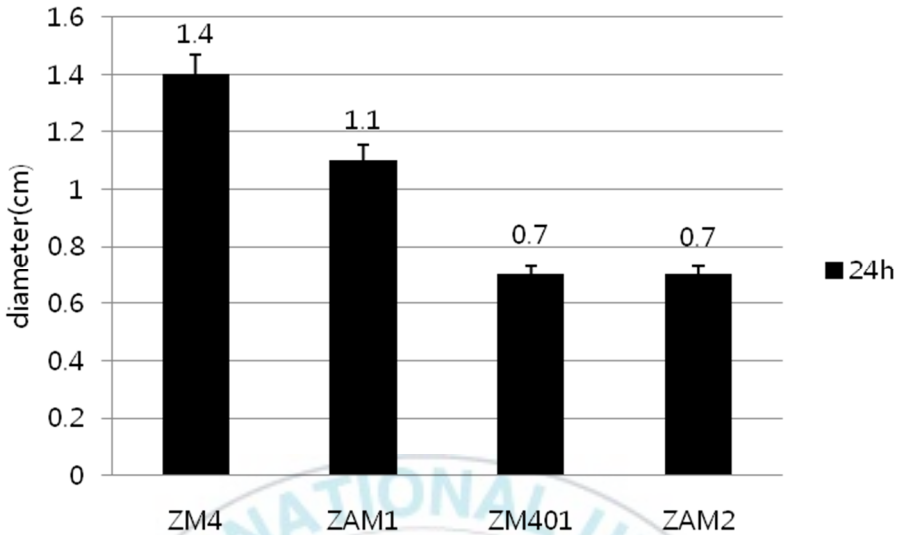


Figure 7. Cell motility assay of *Z. mobilis* strains on semisolid medium. The diameter was measured in both width and length, matched the two lengths.ZM4 wild type was increased cell motility 0.9cm from initial drop diameter.ZAM1 was increased cell motility 0.6cm from initial drop diameter. ZM401 and ZAM2 were increased only 0.2cm from initial drop diameter.

3.3.3 The quantification of bacterial cellulose from phosphodiesterase-knockout strains of *Z. mobilis*.

According to the previous reports, several flagellated bacteria such as *Acetobacter xylium* produces cell-embedded polysaccharide such as bacterial cellulose when the intracellular c-di-GMP concentration was increased [30]. Therefore both *pde* knockout mutants with increased c-di-GMP concentrations were also expected to increase the production of bacterial cellulose. Therefore, the quantification of bacterial cellulose produced from the constructed strains of ZMO1055-phosphodiesterase knockout in *Z. mobilis* was measured in this study. The basic idea of bacterial cellulose quantification was based on the methods previously reported by [23, 31]. When crystal violet, a cationic dye is applied to stain bacterial cells, the cell-embedded polymers such as bacterial cellulose as well as the cell wall component peptidoglycan can be stained as violet color. However, the incorporated dye is removed with D.W and the remaining cooperated dye in bacterial cellulose and peptidoglycan cell wall can be was further extracted by ethanol. The extractants from bacterial cellulose and peptidoglycan were used for semi-quantitative analysis of bacterial cellulose. As can be seen in Fig.8, the absorbance values of crystal violate extracted from both mutants were significantly higher than that from ZM4 indicating high bacterial cellulose synthesis were achieved. The results also suggest that phosphodiesterase gene knockout induced

to increase synthesis of bacterial cellulose which was consistent with various previous results in this study.

