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Statistical Optimization of the Lysis Agents for Gram-negative Bacterial Cells in a Microfluidic Device

Young-Bum Kim[1], Ji-Ho Park[1], Woo-Jin Chang[2], Yoon-Mo Koo[1,2], Eun-Ki Kim[1,2]*, and Jin-Hwan Kim[3,4]*

[1] Department of Biological Engineering, Inha University, Incheon 402-751, Korea
[2] Center for Advanced Bioseparation Technology, Inha University, Incheon 402-751, Korea
[3] Department of Polymer Sci. & Eng., Sungkyunkwan University, Suwon 440-746, Korea
[4] M&B GreenUs Co., Ltd., Seoul 152-021, Korea

Abstract Through statistically designed experiments, lysis agents were optimized to effectively disrupt bacterial cells in a microfluidic device. Most surfactants caused the efficient lysis of Gram-positive microbes, but not of Gram-negative bacteria. A Plackett-Burman design was used to select the components that increase the efficiency of the lysis of the Gram-negative bacteria *Escherichia coli*. Using this experimental design, both lysozyme and benzalkonium chloride were shown to significantly increase the cell lysis efficiency, and ATP was extracted in proportion to the lysis efficiency. Benzalkonium chloride affected the cell membrane physically, while lysozyme destroyed the cell wall, and the amount of ATP extracted increased through the synergistic interaction of these two components. The two-factor response-surface design method was used to determine the optimum concentrations of lysozyme and benzalkonium chloride, which were found to be 202 and 99 ppm, respectively. The lysis effect was further verified by microscopic observations in the microchannels. These results indicate that Gram-negative cells can be lysed efficiently in a microfluidic device, thereby allowing the rapid detection of bacterial cells using a bioluminescence-based assay of the released ATP.

Keywords: bioluminescence, ATP assay, Gram-negative bacteria, statistical optimization, microfluidic device

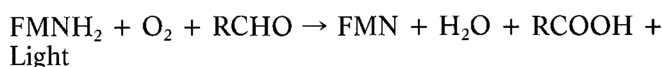
INTRODUCTION

Microbes must be disrupted in order to liberate their internal components that include useful enzymes, antibiotics, peptides, adenosine triphosphate (ATP), *etc* [1]. With the increasing use of intracellular microbial materials in industry, the importance of cell disruption and lysis unit operation has increased [2]. The recovery of an intracellular product requires the disruption of the cell wall and membrane by chemical, enzyme or physical methods. Physical methods are often favored due to economical and operational advantages compared to the other two methods [3]. However, the physical method is inefficient when disrupting a small number of microbes. On the other hand, enzyme and chemical methods have been used in several fields, including the detection of microbial contamination in a substance in liquid or gas phase. These lysis methods have been useful in lysing cells in small facilities such as microfluidic devices.

In addition to their current applications, modern analytical luminescent methods have recently been reviewed with an emphasis on the most sensitive methods that can be used in future micro-analytical systems, such as lab-on-a-chip, as well as point-of-care testing and high throughput screening (HTS) applications [4,18,21].

The detection of microbes in water and foods is very important for maintaining food safety and human hygiene. However, the efficient detection of microorganisms requires the development of efficient detection methods. Although the calibrated loop and serial dilution techniques have been used in many cases, these methods are quite time-consuming [5].

A more rapid detection method has been developed based on adenosine triphosphate (ATP), which exists in all living organisms and can be used to indicate the presence of living organisms [6]. This bioluminescent method is based on the proportionality between the bacterial ATP content and the number of CFU observed in the bacterial cultures [7,8]. A luciferin-luciferase bioluminescence reaction is used to determine the ATP concentration [9,20] through an enzyme-mediated light emission process [10], which occurs as follows:



$$FMNH_2 + O_2 + RCHO \rightarrow FMN + H_2O + RCOOH + \text{Light}$$

***Corresponding authors**
Tel: +82-32-860-7514 Fax: +82-32-872-4046
e-mail: ekkim@inha.ac.kr
Tel: +82-2-6675-6121 Fax: +82-2-6675-6119
e-mail: jhkim@skku.edu

In this reaction, luciferase catalyzes the production of light in the presence of luciferin, magnesium, and oxygen [9,13].

Microbes in drinking water and foods can be detected by bioluminescent assay methods, but the extraction of bacterial ATP must be carried out in a microfluidic device. The general methods for extracting microbial ATP are ultra-sonication and the use of lysis agents such as surfactants, TCA, lysozyme, *etc*.

Surfactants are effective agents for many microbial lysis reactions. However, they have little effect on Gram-negative bacteria, due to their double-layered membranes [6].

Therefore, detecting the Gram-negative cell via a luminescent method is difficult as a result of the low ATP yield, particularly at low bacterial concentrations [11].

