Evaluation of Optimal Condition for Recombinant Bacterial Ghost Vaccine Production with Four Different Antigens of *Streptococcus iniae*-enolase, GAPDH, sagA, piaA

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A vector harboring double cassettes; a heterologous gene expression cassette of pHCE-InaN-antigen and a ghost formation cassette of pλPR-cI-E lysis 37 SDM was constructed and introduced to *E. coli* DH5α. For the production of a bacterial ghost vaccine, bacterial ghosts from *E. coli* / *Streptococcus iniae* with four different types of antigens - enolase, GAPDH, sagA and piaA - were produced by the optimization of fermentation parameters such as a glucose concentration of 1 g/l, agitation of 300 rpm and aeration of 1 vvm. Efficiency of ghost bacteria formation was evaluated with cultures of OD_{600}=1.0, 2.0 and 3.0. The efficiency of the ghost bacteria formation was 99.54, 99.67, 99.99 and 99.99% with inductions at OD_{600}=3.0, 1.0, 2.0 and 1.0 for *E. coli* / *S. iniae* antigens enolase, piaA, GAPDH and sagA, respectively. Ghost bacteria as a vaccine was harvested by centrifugation. The antigen protein expressions were analyzed by SDS-PAGE and western blot analysis, and the molecular weights of the enolase, piaA, GAPDH and sagA were 78, 26, 67 and 26 kDa, respectively. The molecular weights of the expressed antigens were consistent with theoretical sizes obtained from the amino acid sequences.

Key words : Ghost vaccine, enolase, GAPDH, sagA, piaA

Introduction

Streptococcus iniae is a hemolytic, Gram-positive coccus and a main causative agent of streptococcosis in wild and farmed fishes worldwide. The estimated annual impact of infection by *S. iniae* on the aquaculture industry reached 100 million US$ [19]. *S. iniae* has been reported to cause fulminant soft tissue infection in human [20]. The development of a vaccine for *S. iniae* is essential to reduce economic losses in the aquaculture industry and to protect people involved in aquacultural and fisheries activities. For the generation of bacterial ghost vaccine, a vector harboring double cassettes, a heterologous gene pHCE-InaN-antigen and a ghost formation cassette pλPR-cI-E lysis 37 SDM was constructed (Fig. 1).

pHCE is a constitutive expression system, which facilitates the high-level expression of antigen proteins without the induction by chemical inducers like isopropyl-β-D-thiogalactopyranoside (IPTG). InaN (N-terminus of the ice nucleation protein gene from *Pseudomonas syringe*) sequence is capable of displaying antigens on the membrane surface of *Escherichia coli* [8]. The mechanism of *S. iniae* infections is not confirmed, however antigenic proteins related with host cell infection and virulence have been reported in previous studies [3,19]. Streptococcal extracellular proteins (ECPs) have been reported as cell cytosolic and cell wall proteins associated with virulence and immunity to the host. Glyceraldehyde-3-phosphate dehydrogeenase (ed in)been repo and streptolysin S (revi) are the ECPs [9,13,12]. Also, Iron uptake ABC transporter (piaA) lipoprotein have been reported to be probably attached to the outer surface of the cell membrane and induces the protection against many different Streptococcus serotypes [11]. Therefore, GAPDH, enolase, sagA and piaA (iron uptake transporter) in this study were evaluated as antigen candidates and detail functions of these antigens are summarized in Table 1.

The formation of ghost bacteria was carried out with expression of the PhiX174 lysis E gene under transcriptional control with the lambda PR/cI system [15]. The lysis E gene expression in *E. coli* with plasmid pλPR-cI-E lysis 37 SDM was carried out by increasing the temperature from 37°C to 42°C. The lysis process of bacteria for the ghost bacteria formation occurred within 1 hr after the temperature increase and the lysis process was completed after 2 hr of post-induction [5].