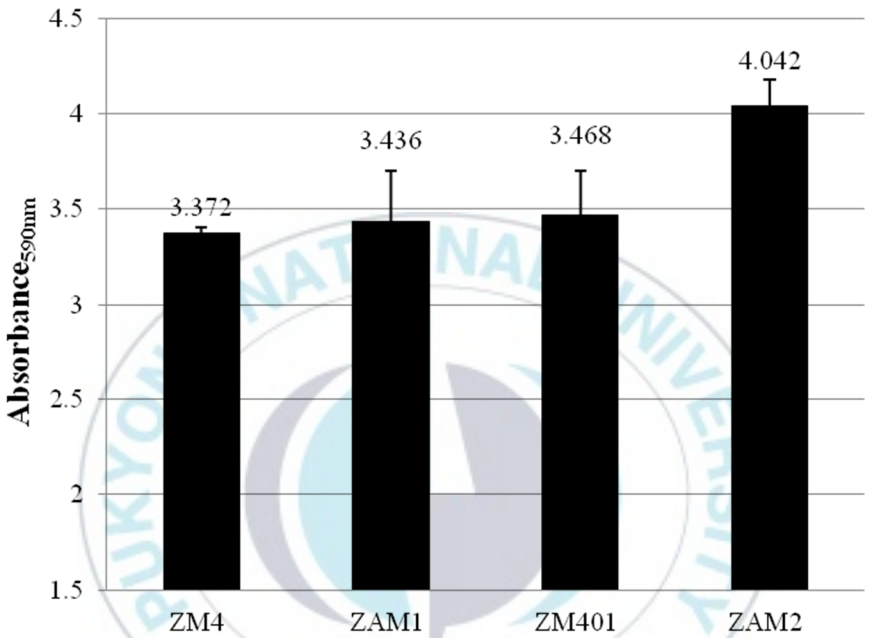
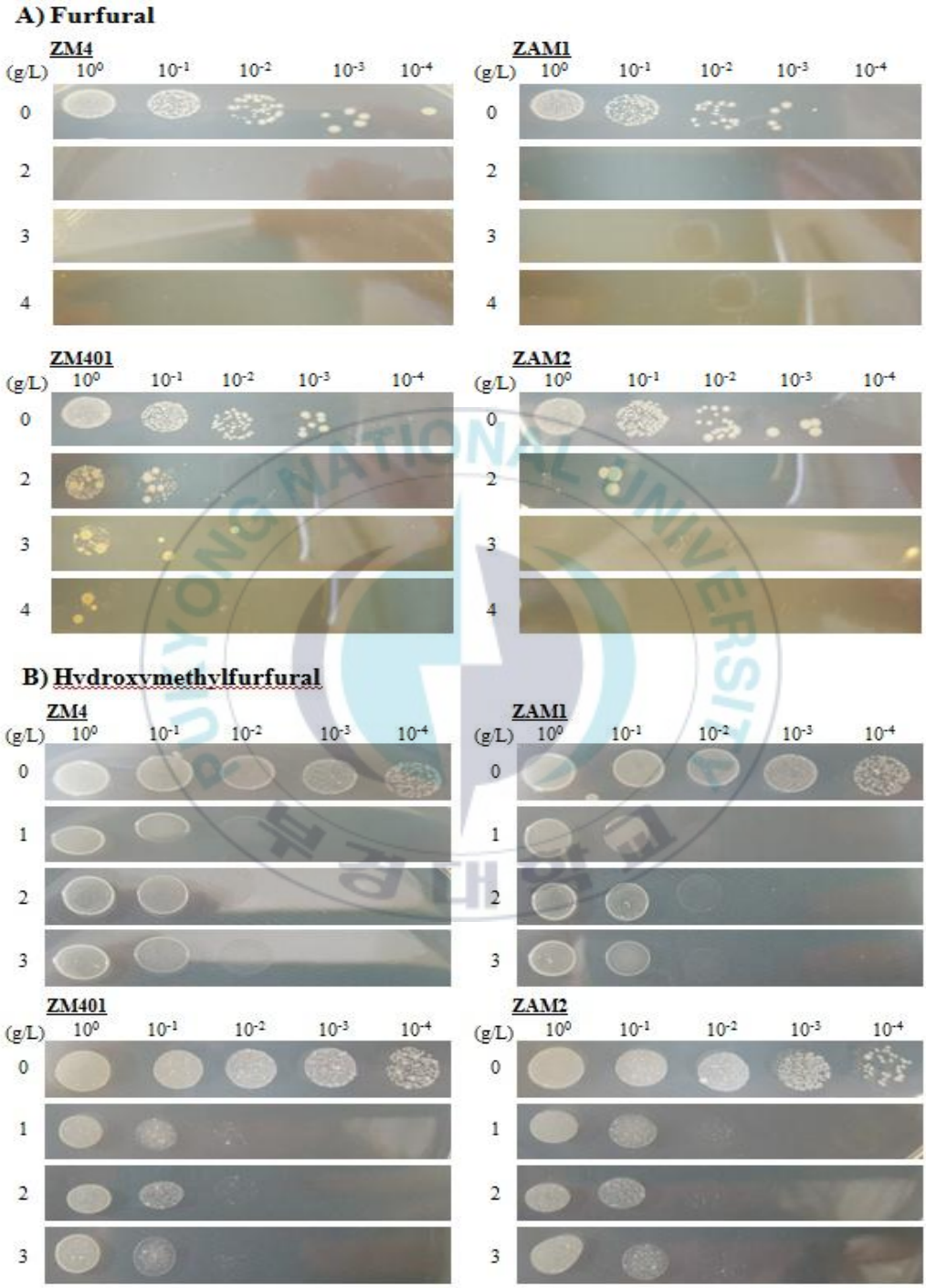


