Thesis for the Degree of Master of Engineering

Mass production of recombinant *E. coli* K-12 ghost vaccine against streptococcal disease

by

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Department of Biotechnology

The Graduate School

Pukyong National University

November 2010

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(연쇄상구균증에 대한 재조합 *E. coli* K-12 고스트 백신의 대량생산)

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연쇄상구균증에 대한 재조합 *E. coli* K-12 고스트 백신의 대량생산

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요 약

어류 단백질 섭취의 증대에 따라 수산 양식업이 다양화되고 생산이 증가함으로써 양식 해양생물의 질병으로 인한 피해가 증대되고 있는 가운데 특히 양식어류는 고밀도 사육 및 사육환경 악화 등 여러 가지 스트레스 요인으로 질병 발생빈도가 높아져 많은 피해를 입고 있다. 이와 같은 어병 피해의 예방대책으로서 대부분 항생제와 같은 항균성 의약품 등의 투여에 의한 피해경감에 의존하고 있는 실정이며, 2008 년도 국내에서는 1 조 625 억원어치의 항생제 중 50% 이상이 축산·수산용으로 사용되고 있다. 그러나, 이러한 항균성 의약품 등의 사용은 약제 내성균의 증가, 약제의 어체 내 잔류에 의한 식품 위생상의 문제 및 환경으로의 확산에 의한 공중위생상의 문제를 내포하고 있으며, 더구나 최근 양식 및 재배어업을 목표로 하는 종묘생산의 현장에 있어서, 항균성 의약품으로는 치료할 수 없는 바이러스성 질병이 만연하여 심각한 사태가 발생하여 질병감염의 문제가 더욱 심화되고 있다. 또한 무분별한 항생제 남용, 약제처리 미숙 등 양식어류의 잔류 항생제 축적에 대한 소비자들의 불신이 팽배해 있으며, 특히 투여된 항생제가 수중에 직접 노출될 수 있는 양식장의 항생제 남용은 생태계 파괴의 논란이 끊이지 않고 있다.

따라서 항생제 사용에 의한 부작용을 최소화하기 위해서는 백신을 통한 질병 예방 전략이 필수적인데 백신은 어류의 세균성, 바이러스성 질병의 발병률을 획기적으로 줄일 수 있는 비용 대비 효과가 가장 큰 의약품으로 알려져 있다. 어류 양식분야에서도 생산성 향상 및 친환경적으로 건강한 어류 생산을 위해 필요한 기술이며 또한 날로 그 중요도가 증가하고 있는 추세이다. 본 연구는 유해한 화합물 및 열처리를 통한 사균 생산 방식에서 완전히 탈피하여 생체의 항원성을 가장 효과적으로 보존시킬 수 있는 기술로서 고스트화 세균, 유전자 및 항원 전달시스템 기술을 통한 해양생물의 세균성 질병에 대한 백신전략으로 사용하고자 한다.

본 연구에서는 연쇄구균증의 주요 원인종의 하나인 *Streptococcus iniae* 에 대한 백신을 개발하기 위해 고스트 박테리아 기술을 도입하였다. 고스트 박테리아 기술이란 세균의 벽에 미세하게 작은 구멍을 인위적으로 유도함으로써 세균의 세포질 성분을 밖으로 유실토록 하는 기술로서 세균 자체는 생존능력이 없으나 세포 표면의 물리적 및 화학적 구성은 완벽히 보존시킬 수 있는 장점을 갖고 있어 우수한 안정성과 동시에 생균에 버금가는 항원성을 보존시킬 수 있다. 또한 다량의 세포질이 제거됨으로써 항원성 전달의 높은 효율과 비특이적인 면역억제 등을 최소화 시킬 수 있다.

이와 같은 기술을 이용하여 생산된 백신을 유가식 배양을 통해 대량생산하여 산업화에 있어 높은 경쟁력을 확보하기위한 연구를 수행하였다.

본 연구에서는 타겟 antigen 으로 GAPDH 를 선택하였고, 이와 함께 항원의 표면 발현을 위한 InaN 신호서열과 고스트 생성을 위한 ghost 27 SDM cassette 가 포함된 재조합 플라스미드를 cloning 하여 *E. coli* K-12 에 형질전환 시켰다. *E. coli* K-12 는 인간이나 동물, 식물 등에 전혀 위해하지 않으며 high volume 및 high cell density 를 목적으로 주로 이용되어져왔다. 따라서 본 연구에서는 고스트 백신의 산업화를 위해 host cell 로 *E. coli* K-12 를 선택하였다. Cloning 된 재조합 균주인 *E. coli* K-12/pHCE-InaN-antigen-ghost27 SDM 를 고스트 백신의 산업화를 위해 5 L 발효기를 이용하여 최적 탄소원, 교반속도, 산소공급 조건 등의 최적 배양조건을 검토하였고, 고스트 발현 유도를 위한 온도조절과 고스트 별현 효율 최적화를 위한 연구를 수행하였다. 그 결과 최종적인 발효조건으로 최적 탄소원은 glucose, 교반속도는 300 rpm, 산소 공급조건은 2.0 vvm 으로 결정하였다.

5 L 발효기를 이용하여 working volume 2.5 L 로 유가식 배양을 수행하였다. 유가식 배양은 총 4 단계로 나누어져 수행되었으며, 1 단계는 intial batch phase, 2 단계는 균의 대량생산을 위하여 fed-batch phase 를 수행하였다. 3 단계는 induction phase 로 온도를 27℃에서 42℃로 증가시켜 Lysis E gene 을 발현시켜 대량생산된 균을 고스트화 시켰다. 4 단계는 high temperature holding phase 로 온도를 42℃에서 47℃로 2 시간동안 증가시켜 고스트 박테리아 백신 생성 효율을 99.9%로 끌어올렸다. 또한 42℃에서 47℃로 온도가 증가함에 따른 표면 단백질에 끼치는 영향을 알아보기 위해 outer-mambrane protien fractionation 실험을 수행하였다. 그 결과 온도가 42℃에서 47℃로 증가함에 따른 표면 발현 단백질에는 크게 영향을 주지 못한 반면, 49℃로 온도를 올렸을 때는 표면 발현 단백질에 영향을 주는 것을 확인 할 수 있었다. 따라서 본 연구에서는 고스트 생성 효율을 증가시키기 위한 최적 증가온도로서 47℃를 선택하였다. 최종적으로 생산된 고스트 백신의 생산량은 34.9 g dcw/L 를 나타내었다. 이는 종래의 *E. coli* XL1-blue 를 이용한 고스트백신의 생산량인 22 g dcw/L 보다 높은 생산성을 나타내었다.