Therefore, four-different clones of *E. coli* DH5α /4 different antigens were constructed; *E. coli* DH5α / pHCE-InaN-

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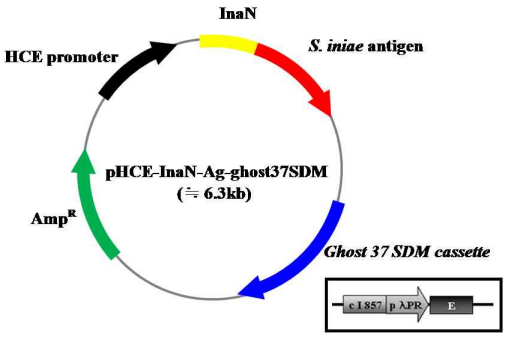

Fig. 1. Recombinant plasmid map of pHCE-InaN-antigen-ghost 37 SDM [5].

enolase-ghost 37 SDM, *E. coli* DH5α /pHCE-InaN-GAPDH-ghost 37 SDM, *E. coli* DH5α /pHCE-InaN-piaA-ghost 37 SDM and *E. coli* DH5α / pHCE-InaN-sagA-ghost 37 SDM. The objective of this study was to determine the optimum fermentation condition that would yield the efficient bacterial ghost formation for industrial application using *E. coli* / four different types of *S. iniae* antigens.

Materials and Methods

Bacterial strains and culture conditions

E. coli DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM cells were grown in LB broth containing 50 μg/ml ampicillin at 37°C. The seed culture was prepared in LB broth containing 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l sodium chloride.

Single colony of *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM was inoculated into 30 ml of medium in an 100 ml Erlenmeyer flask. The seed culture was incubated for 12 hr at 37°C, 200 rpm on a rotary shaker. The subculture was carried out using 150 ml of fresh medium in a 500 ml baffled Erlenmeyer flask with 5% inoculum size, at 37°C and 200 rpm on a rotary shaker for 6 hr. Batch cultures were carried out in a 5-l jar fermenter (Model KF-5, Korea Fermenter Company Ltd., Inchon, Korea) containing 3 l of initial medium. Initial temperature, agitation speed and aeration rate during the fermentation were set at 37°C, 300 rpm and 1.0 vvm, respectively.

Analytical methods

The growth of the ghost bacteria was determined by dry cell weight (g · dcw/l) and optical density (OD at 600 nm) methods [19]. The OD value was converted into cell mass concentration (g/l) using a standard curve. A linear relationship between DCW and OD_{600} was obtained and 0.38 g dcw/l was equivalent to the absorbance of 1.0 at 600 nm. The concentration of glucose was analyzed according to the modified procedure of the phenol-sulfuric acid method [1]. The expression of antigens was confirmed by SDS-PAGE and western blot. The samples were prepared by boiling of washed cells for 5 min in sample buffer. Proteins from the equal amounts of cells measured by optical densities, were loaded on the gel. The part of gel was transferred onto a 0.45 μm pore nitrocellulose membrane (BioTrace, PALL, USA) at 100 V for 1 hr in a Bio-Rad mini Trans-Blot electrophoretic transfer cell for western blot analysis [6,7].

Induction of heterologous protein expression

When the batch cultures were reached to OD of initial exponential phase (OD_{600}=1.0), mid exponential phase (OD_{600}=2.0) and late exponential phase (OD_{600}=3.0), the expression of lysis E gene was induced by the shift of temperature from 37°C to 42°C. The optical density was measured until no further decrease. After the expression of the lysis E gene, 10 μl of fermentation samples were taken at 1 hr interval and spread onto LB agar plate containing 50 μg/ml

Table 1. Characterizations of *Streptococcus iniae* antigen candidates

Name	Characteristics	Ref.
Enolase (78 kDa)	- Essential enzyme of glycolysis pathway - Bound to host proteins such as plasmin, actin, fibronectin and myosin - Virulence of path	[13]
GAPDH (67 kDa)	- Supress a primary immune response against T-cell dependent Ag - One of the important glycolytic enzymes, found in the cytoplasm - Virulence of pathogenic streptococci	[9]
Pia A (26 kDa)	- Iron uptake ABC transporter - Supporting the transport of Fe ions or compounds to bacteria for cell growth	[11]
SagA (26 kDa)	- The structural gene for Streptolysin S - Damage to host cell membrane	[12]

ampicillin for the determination of the efficiency of ghost bacteria formation. The plates were incubated in 37°C for 12 hr and the efficiency of bacterial ghost formation was analyzed by colony counting method [5].

Results and Discussion

Effect of glucose concentration

The effect of various glucose concentration on the growth by *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM cells was carried out using the 5-l fermenter. The glucose concentrations were adjusted to 0, 1, 2 and 3 g/l for carbon source optimization. As showmizatFzg. 2, 1 g/l of glucose showed the hzghest cell mass production.tFurther increase of glucose concentration decreased the cell growth due to inhzbitory effect of substrate [4]. The hzghest cell mass in the cultures were 1.84, 1.63, 1.51 and 1.54 g/l for *E. coli* / *S. iniae* with enolase, GAPDH, sagA and piaA, respectively. Also, the increase of glucose concentration decreased pH of broth from 6.8 to 4.9 during exponential phase. This might be due to the accumulation of organic acids produced from excessive glucose in the broth [16].