Figure 8. Quantification of crystal violet staining to indirectly measure the amount of bacterial cellulose (OD595). Error bars indicate the standard deviation (n = 3).

3.4 Cell robustness from ZMO1055-phosphodiesterase-knockout strains.

In the 2nd generation ethanol production process using lignocellulosic materials such as wheat straw and crop residues derived from plant materials to convert cellulose and hemicellulose to glucose and xylose as monomers, a pretreatment step is necessary to make the cellulose amenable to hydrolysis for cellulase. But the pretreatment step for lignocellulose-base substrates produces potent fermentation inhibitors including acetic acid, furfural, and lignin degradation products as weak acid hydrolysis pretreatment technology was used [32]. Such toxic substrates derived from the acid pretreatment affected the growth of microorganism such as *Z. mobilis* to convert the monomers (glucose and xylose) derived from lignocellulose to ethanol in the fermentation step. Consequently, cell robustness studies with the constructed strains and parent strains were carried out on the RM plates in the presence of such fermentation inhibitory chemicals as described previously. The concentrations of each inhibitory chemical were selected based on other previous studies for *Z. mobilis* [12], [33]. The results were compared with the parental planktonic strain ZM4 under the same conditions together with each strain cultured in the absence of such inhibitors. The results are illustrated in Figure 9. Figure 9A shows the results of cell growth inhibitory effects in the presences of various concentration of furfural. The cell growth inhibitory effects of both knockout mutants ZAM1 and ZMA2 were similar to the planktonic cells of

ZM4 which did not showed any growth in the presence of 2 g l^{-1} furfural, whereas ZM401 showed the better cell growth against furfural which is able to grow up to 10 cfu ml^{-1} in the presence of more than 2 g l^{-1} furfural. However as the cell robustness of both knockout strains and ZM401 against HMF inhibitory chemical was tested, the cell growth inhibitory effects were similar to the planktonic cell of ZM4 as shown Fig 9B. All tested strains showed mild cell growth inhibition in the presence of all HMF concentrations tested. However, in the presence of more than 12 g l^{-1} acetic acid concentrations, all flocculent strains including ZAM1, ZM401 and ZAM2 showed the ability to grow up to 10^3 cfu ml^{-1} whereas the cell growth of planktonic cells ZM4 only shown up to 10^2 cfu ml^{-1} as shown in Fig 9C. In addition, as the growth pattern of each strain against the ethanol concentration was tested, the cell growth effect of ZAM1was similar pattern to ZM4 strain which is progressively decreased as the concentration of acetic acid was increased to 13% ethanol as shown in Fig. 9D. Under such concentrations tested, ZAM2 showed the best cell robustness against ethanol.