국내 양식 어류종인 넙치를 대상으로 *S. iniae* 백신에 대한 효능을 검증하고자 challenge test 를 실시하였다. 2 주일 간 누적 폐사율을 관찰한 결과 최종 누적 폐사율은 positive control, *E. coli* K-12 host strain control group 그리고 *E. coli* K-12/pHCE vector control group 의 경우 누적 폐사율은 100%로 나타났으며, fomalin-killed cell (FKC) vaccine 을 처리한 group 의 경우 65%의 누적 폐사율을 보인 반면, 생산된 고스트 백신을 처리한 group 인 GBV42 와 GBV47 의 경우 50%로 동일한 누적 폐사율을 나타내었다. 이와 같은 백신 효능 검증 결과 본 연구를 통해 대량 생산된 고스트 백신이 FKC vaccine 보다 면역 효과가 크다는 것을 확인할 수 있었고, 이는 *E. coli* K-12 를 이용한 고스트 백신이 양식 산업에 있어 효과적이면서도 상업적으로 유용한 백신으로 사용 될 수 있을 것으로 사료된다.

Ⅰ. INTRODUCTION

Streptococcus iniae is a Gram-positive bacterium and one of main pathogens of farmed fishes. Streptococosis outbreaks under adverse conditions, such as by high water temperature and high density of stocking. *S. iniae* infects a large number of marine and freshwater fishes, including hybrid striped bass (Shoemaker, C.A. *et al*. 2001), channel catfish (Shoemaker, C.A. *et al*. 2001), European sea bass (Kvitt, H. *et al*. 2004), rainbow trout (Eldar, A *et al*. 1994), tilapia (Shoemaker, C.A. *et al*. 2001; Kvitt, H. *et al*. 2004), and olive flounder (Nguyen, H.T. *et al*. 2002). Due to the high mortality, the bacteria, *S. iniae* caused severe economic losses of 150 million US $ annually (Nawawi, R.A. *et al*. 2009). Clinical signs of *S. iniae* infection vary with different fish species. The olive flounder shows haemorrhage, exophthalmia, abdominal distension, ascites, and lesions of the spleen, liver, kidney and intestine [11].

In aquaculture, vaccines of inactivated *S. iniae* (formalin-killed cell, FKC) have been used in Chile, Israel, Russia, and Spain (Hastein, T. R. *et al*. 2005). Conventional formalin inactivation of live cell for the production of vaccine could lead to the denaturation of immunogenic epitopes, thus antibodies produced by the vaccine might not recognize the native antigen leading to low efficacy of the vaccine (Ferguson, M. *et al*. 1993). Comparing to inactivated vaccine, recombinant ghost bacterial vaccine (GBV) is safer, stable and easy to handle (Kwon, S. R. *et al*. 2005).

For the production of GBV, a vector harboring double cassettes (Fig. 1) with a heterologous gene expression cassette (pHCE-InaN-GAPDH) and a ghost 27 SDM cassette was constructed and inserted to *E. coli* K-12 as a host strain (Kwon, S. R. *et al*. 2005).

HCE promoter is a constitutive expression system, which facilitates the high-level expression of antigen proteins without induction. And InaN (N-terminus of ice nucleation protein gene from *Pseudomonas syringe* can display the expressed antigens on the surface of *Escherichia coli* (Li, L. *et al*. 2004).

The induction of ghost bacteria formation was carried out with expression of the PhiX174 lysis E gene under transcriptional control with the lambda PR/cI system (Szostak, M. P. *et al*. 1996; Madeleine, W. *et al*. 2000). The lysis E gene expression in *E. coli* with thermal induction of plasmid pλPR-cI-Elysis 27 SDM was carried out by increasing the incubation temperature from 27℃ to 42℃ (Witte, A. *et al*. 1992).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has multiple binding activities to various mammalian proteins such as fibronectin, lysozyme and cytoskeletal proteins, actin and myosin of the host cell (Madeleine, W. *et al*. 2000; Nakagawa, I. *et al*. 2003). GAPDH is one of the streptococcal antigens with a key role in cell adherence and attachment (Modun, B. *et al*. 1999).

E. coli K-12 used in this study is safe to use as probiotics. The *E. coli* K-12 strains do not cause risks to human, animal, plants, or other microorganisms. The *E. coli* K-12 has been utilized for 70 years for industrial purpose as probiotics with high volumes and high cell densities. Also, the ecological risks associated with the use of

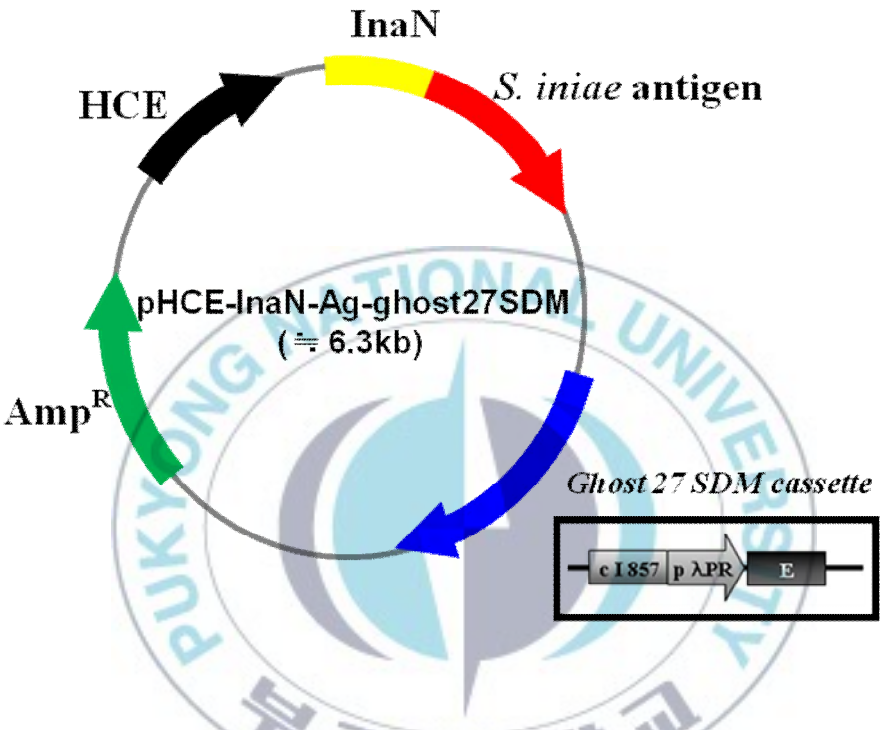

Fig. 1. Recombinant plasmid map used in this study.

E. coli K-12 are low [19]. Therefore, *E. coli* K-12 was used as a host cell for the GBV in this study.

The objectives of this study were to optimize the fed-batch fermentation process for the mass-production of the vaccine using *E. coli* K-12/pHCE-InaN-GAPDH-Ghost 27SDM and the efficient GBV production. Outer-membrane protein fractionation was carried out to evaluate the intactness of the expressed antigen protein on the cell surface after the formation of GBV. The protective efficacy of GBV was determined by the challenge test using live olive flounders (Shin, G. W. *et al*. 2007).