Effect of agitation

Agitation provides proper mixing of the fermentation broth and has a significant effect on the productivity of the fermentation system. Fig. 3 showed the effects of agitation speeds ranging from 100 to 400 rpm for the production of ghost bacteria using *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM cells. Cell density was low due to the low concentration of dissolved oxygen by

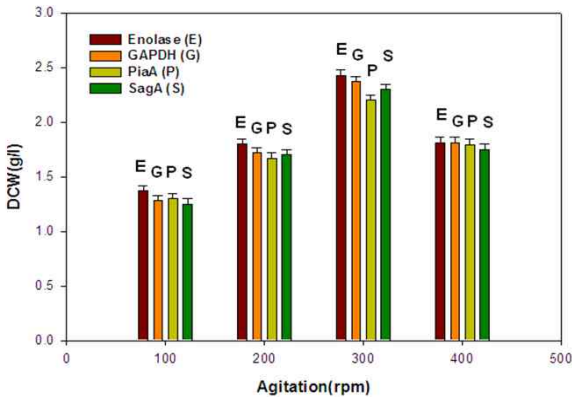

Fig. 3. Effect of agitation speed on the growth of *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM using 5-l Fermenter.

the insufficient mixing at 100 rpm. The maximum production of cell mass was obtained at the agitation speed of 300 rpm with the cell densities of 2.58, 2.37, 2.2 and 2.1 g/l for *E. coli* / *S. iniae* antigens enolase, GAPDH, sagA and piaA, respectively. Further increase in agitation speed adversely affected the cell density as well as specific growth rate. Therefore, the agitation speed of 300 rpm was chosen as the optimal agitation speed.

Effect of aeration

Aeration provides dissolved oxygen transfer as well as mixing effect to the fermentation system. During the fermentation, the transfer of oxygen occurs from air bubble into the medium and then to the cells. Thus, the oxygen transfer from air bubble to microbm ancells through the liquid medium is essenum anfor cell growth and productnformatiomatis shown in Fig. 4, the effect of aeration rate on cell density was evaghated with the aeration rates of 0.5, 1.0, and

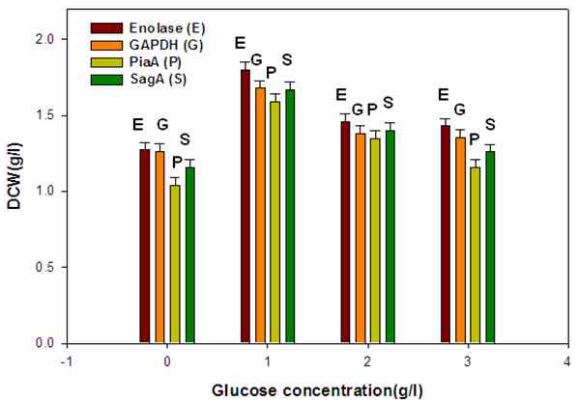

Fig. 2. Effect of glucose concentration on the growth of *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM using 5-l Fermenter.

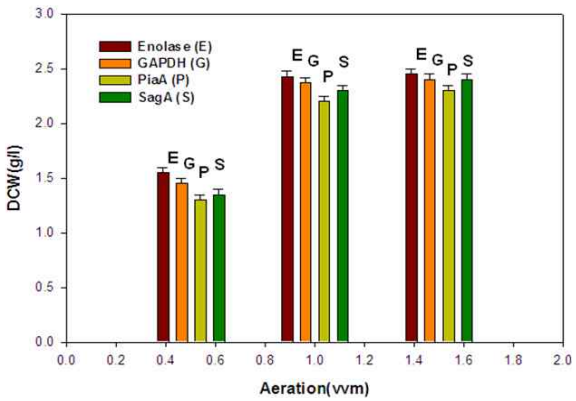

Fig. 4. Effect of aeration on the growth of *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM using 5-l Fermenter.