A) Furfural
ZM4
ZAM1
ZM401
ZAM2
(g/L)
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
0
2
3
4
B) Hydroxymethylfurfural
ZM4
ZAM1
ZM401
ZAM2
(g/L)
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
0
1
2
3

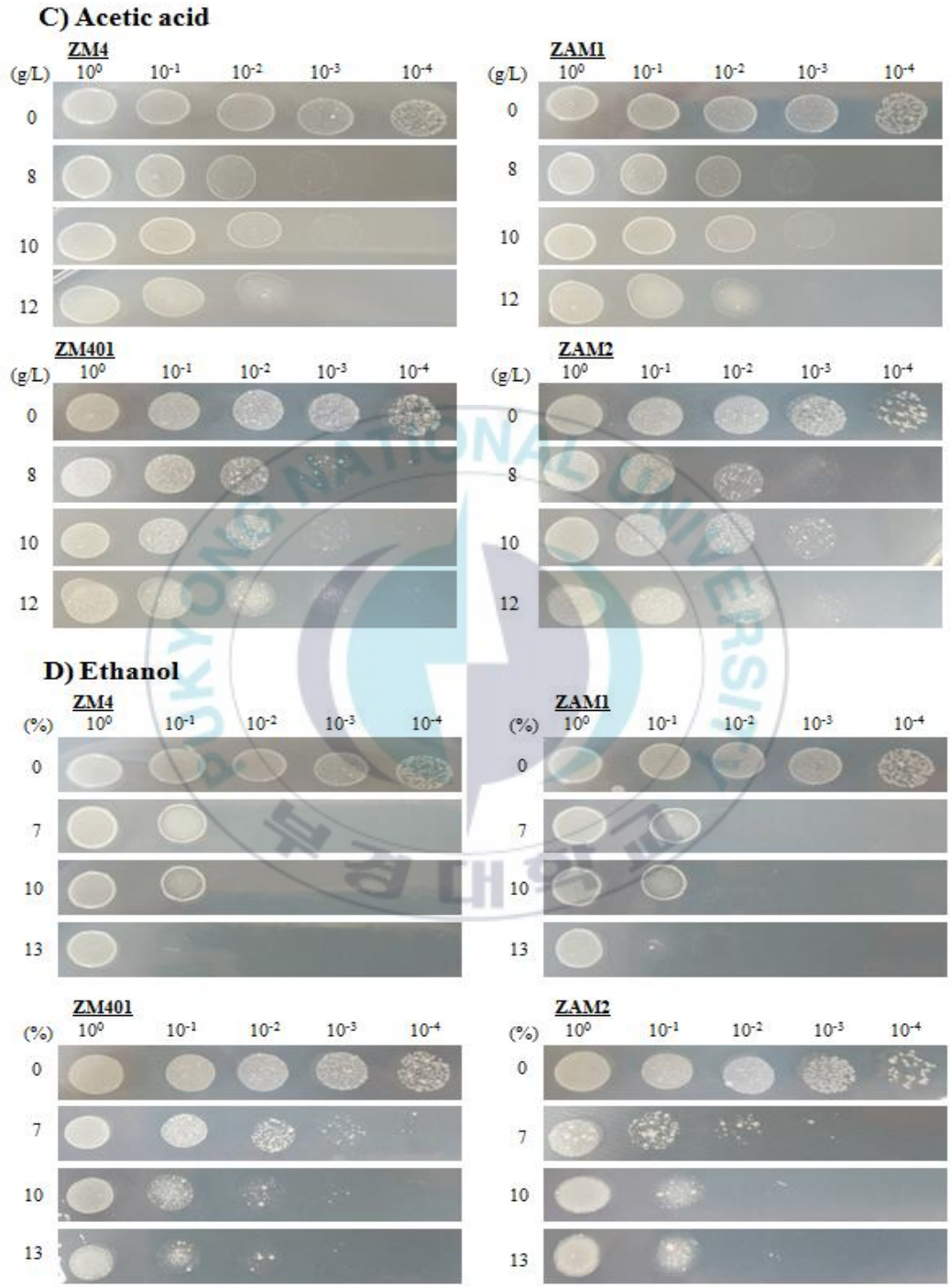

C) Acetic acid
ZM4
(g/L)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
8
10
12
ZAM1
(g/L)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
8
10
12
ZM401
(g/L)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
8
10
12
ZAM2
(g/L)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
8
10
12
D) Ethanol
ZM4
(%)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
7
10
13
ZAM1
(%)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
7
10
13
ZM401
(%)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
7
10
13
ZAM2
(%)
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
0
7
10
13