Ⅱ. MATERIALS AND METHODS

1. Bacteria strains and culture medium

The bacteria strain used in this study was recombinant *E. coli* K-12/pHCE-InaN-GAPDH ghost 27 SDM constructed (Fig 1). The recombinant *E. coli* K-12/pHCE-InaN-GAPDH ghost 27 SDM was cultured in modified Riesenberg medium (Riesenberg, D. *et al*. 1991). The medium was composed of: 13.5 g KH_2PO_4, 4.0 g $(NH_4)_2HPO_4$, 1.4 g $MgSO_4$-$7H_2O$, 1.7 g citric acid, 10 g glucose, 5 g yeast extract, 50 mg thiamine, and 10.0 mL trace metal solution per 1 L. Trace metal solution consisted of 10.0 g $FeSO_4 7H_2O$, 2.0 g $CaCl_2$, 2.2 g $ZnSO_4$-$7H_2O$, 0.5 g $MnSO_4$-$4H_2O$, 1.0 g $CuSO_4$-$5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}$-$4H_2O$, and 0.02 g $Na_2B_4O_7$-$10H_2O$ per 1 L of 5M HCl. The feeding solution during the fed-batch cultures consisted of 700 g glucose, 20 g $MgSO_4$-$7H_2O$ and 10.0 mL trace metal solution per L (Table 1).

Table 1. Medium composition

Components	Batch medium (per L)	Feeding solution for fed-batch fermentation (per L)
KH_2PO_4	13.5 g	-
$(NH_4)_2HPO_4$	4.0 g	-
$MgSO_4.7H_2O$	1.4 g	20 g
Citric acid	1.7 g	-
Yeast extract	5 g	-
Glucose	10 g	700 g
Thiamine	50 mg	-
Trace metal solution	10 ml (per L of 5M HCl)	10 ml
$(FeSO_4.7H_2O)$	(10.0 g)	(10.0 g)
$(CaCl_2)$	(2.0 g)	(2.0 g)
$(ZnSO_4.7H_2O)$	(2.2 g)	(2.2 g)
$(MnSO_4.4H_2O)$	(0.5 g)	(0.5 g)
$(CuSO_4.5H_2O)$	(1.0 g)	(1.0 g)
$((NH_4)_6M_{O7}O_{24}.4H_2O)$	(0.1 g)	(0.1 g)
$(Na_2B_4O_7.4H_2O)$	(0.02 g)	(0.02 g)

2. Culture conditions

For the incubation of seed culture, 10 ml LB medium including 50μg/mL ampicillin in 100 ml baffled flasks were inoculated with single colony of *E. coli* K-12 /pHCE-InaN-GAPDH ghost 27 SDM from the preservation slant. The seed culture was placed on a rotary shaker at 27°C, 200 rpm for 10 – 14h. The second seed culture with 125 ml medium in 1 L baffled flask was prepared with the inoculation of first seed culture (5% v/v) on a rotary shaker at 27°C for 8 h. For the preparation of 2.5 L batch medium, $(NH_4)_2HPO_4$, KH_2PO_4, citric acid and trace elements were dissolved in 2.3 L distilled water in the bioreactor. pH of the broth was adjusted to 6.3 with 5N NaOH and sterilized for 30 min at 121°C. Stock solutions of $MgSO_4$, yeast extract and glucose were sterilized for 30 min at 121°C, separately. Thiamine was sterilized by the filtration. After cooling sterilized broth, the pH of the broth was adjusted to 6.7 with aqueous NH_3 (28% w/w) prior to the inoculation of seed culture. The inoculation (5%, v/v) to the broth was carried out to start the fermentation.

3. Batch and fed-batch fermentation

Batch and fed-batch fermentations were carried out in 5 L Bioreactor (KF-5, KFC, Inchon, Korea) with 2.5 L working volume. Temperature, agitation rate and aeration rate during the culture were adjusted to 27°C, 300 rpm and 2.0 vvm.

The fed-batch fermentation process was carried out in 4 phases. The phase 1 the initial phase, was the batch fermentation phase. Phase 2 was a fed-batch fermentation phase with intermittent feeding strategy. The intermittent feeding was used for the fed-batch operation based on the measurement of glucose concentrations. When the exhaustion of glucose was detected, 25 ml of nutrient feeding solution was added, and the glucose concentration was controlled below 8 g/L. The dissolved oxygen concentration was maintained above 20% of saturation by adjusting the agitation rate, aeration rate and supplying pure oxygen when required. Phase 3 was induction phase by temperature increase to 42°C to induce lysis E gene for the ghost formation. The induction was carried out at stationary phase (OD_{600} = 100.5) in fed-batch operation by the increase of temperature from 27°C to 42°C. The optical density was decreased slowly due to the ghost formation. When the DO reached to 100%, it was presumed that most of the cell became ghost by pore formation with the action of protein E. Phase 4 was heat shock phase at 47°C for 2 h to increase the efficiency of GBV formation after the induction phase**.** To increase the efficiency of the GBV formation by the induction of lysis E gene at 42°C at phase 3, heat shock treatment was carried out in 5 L fermenter by increasing temperature to 47 and 49°C for 2 hours. The efficiency of GBV formation was determined by live cell count with CFU method.

4. Analytical methods

4.1. Measurement of cell growth

Cell growth was monitored by measuring the optical density of the 10 ml of culture samples at 600 nm (OD_{600}) using a spectrophotometer (Ultrospect 6300 pro, Biochrom Ltd., England) at the interval of 1 h. Cell concentration was also determined by measuring dry cell weight (DCW). A linear relationship between DCW and OD_{600} was obtained and 0.38 g dcw/L was equivalent to the absorbance of 1.0. After the expression of lysis E gene, culture samples were taken at the interval of 1 h and spread on LB agar plate containing 50μg/mL ampicillin to determine the efficiency of GBV formation. The plates were incubated in 27°C for 8-14 h and the efficiency of GBV formation was analyzed by colony forming unit (CFU) method. The glucose concentration was analyzed according to the 3,5-dinitrosalicylic acid (DNS) method (Dubois, M. *et al*. 1956).

4.2. Outer-membrane protein fractionation of GBV

Hundred mL of culture broth were centrifuged at 8000 rpm, 4°C for 6 min. Pellets

were suspended in 4 mL 20 mM tris-HCl (pH 8.6). The sonication of the suspended pellet was carried out for 1.5 min and then centrifuged at 8000 rpm, 4°C for 6 min. Supernatant was collected and recentrifuged at 15,000 rpm, 4°C for 1 h. Pellet containing total envelop protein was suspended in 4 mL of 20 mM tris-HCl buffer (pH 8.6, contained 1% Lauroyl sarcosyl). The pellet suspension was centrifuged at 15,000 rpm, 4°C for 1 h. Pellet was resuspended in 4 ml of 20 mM Tris-HCl buffer and used as the outer membrane protein fraction (pH 8.6). The outer-membrane fraction sample was used for analysis of SDS-PAGE and western blot.