Lysozyme is a bacteriolytic enzyme discovered by Fleming in 1922 that lyses suspensions of certain bacterial species. Its lytic action occurs via the cleavage of β-1,4-glycosidic linkages in the cell wall [12]. When used concurrently, a surfactant and lysozyme can interact and affect the lysis efficiency by altering the reaction mechanism. However, few studies have reported the effects of lysozyme and surfactants on cell lysis [1,2,4,6].

In this work, we describe optimization of the microbial lysis reagent through statistically designed experiments for application in microfluidic devices. The optimized reagent increased the lysis efficiency with no reduction in the bioluminescence.

MATERIALS AND METHODS

Strains

One Gram-positive bacteria, *Bacillus subtilis*, and two Gram-negative bacteria species, *Escherichia coli* and *Pseudomonas aeruginosa*, were used. The strains were maintained frozen at −70°C in LB broth containing 30% glycerol, 0.5% yeast extract, 1.0% tryptone, and 0.5% NaCl. Prior to use, these strains were propagated in LB broth at 37°C for 24 h.

Optimization of ATP Extraction Solution

Among the six cell-lysis reagents (alpha olefin sulfonate, benzalkonium chloride, Triton X-100, lysozyme, EDTA, SDS), the two optimum components were ultimately selected based on lysis efficiency, as well as the intensity and the stability of bioluminescence using the Plackett-Burman design [16]. In addition, the optimum concentration of these lysis reagents was determined using the two-factor response-surface method. Statistical analyses were carried out using Minitab software (Version 14, Minitab Co., PA, USA)

Bioluminescence Assay

Using a bioluminescence detection kit (Kikkoman International Co., Japan), a 0.1 mL sample was combined with 0.1 mL of the cell-lysis reagents and incubated at room temperature for 1 min. 0.1 mL of the luciferin-luciferase reagent was then added. The samples were mixed, and the bioluminescence was quantified using a luminometer (Kikkoman International Co., Japan). The complete lysis of the tested cells was confirmed by microscopic examination.

Table 1. Effect of the various buffers on the lysis of *E. coli* (1.5×10^4 cfu/mL)

Buffer	Bioluminescence intensity (RLU; relative light unit)
Distilled water	485
Tris-HCl (250 mM)	88
Tricine (25 mM)	1,725
HEPES (10 mM)	3,350

Fabrication and Structure of Microfluidic Device

The master for casting the polydimethyl siloxane (PDMS) was fabricated by a soft-lithographic method using a 4-inch silicon wafer and a negative photoresist, SU-8 25. The soft-lithographic fabrication of PDMS-based microfluidic devices is described elsewhere [14,15,19]. Briefly, a positive structure of SU-8 25 was patterned on the silicon wafer using a polyimide mask and a Karl-Suss mask aligner (MA3, Tempe, AZ, USA). The surface of the master was silanized by trichloro(3,3,3-trifluoropropyl) silane (Aldrich Chemical Co., USA) in order to prevent bonding of the PDMS during casting. The PDMS pre-polymer was then cast on this master. Next, the patterned PDMS plate was bonded with the flat PDMS slab after peeling the polymer off from the master. The surfaces of the PDMS plate and the flat PDMS slab were then treated with a plasma discharger for permanent bonding. Finally, the connection ports to the syringe pump and sampling reservoirs were made using a stainless-steel hole-puncher.

RESULTS AND DISCUSSION

Selection of Buffer Solution

Three buffer solutions were used: 10 mM tris-HCl (pH 7.7), 10 mM HEPES (pH 7.7), and 25 mM Tricine (pH 7.7). Table 1 shows the characteristics of the three buffer solutions, in terms of the bioluminescence intensity and the lysis efficiency. Both HEPES (pH 7.7) and Tricine (pH 7.7) resulted in high bioluminescence intensity, but the lysis efficiency of the Tricine buffer was lower than that of the HEPES buffer. Therefore, the HEPES buffer was selected as the buffer for cell lysis.

Optimization of Lysis Reagent Solution

Approximately 15 times more ATP was extracted from the Gram-positive bacterial species than from the Gram-

Table 2. ATP extraction efficiency

Microbe	RLU per cell count after surfactant treatment	RLU per cell count after sonication
E. coli	0.09	1.54
B. subtilis	15.00	14.71