Figure 9. The resistance of *Z. mobilis* to various concentrations of hydroxymethylfurfural, furfural, acetic acid, and ethanol. A) Furfural. The concentration was used 0, 2, 3, and 4 g / l, and a zero as the control. B) Hydroxymethylfurfural. The concentration was used 0, 1, 2, and 3 g / l. C) acetic acid. The concentration was used 0, 8, 10, and 12 g / l. D) Ethanol. The concentration was used 0, 7, 10, and 13 g / l. Dilutions were used up to 10^{-4}, the tolerance to stress substances can be confirmed for each strain.

4. Discussion

The ability to form stable cell flocs is one of the potential advantageous characteristics of ethanologens used in ethanol fermentation industry because it provides cost-effective and simple ways to separate cell biomass from ethanol fermentation broth or to recycle the cell biomass for the next batches for ethanol production [16, 34]. In addition, cell flocculation can confer industrial robustness against external stress environments which has been demonstrated by other studies for various biotechnological purposes [7, 35]. Due to these advantages, many studies have been conducted to develop industrial strains with cell flocculation. In this respect, our study was motivated to investigate the genetic mechanism of cell flocculation. Accordingly, we investigated the crucial factor of involved in cell flocculation through previous literature and found a cell signaling molecule called c-di-GMP. Many of the cellular functions controlled by c-di-GMP are crucial for the transition between the phenotypically distinct motile and sessile lifestyles. The molecular mechanism of c-di-GMP signaling has been under intensive investigation. Many c-di-GMP signaling proteins and pathways from a diversity of bacterial species have been unveiled. Through binding to effector molecules, c-di-GMP regulates diverse cellular processes, including motility, adherence, biofilm formation, virulence, development and cell cycle progression [36]. Most importantly, diguanylate cyclase (DGC) and phosphodiesterase (PDE) family proteins responsible for the synthesis and degradation of the cyclic dinucleotide

were discovered [37]. C-di-GMP is synthesized from GTP by diguanylate cyclases (DGC) characterized by the GGDEF domain (this motif represents the conserved active site or A-site). Degradation of c-di-GMP is mediated by specific phosphodiesterases (PDEs). Many DGCs and PDEs actually feature GGDEF and EAL domains in the same protein, with usually one domain being enzymatically active and the other being degenerate and exerting a regulatory influence [38].

Based on these investigations, we investigated strains for producing cell flocculation in *Zymomonas mobilis* and the transcriptomic analysis of flocculant mutant ZM401 which was previously isolated by the conventional chemical mutagenesis using NTG. Among the mutation of the gene, ZMO1055 of putative encode diguanylate cyclase and phosphodiesterase gene up-regulated and confirmed that one of the amino acids of the EAL domain was substituted. The mutation was suggested that cell flocculation might be induced by the concentration of c-di-GMP in the cells. In the case of ZMO1055, diguanylate cyclase includes the A-site and I-site motif in GGDEF domain and phosphodiesterase include the EAL domain are hybrid in a row, and various strains with this type of protein have been studied [39]. Therefore, the concentration of intracellular c-di-GMP will be controlled by the antagonistic enzyme of diguanylate cyclase and phosphodiesterase protein of ZMO1055. So, we inactivated the putative phosphodiesterase to observe changes in phenotype and physiological changes. Our study clearly demonstrated that cell flocculation

induction can be achieved by a gene encoding phosphodiesterase of the ZMO1055 being inactivated in *Z. mobilis*. In addition, ZAM1 and ZAM2 showed stable mutation characteristics throughout all experiments were carried out in this study. This indicates that the gene encoding phosphodiesterase in *Z. mobilis* is not essential.