4.3. Analysis of SDS-PAGE and Western blot

The outer-membrane protein fraction was subjected to two sets of SDS-PAGE. Outer-membrane proteins were separated in a 10% SDS-PAGE gel and the gel with the outer-membrane proteins was stained with Coomassie brilliant blue R-250. Outer-membrane proteins from SDS-PAGE gel were transferred onto a 0.45 μm pore nitrocellulose membrane (BioTrace, PALL, USA) at 100 V for 1 h in a Bio-Rad mini Trans-Blot electrophoretic transfer cell for western blot analysis. The blotted membrane was rinsed with TTBS (0.02 M Tris-HCl, 0.5 M NaCl, 0.05% Tween-20, pH 7.5) 3 times for 10 min and then blocked in TBS (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) containing 3% (w/v) BSA for 2 h at room temperature. Then, the membrane was washed with TTBS (Tween-Tris Buffered Saline: 20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 3 times for 10 min and then cut into several strips according to the sample lane. These strips were put into polyclonal rabbit antiserum

diluted 1 : 1000 in TTBS containing 1% BSA and then incubated for 2 h at room temperature. After 3 times washing for 15 min in TTBS, the membrane was treated for 2 h with alkaline phosphatase conjugated goat anti-rabbit IgG (1 : 1000 dilution, Santa Cruz Biotechnology, USA) in TTBS containing 1% BSA. Then, the membrane was washed 3 times for 10 min in TTBS and developed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma, USA) for 1 min. The developing was stopped by washing strips with distilled water.

5. In vivo vaccination test

5.1. Fishes for challenge test

Healthy olive flounders (*Paralichthys olivaceus*, 16-18g) were purchased from a commercial fish farm (Geoje-si, Gyeongsangnam-do, Korea) and acclimated in the aquarium for 2 weeks before using for the test. Fishes were maintained at 18-20°C in 200 L tank aquarium with changing the seawater twice a day. Fishes were fed by commercial dry pellets with feeding rate of 0.36 g/day.

5.2. Preparation of formalin-killed *S. iniae* (FKC)

S. iniae was grown for 24 h at 27℃ in tryptic soy broth (TSB, Sigma) containing 1.5% NaCl. For FKC preparation, formalin was added to a 24 h culture of the bacterium to the final concentration of 0.5%. After 24 h incubation, cells were washed

three times with phosphate buffered saline (PBS, pH 7.2) and resuspened in 10 ml PBS. The sus-pensions were streaked on tryptic soy agar containing 1.5% NaCl for checking sterility and stored at 4℃ until use.

5.3. Challenge tests

The group sizes for the vaccination were 20 fishes. Group 1 as positive control and 2 as negative control were injected with 50 μl PBS. Group 3 and 4 were injected with GBV prepared at 42°C and 47°C (heat treatment for the increase of GBV efficiency), respectively. Group 5 and 6 were injected with *E. coli* K-12 host strain as a host control and recombinant *E. coli* K-12/pHCE vector strain as a host with plasmid control. Group 7 was formalin killed *S. iniae* (FKC) vaccine as a comparison control as shown in Table 2. The treated GBV vaccines groups, the host control group, the vector control group and treated FKC vaccine group were injected concentration of 4 mg per 50 μl PBS. Challenge tests were performed at 2 weeks of post-immunization. *S. iniae* had been passaged in live olive flounder to enhance the virulence by intraperitoneal injection to *S. iniae*. The bacteria were reisolated from the kidney of moribund fishes at 3 days after the injection of live *S. iniae* and cultured on TSA plates supplemented with 1.0% NaCl at 27°C for 24 h. *S. iniae* was confirmed by colony PCR. Fishes in each group were challenged by the injection of 100 μl (1.88×10^7 cells/ml) of the bacterial suspension except negative control (Table 2). Mortalities were recorded daily for 14 days. Dead fishes were collected daily and necropsied. The

kidney samples were streaked on TSA containing 1.0% NaCl to confirm the presence of *S. iniae* by colony PCR.

Table 2. Setup for vaccination by injection

Group	Injection (mg/ml)	Challenge (1.88x107 cells/ml)	#Olive flounder
Positive control	50 ㎕ PBS	100 ㎕ *S. iniae*	20
Negative control	50 ㎕ PBS	100 ㎕ PBS	20
Antigen GAPDH ghost (42℃)	50 ㎕ ghost vaccine 42℃	100 ㎕ *S. iniae*	20
Antigen GAPDH ghost (47℃)	50 ㎕ ghost vaccine 47℃	100 ㎕ *S. iniae*	20
E. Coli K-12 host strain	50 ㎕ *E. Coli* K-12 host	100 ㎕ *S. iniae*	20
E. Coli K-12/pHCE vector control	50 ㎕ E. Coli K-12/pHCE	100 ㎕ *S. iniae*	20
Fomalin-killed cell (FKC)	50 ㎕ FKC	100 ㎕ *S. iniae*	20

Ⅲ. RESULTS AND DISCUSSION

1. Optimization of culture condition

1.1. Effect of carbon source

The selection carbon source for the growth of *E. coli* K-12 / pHCE-InaN-GAPDH-ghost 27 SDM was determined by carrying out the 1 L baffled flask. Various carbon sources were used to fructose, glucose, maltose, mannose and sucrose for carbon source optimization. As shown in Fig. 2, glucose as carbon source showed high cell mass production. Culture sample of used to glucose showed 2.6 g dcw/L and 0.7 g/g yield coefficient for *E. coli* K-12 / pHCE-InaN-GAPDH-ghost 27 SDM. Therefore, glucose was selected for high cell mass production.