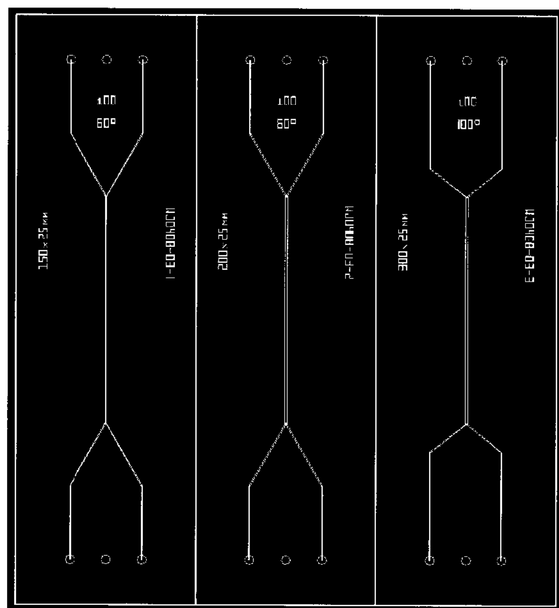


Fig. 1. The shape and dimensions of the microfluidic device used for the cell lysis reaction: Height: 90 μm, width: 400 μm, length of reaction channel: 28 mm.

negative bacteria (Table 2). This variation was caused by the structural differences between Gram-positive and Gram-negative cells. However, the detection of Gram-negative bacteria is very important for water safety and human hygiene, particularly because *E. coli* is an indicating strain [17].

The Plackett-Burman (P-B) design was used to select the components that would efficiently increase the level of cell lysis in Gram-negative cells by detecting the level of bioluminescence with differing combinations and concentrations of the six lysis components. Table 3 lists the high and low concentrations of the six components, as well as the combinations used. The P-B design experiment was composed of 15 runs: 12 runs contained the indicated combinations of high and low concentrations of each component, and 3 runs served as the control, containing the averages of the high and low values for each component. The efficiency of cell lysis was determined by ATP analysis using the bioluminescence assay and is summarized in Fig. 2. High concentrations of lysozyme and benzalkonium chloride were found to increase the cell lysis efficiency. Benzalkonium chloride disturbs the membrane stability because it has a similar structure to the cell membrane construction unit, *i.e.*, phospholipids. On the other hand, lysozyme cleaves the β-1,4-glycosidic linkages in the

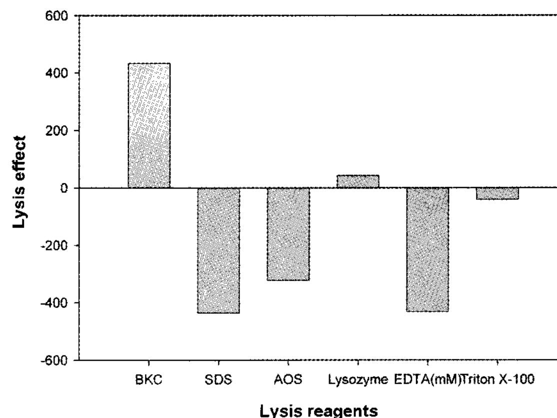


Fig. 2. Lysis effects of reagent components according to P-B design.

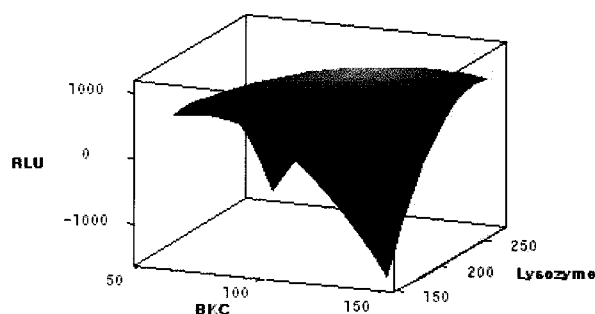


Fig. 3. Response-surface plot of effects of benzalkonium chloride and lysozyme on cell lysis.

cell wall. Therefore, a synergistic interaction between these two reagents might increase the level of ATP extraction. In order to maximize this interaction, as well as the ATP extraction and bioluminescence, the two-factor response-surface design method was used to determine the optimum concentrations of lysozyme and benzalkonium chloride. The concentrations of the two components used in the surface design method are shown in Table 4. Using statistical analysis, the ATP extraction efficiency, as expressed by the bioluminescence, was determined using the following second-order polynomial function:

$$\text{Lysis efficiency (bioluminescence)} = -3490.98 - 9.62B + 47.29L - 0.38B^2 - 0.22L^2 + 0.42BL$$

where B = concentration of benzalkonium chloride (ppm) and L = concentration of lysozyme (ppm)

Fig. 3 illustrates the existence of the optimum point. The optimum concentrations of lysozyme and benzalkonium chloride that maximized the ATP extraction efficiency were calculated by partial differentiation with respect to each component. The optimum concentrations were found to be 202 ppm lysozyme and 99 ppm benzalkonium chloride.

The optimized lysis solution increased the intensity of the bioluminescence more than 3 times compared to surfactant (Fig. 4).