In addition, the concentration of c-di-GMP from ZAM1, ZM401, and ZAM2 was higher than that from ZM4 indicating the EAL domain from ZMO1055 has a phosphodiesterase function. The accumulation of intracellular c-di-GMP, which induces bacterial cell aggregation, has been found in other bacterial strains such as *Caulobacter crescentus, Acetobacter xylinum,* and *Pseudomonas aerugnosa* as the particular gene encoding phosphodiesterase genes were inactivated in such bacteria. Such gene knockout strains also showed c-di-GMP accumulation with bacterial cell aggregation. Our results also support the previous studies that the *pde* knockout mutants of ZAM1 and ZAM2 showed the higher intracellular concentration of c-di-GMP than that of the parental strain of ZM4. Although our results suggest that the strain with an EAL domain gene knockout showed the accumulation of c-di-GMP, the level of c-di-GMP change was not more than 3 nmol ug^{-1} as compared to ZM4. Other studies reported that as phosphodiesterase genes were inactivated, its concentrations were six-fold increased in particular other bacteria such as *Caulobacter crescentus* [40]. Therefore, we investigated other c-di-GMP regulatory genes in *Z. mobilis* apart from other ZMO1055 may exist in this

bacterium. Throughout bioinformatic analysis with NCBI (National Center for Biotechnology Information), five diguanylate cyclase and phosphodiesterase genes were found, exists ZMO0919 and ZMO1365 have the encoding single diguanylate cyclase with beta propeller sensor with a single GGDEF domain, and exists ZMO1487 have the encoding single diguanylate phosphodiesterase with EAL domain and exists ZMO0401 have the encoding an allosteric gene for diguanylate cyclase/phosphodiesterase with GGDEF and EAL domain (NCBI). It is possible to predict that such genes other than ZMO1055 will regulate the biosynthesis of intracellular c-di-GMP, and further gene functional studies on these candidates should demonstrate their activity.

Previously, we mentioned industrial robustness as an advantage of cell flocculation. This is an important feature for cell viability and maximizing ethanol production in ethanol fermentation industry. Therefore, first of all, typical inhibitory substances produced in ethanol fermentation process using lignocellulose were investigated. A variety of inhibitors other than hydroxymethylfurfural, furfural are produced in the lignocelluloses pretreatment used acid catalyst as sulfuric acid, and acetic acid from acetylated hemicellulose [41]. Among them, hydroxymethylfurfural and furfural inhibit the enzymatic activity used in the ethanol fermentation, and acetic acid reduces the pH of the culture medium, which causes the cells to consume energy (ATP) to release proton penetrating into the cell thereby reducing ethanol fermentation efficiency [42]. In addition, the final product

ethanol effect on ethanol fermentation is also known [10]. Therefore, the resistance of various inhibitors is important in fermentation strains. In this study, we found that ZM401 and ZAM2 are more resistant than ZM4 wild type to four inhibitors. These results show that cell flocculation affects an important role in the increase of robustness of *Z. mobilis*. In particular, resistance to ethanol was shown to be highest, indicating that ethanol infiltration was inhibited by cell flocs formation. However, the tolerance of ZAM1 was lower than that of ZM401 and ZAM2. These results suggest that ZAM1 flocculation is not as tight as ZM401 and ZAM2, and therefore, it appears to be affected by the inhibitor and there is a difference in tolerance depending on the level of flocculation.

In this study, we investigated the genes that induce flocculation in *Z. mobilis* and constructed stable mutant strains ZAM1 and ZAM2 through phosphodiesterase knockout of the ZMO1055 domain. Also, it was confirmed that flocculation and c-di-GMP accumulation occurred by gene knockout of phosphodiesterase of ZMO1055. In addition, we confirmed the robustness to effect of inhibitors by flocculation, indicating that gene manipulation strains with higher robustness against with inhibitors can be produced by increasing flocculation in *Z. mobilis*.