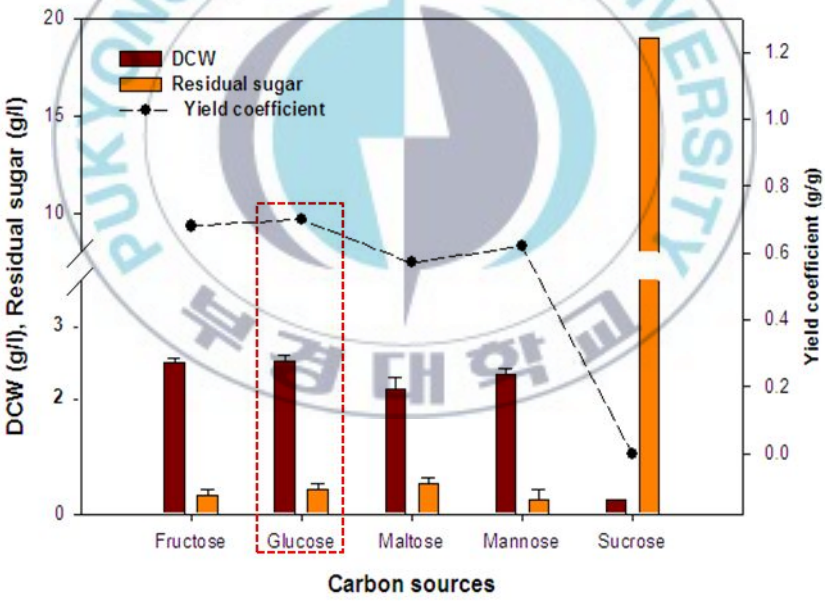

Fig. 2. Effect of carbon source on the dry cell weight, residual sugar and yield coefficient for *E. coli* K-12/*S. iniae* antigen GAPDH.

1.2. Optimization of culture medium in batch fermentation

Optimization of culture medium was carried out one-at-a-time analysis. *E. coli* K-12 / pHCE-InaN-GAPDH-ghost 27 SDM was cultured with 200 rpm, 27°C in 1L baffle flask (walking volume – 100mL). As shown in Table 3, the result of optimization of culture medium was determined as 10 g/L glucose, 5 g/L yeast extract, and 50 mg/L thiamine, as the composition modified from Riesenberg et al., 1990. Maximum cell growth was 5.14 g dcw/L in composition of 10 g/L glucose, 10 g/L yeast extract, and 50 mg/L thiamine. Therefore, final optimization of culture medium was selected Exp. No 1 as shown in Table 3.

Table 3. Effects of main factors of R medium on the cell growth by orthogonal array (n=3)

Exp. no.	Factors and leverls				Cultivation results			
	Yeast extract ($g\ l^{-1}$)	Glucose ($g\ l^{-1}$)	Thiamine ($mg\ l^{-1}$)	OD_{600}	Specific growth rate (hr^{-1})	Dry cell weight ($g\ l^{-1}$)	Residual sugar ($g\ l^{-1}$)	Yield coefficient ($g\ g^{-1}$)
1	5.0	10.0	50.0	13.0	0.298	4.94	0.32	0.508
2	5.0	20.0	100.0	12.9	0.299	4.90	0.30	0.248
3	5.0	30.0	150.0	10.2	0.289	3.88	0.33	0.130
4	10.0	10.0	50.0	13.5	0.289	5.13	0.31	0.527
5	10.0	20.0	100.0	12.2	0.284	4.64	0.21	0.233
6	10.0	30.0	150.0	12.7	0.287	4.83	0.25	0.161
7	15.0	10.0	50.0	12.8	0.278	4.86	0.30	0.498
8	15.0	20.0	100.0	12.2	0.270	4.64	0.44	0.235
9	15.0	30.0	150.0	12.3	0.273	4.67	0.28	0.156

1.3. Effect of agitation

Agitation provedes proper mixing of the fermentation broth and has a tremendous effect on the productivity of the system. Fig. 3 showed the effect of agitation speeds ranging from 100 to 400 rpm for the production of ghost bacteria by *E. coli* K-12 / pHCE-InaN-GAPDH-ghost 27 SDM. At 100 rpm, cell mass was comparatively low. This could be attributed to the dearth of oxygen being experienced by the organisms due to the insufficient mixing. The maximum production of cell mass was obtained at

the agitation speed of 300 rpm with the cell density of 3.9 g/L for *E. coli* / *S. iniae* antigen GAPDH.

Although the cell grew faster at higher agitation, the cell growth decreased at 400 rpm by the high shear, thus, the shear caused adverse effect to the cell mass formation. Hence, the agitation speed of 300 rpm was chosen as the optimal agitation speed.

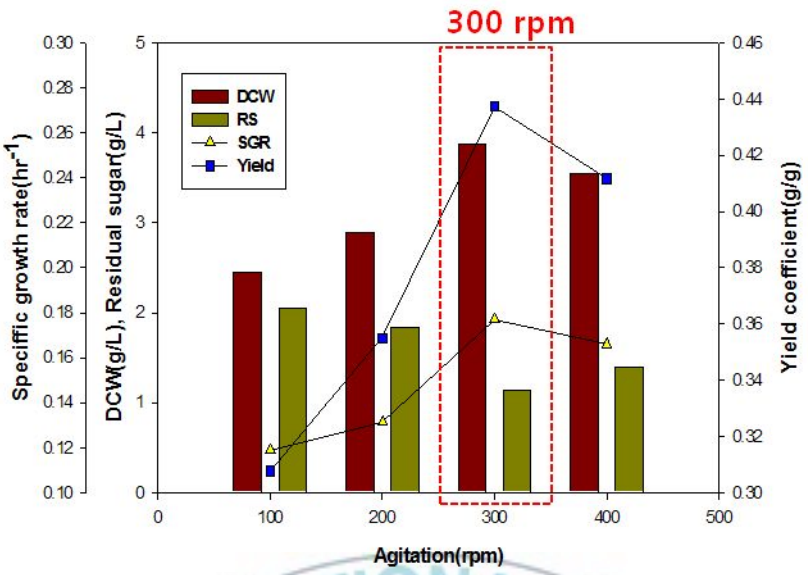

Fig. 3. Effect of the agitation in semi-defined medium with *E. coli* K-12/ pHCE InaN GAPDH-ghost27SDM.

1.4. Effect of aeration

Aeration provides mixing effect to the fermentation broth as well as oxygen transfer. During fermentation, the transfer of oxygen occurs from air bubble into the medium and then to the cell. Thus, the oxygen transfer from air bubble, through the liquid medium, to microbial cells is essential for the cell growth and the product formation.

As shown in Fig. 4, the effect of aeration rate on cell mass was examined with the different aeration rates of 1.0, 1.5, 2.0 vvm at the agitation speed of 300 rpm. At the aeration rate of 2.0 vvm, maximum production of cell mass was obtained with the cell density of 5.8 g/L for *E. coli* / *S. iniae* antigen GAPDH. Therefore, 2.0 vvm was chosen as the optimal aeration rate.