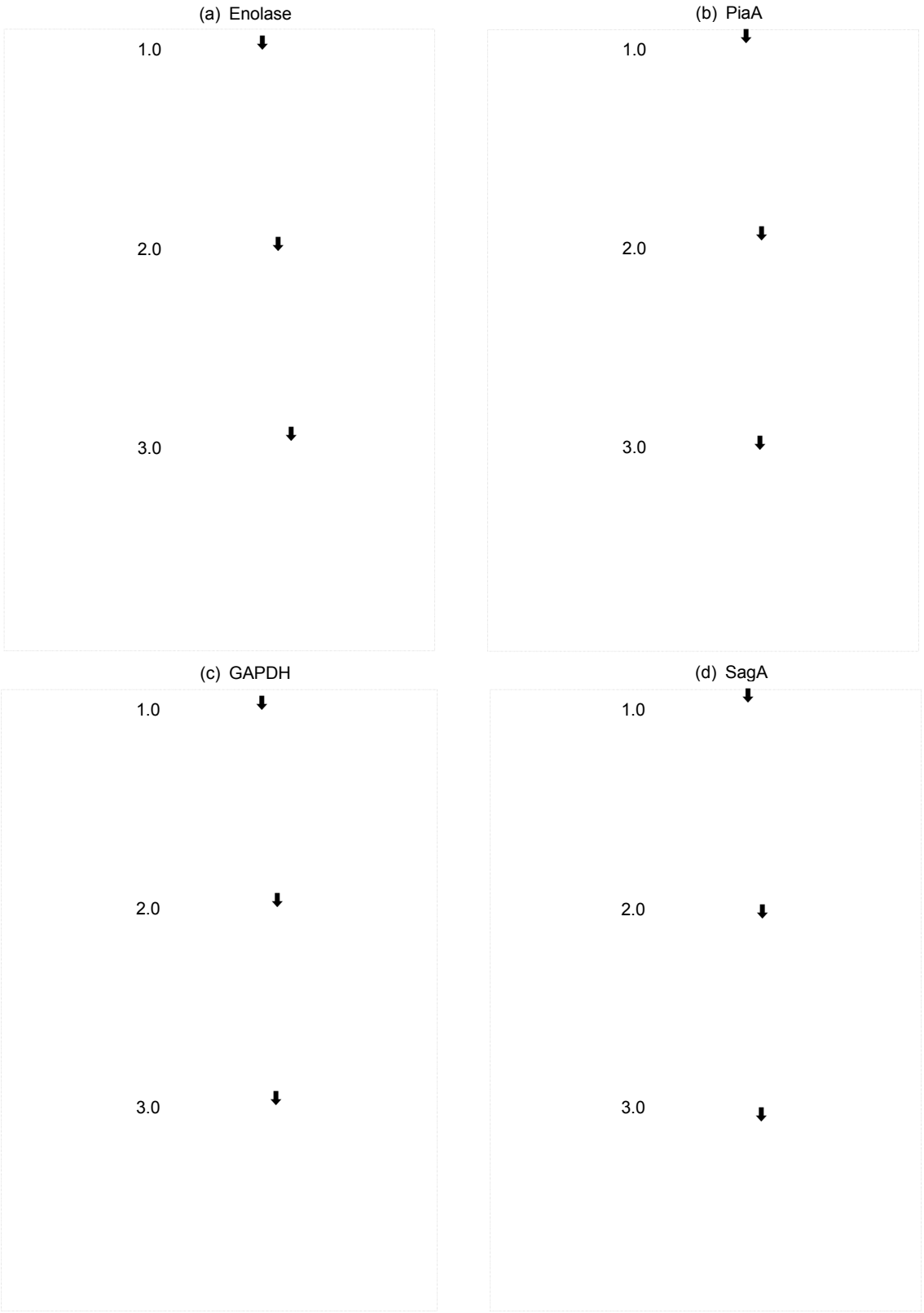

Fig. 5. Efficiency of the ghost bacteria formation efficiency and growth of *E. coli* DH5α / pHCE-InaN-antigens-ghost 37 SDM by temperature induction of lysis E gene expression. (a) enolase, (b) piaA, (c) GAPDH, (d) sagA. Temperature induction point: early log phase (OD_{600}=1.0), mid log phase (OD_{600}=2.0), late log phase (OD_{600}=3.0), The arrow indicates temperature shifted from 37°C to 42°C.