Table 3. Plackett-Burman design for the screening of effective agents for cell lysis

Run[a]	Components (ppm)					
	Benzalkonium chloride	SDS	Alpha olefin sulfonate	Lysozyme	EDTA (mM)	Triton X-100
+	400	300	400	300	1	400
0	200	150	200	150	0.5	200
–	0	0	0	0	0	0
1	–	–	–	–	–	–
2	–	–	–	–	–	–
3	–	–	–	–	–	–
4	–	–	–	–	–	–
5	–	–	–	–	–	–
6	–	–	–	–	–	–
7	–	–	–	–	–	–
8	–	–	–	–	–	–
9	–	–	–	–	–	–
10	–	–	–	–	–	–
11	–	–	–	–	–	–
12	–	–	–	–	–	–
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0

[a]Run 1~12: with the different combinations of high and low concentrations of each component.
Run 13~15: with the averaged concentrations of each component as a control.

Table 4. 2-Factor response-surface design method for determining the optimum concentrations of lysozyme and benzalkonium chloride (coded values)

	Benzalkonium chloride	Lysozyme
1	0	1.41421
2	-1	1
3	1	-1
4	1	1
5	-1	-1
6	0	0
7	1.41421	0
8	0	0
9	-1.41421	0
10	0	0
11	0	-1.41421
12	0	0
13	0	0

Code for component concentrations: BKC (1.41421 = 420 ppm, 1 = 300 ppm, -1 = 200 ppm, -1.41421 = 80 ppm, 0 = 250 ppm), lysozyme (1.41421 = 280 ppm, 1 = 200 ppm, -1 = 100 ppm, -1.41421 = 20 ppm, 0 = 150 ppm).

Lysis on Chip by Optimized Lysis Reagent Solution

A microfluidic device with two inlets and two outlets was used. The height, width and length of the reaction-channel were 90 μm, 300 μm and 28 mm, respectively. The total volume of the reaction-channel was approximately 1.0 μL. In order to achieve the maximum lysis effect in the microchannel, the range of appropriate flow rates of the lysis reagent was determined by microscopy. The optimum flow rate was 0.1 μL/min (retention time: 10 min). Once this had been determined, micro-syringe pumps were used to control the optimum flow rates in the microfluidic devices.

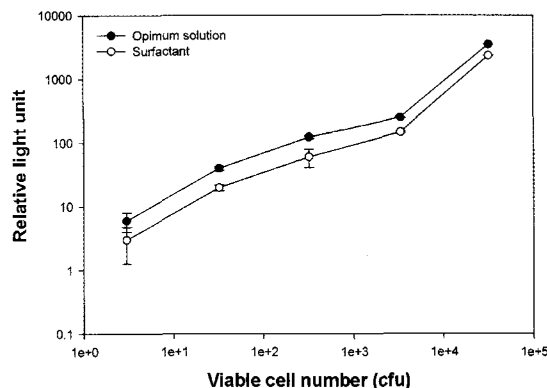


Fig. 4. Comparison of lysis effects of optimum lysis reagent and general surfactant solution (BKC 250 ppm).

Optical micrographs of cell solutions within the micro channel are shown in Fig. 5. Micrograph A (taken from position A) shows the bacterial cells prior to mixing with the lysis solution. Lysed cells were observed in the micro-channel approximately one minute after the lysis solution and cells were mixed (position B).

CONCLUSIONS

The efficient lysis of microbial cells is essential for the

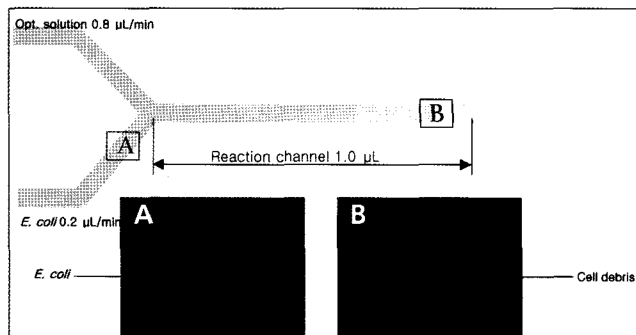


Fig. 5. Micrographs of cells in microfluidic device using optimum cell lysis reagents; A: *E. coli* cells before mixing with lysis solution, B: lysed cells approximately 1 min after mixing with lysis solution.

rapid detection of microbial cells, which is very important for food safety and public hygiene. This study developed an efficient lysis reagent for Gram-negative bacteria, which have a cell wall structure that is resistant to conventional lysis methods.

The optimum lysis reagent for Gram-negative bacteria was developed using a statistical design method. Among the various reagents examined, lysozyme and benzalkonium chloride displayed synergistic effects. After further optimizing this combination, a lysis efficiency approximately three times higher than that of control was achieved. In addition, the lysis of Gram-negative cells was observed in a microfluidic device using this reagent. This indicates that Gram-negative cells can be lysed efficiently in a microfluidic device, thereby allowing the rapid detection of ATP released from the lysed cells with a bioluminescence-based assay.

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