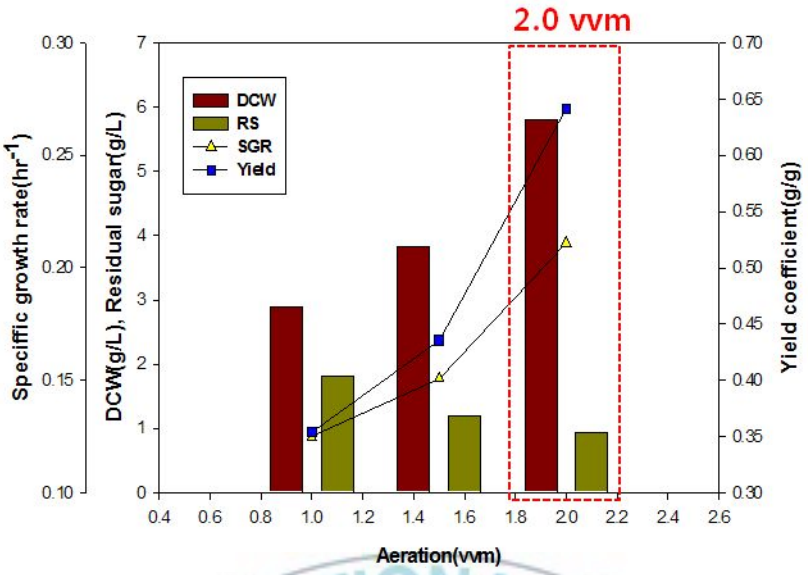

Fig. 4. Effect of the aeration in semi-defined medium with *E. coli* K-12/ pHCE-InaN-GAPDH-ghost27SDM.

2. Enhancement of efficiency of GBV formation

After the optimization of induction phase in batch fermentation, the efficiency of

GBV formation was evaluated. The efficiencies of GBV formation after the thermal induction at 42°C were determined by heat shock treatments at 42, 47 and 49°C. The increases of temperature after the induction at 42°C were performed to reduce live cell number. After the induction at 42°C for 10 h, the temperatures of the cultures were held at 42 or increased 47 and 49°C for 2 h. GBV formation efficiency of the holding temperature of 42°C was low with high cell survival ratio of 5.8%. However, cell survival ratios decreased to 0.14 and 0.12% at 47 °C and 49°C, respectively. There was no significant difference in the cell survival between 47°C and 49°C. Increase of temperature in mass production costs energy, therefore, the temperature holding at 47°C for 2 h was selected to increase the efficiency of GBV formation in high cell density culture by fed-batch fermentation as shown in Fig. 5.

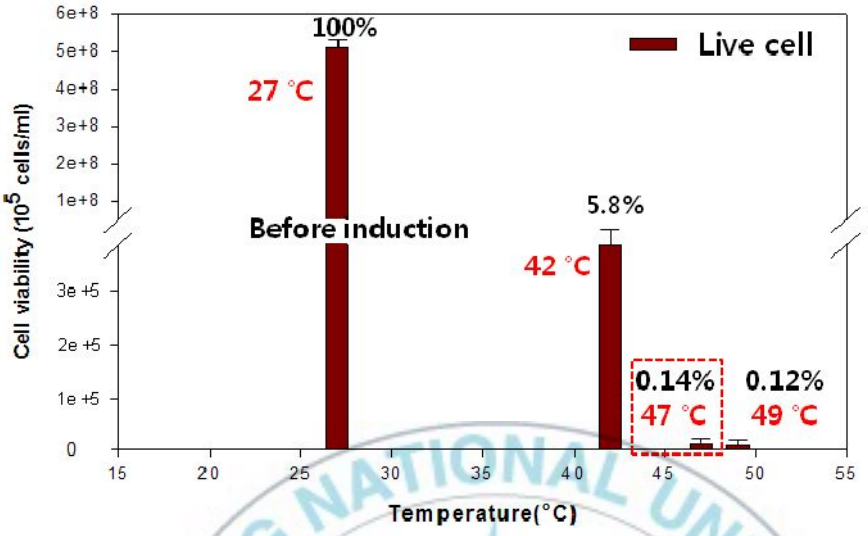

Fig. 5. Efficiency of ghost bacteria vaccine (GBV) formation in defined medium with *E. coli* K-12 / pHCE- InaN-GAPDH-ghost27SDM by heat shock treatments with 42°C, 47°C and 49°C.

3. Outer-membrane protein fractionation of GBV

GBV was produced by batch fermentation with 300 rpm, 2.0 vvm and culture medium containing 10 g/L glucose. The temperature was shifted to 42°C for the induction of lysis E gene at OD600 = 3.0 for GBV formation. At the end of the lysis process at 42°C, the temperature was increased to 47°C and 49°C and held at the temperatures for 2 h. The ghost bacteria were harvested. The outer-membrane protein fractionation from the ghost cell was carried out. The outer-membrane protein fraction was analyzed by SDS-PAGE and western blot. SDS-PAGE analysis showed that molecular weights of the outer-membrane protein fraction was 40KDa indicating the existence of GAPDH on the outer-membrane of ghost cell as shown Fig. 6(a). Lane 1 as *E. coli* K-12 host strain showed no GAPDH antigen protein band due to the lack of GAPDH plasmid. Lane 2, 3, 4 and 5 showed bands of GAPDH at 40KDa.

Western blot analysis showed that molecular weights of the outer-membrane protein fraction were 40KDa in lanes 2, 3, 4 and 5 as shown Fig. 6(b). Lane 1 did not showed band of GAPDH antigen protein as expected. Lane 2, 3, and 4 clearly showed bands of GAPDH. However, lane 5 showed thin band of GAPDH. This indicates that heat shock at 49°C decreased the amount of GAPDH protein on the surface of cell membrane. Heat shock at 47°C did not affect on amount of GAPDH protein on the surface of cell membrane. Therefore, 47°C of heat shock condition was selected as an optimal condition for the production of GBV with high ghost formation without losing surface antigen GAPDH on the cell membrane.

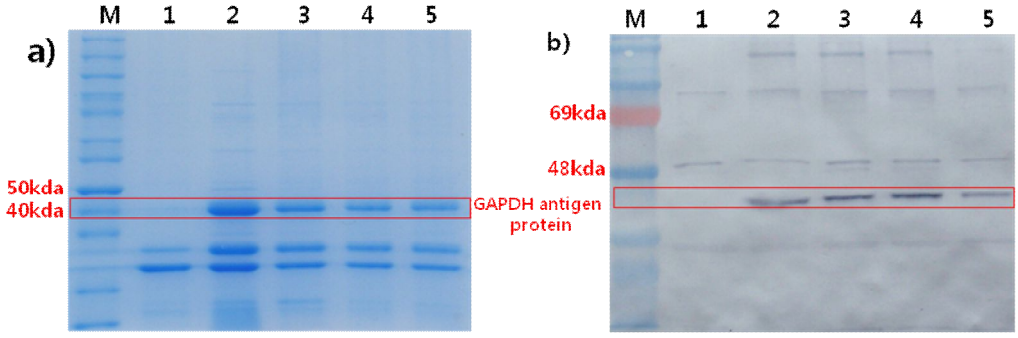

Fig. 6. Identification of the antigen GAPDH expression on the ghost cell membrane by SDS-PAGE(a) and western blot(b) through the fractionation of outer-membrane protein from *E. coli* k-12/pHCE-InaN-GAPDH-ghost27SDM.