Table 2. Summary of ghost bacteria formation efficiency

Antigen candidates	Ghost bacteria formation efficiency (%)/Ghost bacteria vaccine (g/l)		
	Early log phase induction at (OD=1.0)	Mid log phase induction at (OD=2.0)	Late log phase induction at (OD=3.0)
Enolase (78 kDa)	*99.99/0.39 (±0.02)	*99.92/0.78 (±0.02)	*99.54/1.14 (±0.02)
GAPDH (67 kDa)	*99.99/0.37 (±0.02)	*99.67/0.77 (±0.02)	98.71/1.16 (±0.02)
PiaA (26 kDa)	*99.99/0.38 (±0.02)	97.72/0.79 (±0.02)	97.13/1.17 (±0.02)
SagA (26 kDa)	*99.99/0.37 (±0.02)	95.33/0.83 (±0.02)	94.95/1.28 (±0.02)

*Acceptable efficiency of ghost bacteria formation. >99%.

1.5 vvm at the agitation speed of 300 rpmatit the aeration rates of both 1.0 vvm and 1.5 vvm, maximum productions of cell densities were obtained with 2.58, 2.37, 2.2 and 2.1 g/l for *E. coli* / *S. iniae* antigens enolase, GAPDH, sagA and piaA, respectively. One and 1.5 vvm of aeration rates showed similar growth trend. Therefore, 1 vvm was chosen as the optimal aeration rate.

Efficiency of bacterial ghost formation

As shown in Fig. 5, the induction efficiency of lysis E gene expression was evaluated as the efficiency of bacterial ghost formation in three exponential phases, initial exponential phase (OD_{600}=1.0), mid exponential phase (OD_{600}=2.0), and late exponential phase (OD_{600}=3.0). The ghost bacteria formation was occurred at 1 hr after temperature shift to 42°C and lysis process was completed at 3 hr after induction. The lysis E kinetics was reported by the methods described in previous report [5].

The efficiencies of the ghost bacteria formations with *E. coli* DH5α / pHCE-InaN-enolase-ghost 37 SDM cells were 99.99, 99.92 and 99.54% with the inductions at OD_{600}=1.0, 2.0 and 3.0, respectively (Fig. 5(a)). The efficiencies of ghost bacteria formations were high regardless of induction phase. Efficiencies of the ghost bacteria formation with *E. coli* DH5α / pHCE-InaN-piaA-ghost 37 SDM cells were 99.99, 97.72 and 97.13% with the inductions at OD_{600}=1.0, 2.0 and 3.0, respectively (Fig. 5(b)). The efficiency of ghost bacteria formation was acceptable only at initial exponential phase (OD_{600}=1.0).

Efficiencies of the ghost bacteria formations with *E. coli* DH5α / pHCE-InaN-GAPDH-ghost 37 SDM cells were 99.99, 99.67 and 98.71% with the inductions at OD_{600}=1.0, 2.0 and 3.0, respectively (Fig. 5(c)). The acceptable efficiencies of bacterial ghost formation were obtained at initial and mid exponential phase (OD_{600}=1.0, 2.0). Efficiencies of the ghost bacteria formations with *E. coli* DH5α / pHCE-InaN- sagA-ghost 37 SDM cells were 99.99, 95.33 and 94.95% with the inductions at OD_{600}=1.0, 2.0 and 3.0, respectively (Fig. 5(d)). The efficiency of bacterial ghost formation was acceptable at initial exponential phase (OD_{600}=1.0).

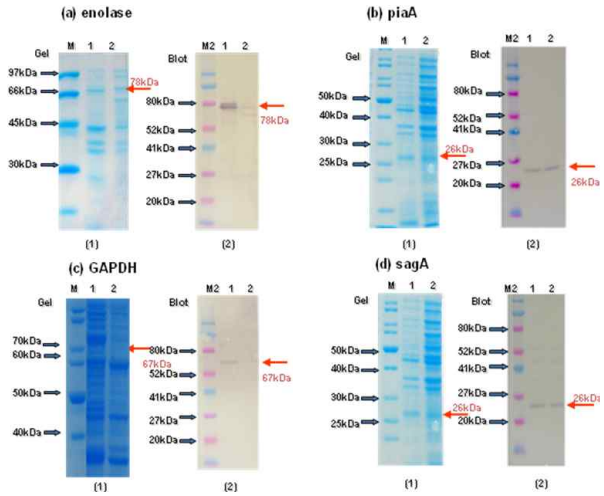

Fig. 6. Analysis of antigen expressions by SDS-PAGE (1) and western blot (2) with enolase (a), piaA (b), GAPDH (c) and sagA (d). M1: Protein ladder marker, lane 1: Insoluble fraction, lane 2: Soluble fraction, M2: ProSieve-Color Protein Markers (Cambrex, Rockland, USA).