5. Acknowledgement

2 년의 짧고도 긴 시간이 훌쩍 지나 석사 과정의 마침표를 앞두고 있습니다. 학부 생활의 마무리에서부터 지금까지 항상 끊이지 않는 열정과 따스함으로 저를 이끌어 주신 전용재 교수님께 감사하다는 말씀 전해드립니다. 제가 헤맬 때면 언제나 길을 찾아 주신 덕에 지금의 제가 있었다고 생각합니다. 또한 부족한 저의 논문을 마무리 할 수 있도록 지도와 조언을 주신 최태진 교수님과 김군도 교수님께도 감사의 말씀을 드립니다. 그리고 학부 실험실 생활 동안 많은 지도와 가르침을 주셨던 김경호 교수님, 언제나 저를 챙겨주신 이명숙 교수님 그리고 송영환 교수님과 김영태 교수님께도 감사의 말씀을 드리고 싶습니다.

4 년이라는 실험실 생활 동안 저에게 많은 것을 가르쳐 주고, 힘들고 지칠 때 든든하게 항상 뒤를 지켜주었던 실험실 선배, 후배, 친구들에게도 고맙고 감사하다는 말을 전하고 싶습니다.

길었던 학과 생활을 마지막 순간 앞에서 돌이켜 보며 배웠던 많은 가르침들과 소중한 사람들을 떠올려 보면서 아직도 많이 채우지 못했다는 생각이 들어 아쉬운 마음이 들기도 합니다. 하지만 이런 부족함을 계기로 더 발전할 수 있을 것이라고 생각합니다.

이 자리를 빌어 같은 석사 생활을 하면서 언제나 든든한 기둥이 되어준 다래와 경하에게도 고맙다는 말을 전하며 아낌없는 응원과 격려를 보내준 친구들에게도 고맙다는 말을 전하고 싶습니다. 마지막으로 항상 부족함 없이 저를 사랑으로 지켜봐주신 부모님께 언제나 사랑한다는 말을 이 기회를 통해 전합니다.

6. 국문초록

Zymomonas mobilis는 lignocellulose를 사용하는 바이오 에탄올 생산 공정에서 중요한 효과적인 에탄올 생성 균주로 연구되어왔다. 그러나 lignocellulose를 이용한 에탄올 생산 공정 중에서 셀룰로오스 분해 효소를 이용해 식물 잔류물을 처리할 때 보다 쉽게 이용할 수 있도록 하는 전처리 단계에서 다양한 저해제가 생성된다. 이러한 저해제는 세포 성장뿐만 아니라 전반적인 에탄올 생산성에 영향을 준다. 따라서 저해제의 분리 또는 내성 균주의 제작은 에탄올 생성 공정에서 중요한 산업적 목적이다. 이와 관련하여, 이전에 세포 응집 돌연변이 균주인 Zymomonas mobilis ZM401이 비교적 비용 효율적인 세포 회복 특성을 갖는 세포 응집 균주로써 분리되었다. 이 응집 균주의 이전 연구 중 transcriptomic 분석은 ZMO1055에서 발생한 단일 점 돌연변이가 응집에 관한 주요 원인 중 하나로 가정하였다. 이 단백질들은 cyclic-di-GMP의 농도를 조절함으로써 세포 응집을 조절할 것으로 예측된다. 따라서 Z.mobilis ZM4와 응집 돌연변이 균주인 ZM401로부터 각각 ZAM1과 ZAM2의 두 개의 유전자 knockout 돌연변이를 분리했다. 그 결과로, knockout 돌연변이 균주의 세포 내 c-di-GMP의 농도가 감소하고, 저해제에 대한 견고성이 증가하였으며 다양한 수준의 세포 응집이 나타나는 것을 확인하였다. 결과적으로 이 연구는 ZMO1055의 phosphodiesterase knockout이 세포 응집을 유도하고, 이로 인해 생성된 안정한 세포 응집이 균주의 산업적 견고성 및 효과적인 에탄올 생산 전략을 제시하는 것으로 나타났다.

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