M: protein maker, lane 1: fraction of outer-membrane proteins from *E. coli* K-12 host, lane 2: fraction of outer-membrane proteins before ghost induction (27°C), lane 3: fraction of outer-membrane proteins after the ghost induction (42°C), lane 4: fraction of outer-membrane proteins after heat shock treatment (47°C), lane 5: fraction of outer-membrane proteins after heat shock treatment at 49°C.

4. Optimization of fed-batch fermentation

The fed-batch fermentation was carried out in four phases with optimal conditions as shown in Fig. 7. Total fed-batch fermentation took 40 hours with 4 phases of fermentation. Phase 1 was batch fermentation during first 9 h of fermentation. As a typical batch fermentation, the dissolved oxygen (DO) value decreased to 20 % of saturation. The residual sugar concentration rapidly fell down after 3 h of fermentation due to the initiation of cell growth. The residual sugar concentration went down to 1.1 g/L after 9 h of fermentation. The cell density reached to 4.6 g dcw/L with OD_{600}=12.2. Next phase was fed-batch phase. The fed-batch fermentation phase with intermittent feeding strategy was carried out after phase 1. Duration of fed-batch fermentation was 19 h and feeding solution was fed to the fermenter at the average interval of 2 hours by monitoring DO and glucose concentration in the fermenter. The maximum cell density reached to 38.2 g dcw/L with OD600=100.5. The cell density of *E. coli* K-12/pHCE-InaN-GAPDH-ghost 27 SDM in this trial was higher than that of previously published result using *E. coli* XL1-Blue/pHCE-InaN-GAPDH-ghost 37 SDM (Ra, C. H. *et al*. 2010). The increase of cell density was declined at 14 h of fed-batch phase due to high cell density and reached to stationary phase at 19 h of fed-batch phase. Therefore, the fermentation phase was shifted to the induction phase to make ghost cell by the expression of lysis E gene. After the fed-batch fermentation for the high cell density culture, the thermal induction was carried out by the temperature increase to 42°C to induce lysis E gene for the formation of GBV during 10 h in phase 3. In this phase, the cell growth was stopped by the ghost cell formation by the expressed protein E. This indicated that pores on the cell

membrane were made by protein E and most of the cells became the ghost cell by the secretion of the cytoplasmic materials through the pores. High temperature holding at 47°C for 2 h in phase 4 was carried out to increase the efficiency of GBV formation to 99.9% in high cell density fermentation as shown in the last part of fermentation of Fig. 7. Final GBV cell density of 34.9 g dcw/L with OD_{600}=91.8 could be obtained in 4 phases of fed-batch fermentation. The produced GBV was dried by lyophilization and made to a powder.

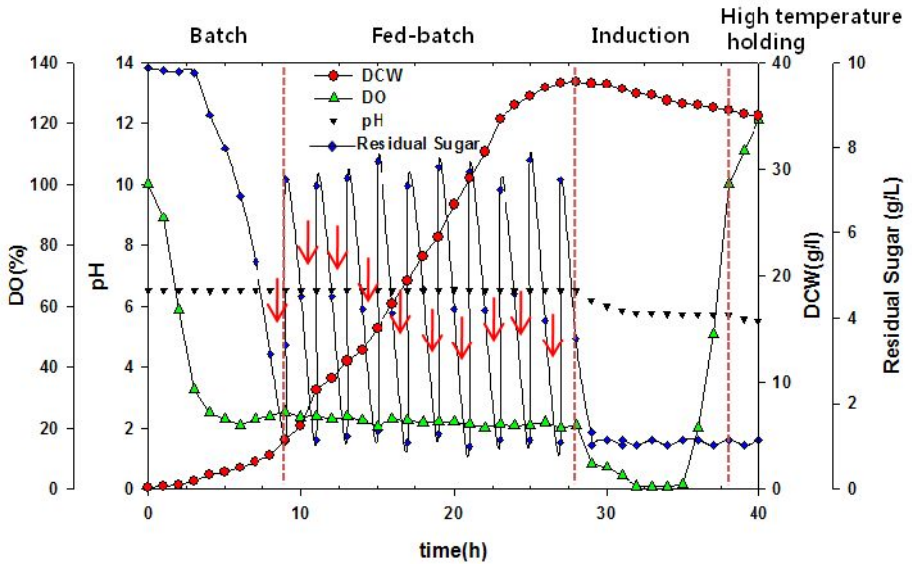

Fig. 7. High cell density production of GBV in defined medium with *E. coli* K-12/pHCE-InaN-GAPDH-ghost27SDM by fed-batch fermentation. The arrow indicate the time when nutrient feeding and pure oxygen were supplied to the bioreactor (↓feeding point)

5. Protective efficacy of ghost bacterial vaccine by challenge test

The efficacy of GBV was evaluated by challenge test with olive flounder for 14 days as shown in Fig. 8. Hundred μl (1.88×10^7 cells/ml) of live *S. iniae* were injected to the each groups of fishes except the negative control. The mortalities of each groups we monitored daily basis and the existence of *S. iniae* in dead fishes were confirmed by colony PCR. Usually, the death of fishes occurs after 2 days and 4 days of infection. In this challenge, the mortalities increased at day 2 and 4. The dead fishes showed dark pigmentation, abdominal extension, hemorrhagic ascites and necrosis of organs, particularly the liver.

Positive control without any treatment showed 100% of mortality at day 10. *E. coli* K-12 host control also showed 100% of mortality at day 13. *E. coli* K-12 with pHCE control showed 100% of mortality at day 12. All of the positive control groups showed 100% mortality in 10-13 days of challenge test. The group immunized with FKC vaccine showed low cumulative mortality comparing to the positive control groups. The final cumulative mortality of fishes vaccinated by FKC vaccine reached to 65%. Groups immunized with GBV vaccine produced at 42°C(GBV42) and GBV vaccine with heat shock at 47°C for 2 h (GBV47) showed low cumulative mortalities comparing to those of FKC vaccine as well as control groups. Both groups treated with GBV42 and GBV47 showed 50% of final cumulative mortalities. The challenge test indicates that GBV47 showed the efficacy of the vaccine similar to that of GBV42 even though the heat shock at 47°C for 2 h to increase the efficiency of ghost formation. The GBV with intact GAPDH antigen on the surface of the cell provided better immunization activity than FKC vaccine with denatured antigen on the cell

surface. This could be confirmed by the cumulative mortality measurement as shown in Fig. 8.

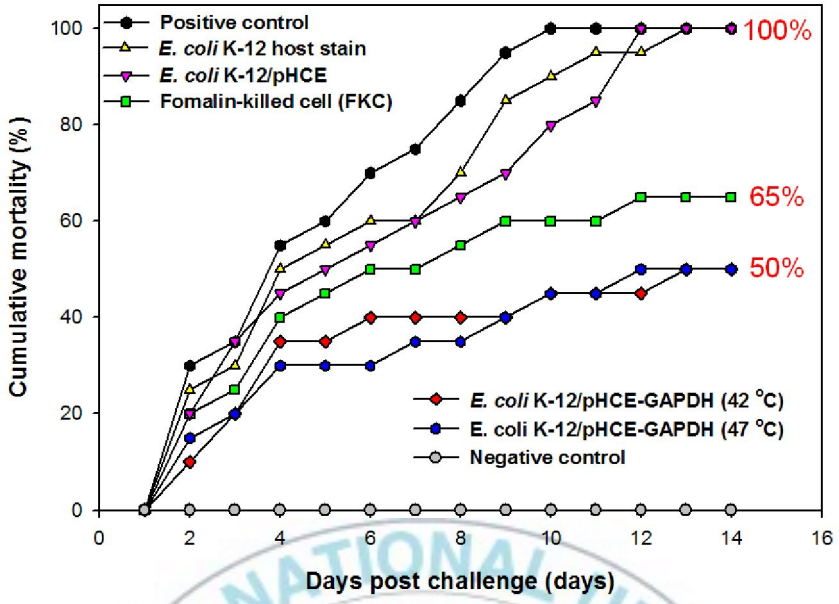

Fig. 8. Cumulative mortalities of olive flounders by challenge tests with live *S. iniae* after the immunization by ghost bacterial vaccines with the heat shocks of 42°C and 47°C, FKC vaccine, *E. coli* K-12 as a host strain, *E. coli* K-12/pHCE vector control, positive and negative controls.

Ⅳ. CONCLUSION

The high cell density culture of GBV reached 34.9 g dcw/L and GBV formation efficiency was 99.9% using heat shock treatment at 47°C for 2 h in fed-batch fermentation. The antigen, GAPDH expression on the outer membrane of ghost cell after heat shock phase was confirmed by outer-membrane protein fractionation analysis. There was no difference before and after the heat shock treatment at 47°C. The efficacy of ghost bacterial vaccine was evaluated by the challenge test with live *S. iniae* to immunized olive flounder. Fishes immunized with GBV showed higher potential to induce protective antibodies than FKC vaccine by the measurement of cumulative mortalities.

The present results especially regarding the optimization of condition parameters to produce GBV by fed-batch fermentation could provide a new opportunity to improve the production efficiency of ghost bacteria as a vaccine for streptococcal disease. In addition, mass production through optimized fed-batch fermentation process could provide the basis for the industrial production of GBV.

V. ACKNOWLEDGMENT

철없던 학부시절부터 아버지처럼 저를 이끌어 주시고 4년이란 긴 시간 동안 한결같이 저의 앞길에 불빛이 되어 주셨던 김성구 교수님께 진심으로 감사의 말씀을 올립니다. 몸이 아파 집에서 꼼짝할 수 없었을 때 집까지 손수 찾아오셔서 밥까지 챙겨주셨던 그 따뜻했던 마음 덕분에 제가 여기까지 올 수 있었던 것 같습니다. 또한 어머니와 같이 실험실 식구를 항상 걱정해주시며 보듬어주셨던 강향숙 사모님께도 감사의 맘을 전합니다.

또한 지금까지 많은 가르침과 도움을 주신 생물공학과의 김중균 교수님, 정귀택 교수님, 공인수 교수님, 이형호 교수님, 홍용기 교수님, 박남규 교수님께도 감사의 말씀을 드립니다.

비록 다른 실험실이었지만 4년 동안 제 옆에서 기쁠 때나 아플 때나 항상 함께 해주며 여기까지 올 수 있게 가장 큰 버팀목이 되어준 혜림이에게 진심으로 고마움과 감사함을 전합니다.

처음 실험실에 들어왔을 때 아무것도 모르던 저에게 실험을 처음 가르쳐 주었던 유미누나, 수정이, Hoai, 실험에 대한 모든 걸 늘 친절하게 가르쳐 주셨으며 졸업하고도 항상 이것 저것 챙겨주셨던 채훈선배, 지금까지 함께 실험하면서 친형처럼 서로 의지하며 큰 힘이 되어 준 석주형, 지금은 인천에서 열심히 일하고 있을 미란이, 그리고 힘든 일 굳은 일 마다 않고 도와준 지숙이, 비록 타 과에서 왔지만 열정 하나만큼은 남 못지 않았던 유경이, 몇 달 보진 못했지만 막내로 들어와 열심히 노력하던 혜진이, 항상 맏이와 같이 실험실을 잘 이끌어가기 위해 노력하던 멋진 수근선배, 그리고 학부과정에서 실험실에 들어와 선배들을 열심히 도와주었던 설희, 해리, 종천에게도 고마운 마음을 전합니다.

지금은 다른 길을 걷고 있지만 옆에서 힘이 되어주었던 승훈이 그리고 이젠 어엿한 트레이너로써 스스로의 삶을 찾아간 멋진 동생 성재, 함께 대학원에 들어와 고생하며 서로간에 힘이 되어주었던 우리 동기생 정수, 경은이, 유리, 아람이에게 고마움을 전합니다.

짧은 시간이었지만 실험에 대해 많은 조언과 충고를 아끼지 않았던 김영진

박사님과 김삼웅 박사님의 도움이 없었더라면 어쩌면 학위과정을 무사히 마치지 못했을 것입니다. 몸은 멀리 떨어져있지만 마음은 항상 옆에 있었던 나의 가장 친한 친구 기봉이, 항상 힘들 때마다 찾아와 술 한잔 사주며 웃어주었던 이제 태영이라고 불릴 기봉이에게 너무나 고맙고 미안한 마음을 전합니다. 연락도 자주 하지 못했지만 항상 친구라는 이름으로 큰 힘이 되어준 지혜와 정례, 보현누나 그리고 보혜에게도 감사의 마음을 전합니다.

저 대신 어머니를 모시며 가족을 이끌어갔던 착한 여동생 소란이, 못난 아들 덕에 늘 고생만했던 우리 어머니, 그리고 어디선가 지켜보고 있을 아버지에게 정말 미안하고 감사하며 고마운 맘을 전합니다. 지금까지 늘 사랑과 관심으로 믿음을 잃지 않았던 가족들의 지원이 아니었다면 제가 오늘 여기까지 올 수 없었을 것 입니다.

끝으로 지금까지 제가 대학원 생활을 하면서 힘이 되어주신 모든 분들께 감사드리며 대학원에서 보고 배운 것을 바탕으로 이 세상을 부끄럽지 않게 살아가겠습니다. 감사합니다.

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