The lysis E kinetics in *E. coli* / *S. iniae* antigens piaA and sagA were weaker than those of *E. coli* / *S. iniae* antigens enolase and GAPDH (Fig. 5). The efficiency of ghost bacteria formation was summarized Table 2. This result indicates that the action of protein E which makes holes was related to the cell divisions [2] and the low efficiency of ghost bacteria formation might be caused by either reduced susceptibility of the *E. coli* DH5α cell wall to the non-enzymatic E protein or reduced power of the λP_R promoter in *E. coli* DH5α.

Three hours after the induction at 42°C, ghost bacteria was harvested. The antigen expression was analyzed by SDS-PAGE and western blot. The molecular weights of the 78 kDa of enolase, 26 kDa of piaA, 67 kDa of GAPDH and 26 kDa of sagA bands could be detected (Fig. 6). The molec-

ular weights of those proteins were consistent with theoretical sizes obtained from the amino acid sequences.

In conclusion, production of ghost bacteria as vaccine from *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM cells was optimized by controlling the fermentation parameters as glucose concentration of 1 g/l, agitation speed of 300 rpm, aeration of 1 vvm. The highest ghost bacteria production was obtained at OD_{600}=3.0, 1.0, 2.0 and 1.0 with the concentration of 1.14, 0.38, 0.77 and 0.37 g/l for *E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM cells, respectively.

The optimization of fermentation parameters could provide a new opportunity to improve the production efficiency of ghost bacteria as a vaccine against Streptococcal disease.

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References

1. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimeteric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350-356.
2. Eko, F. O., A. Witte, V. Huter, B. Kuen, and S. Furst-Ladani. 1999. New strategies for combination vaccines based on the extended recombinant bacterial ghost system. *Vaccine* **17**, 1643-1649.
3. Georgiou, G., C. Stathopoulos, P. S. Daugherty, A. R. Nayak B. L. Iverson, and R. Curtiss. 1997. Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines. *Nat. Biotechnol.* **15,** 29-34.
4. Haslberger, A. G., G. Kohl, D. Felnerova, U. B. Mayr, S. Furst-Ladani, and W. Lubitz. 2000. Activation, stimulation and uptake of bacterial ghosts in antigen presenting cells. *J. Biotechnol.* **83,** 57-66.
5. Kwon, S. R., Y. K. Nam, S. K. Kim, D. S. Kim, and K. H. Kim. 2005. Generation of *Edwardsiella tarda* ghosts by bacteriophage PhiX174 lysis gene E. *Aquaculture* **250,** 16-21.
6. Kim, M. S., S. H. Choi, E. H. Lee, Y. K. Nam, S. K. Kim, and K. H. Kim. 2007. α-Enolase, a plasmin(ogen) binding and cell wall associating protein from a fish pathogenic *Streptococcus iniae* strain. *Aquaculture* **265**, 55-60.
7. Korz, D. J., U. Rinas, K. Hellmuth, E. A. Sanders, and W. D. Deckwer. 1995. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J. Biotechnol.* **59**, 59-65.
8. Lin, Li, D. G. Kang, and H. J. Cha. 2004. Functional display of foreign protein on surface of *Escherichia coli* using N-terminal domain of ice nucleation protein. *Biotechnol. Bioprocess Eng.* **85,** 214-221.
9. Modun, B. and P. Wiliams. 1999. The staphylococcal transferring-binding protein is a cell wall glyceraldehydes-3-phosphate dehydrogenase. *Infect Immun.* **67**, 1086-1092.
10. Marchart, J., G. Dropmann, S. Lechleitner, T. Schlapp, G. Wanner, M. P. Szostak, and W. Lubitz. 2003. *Pasteurella multocida* and *pasteurella haenolytica* ghosts: new vaccine candidates. *Vaccine* **21,** 3988-3997.
11. Nikaido, H. and J. A. Hall. 1998. Overview of bacterial ABC transporters. *Methods Enzymol.* **292,** 3-20.
12. Nizet, V., B. Beall, D. J. Bast, V. Datta, L. Kilburn, D. E. Low, and J. C. DeAzavedo. 2000. Genetic locus for streptolysin S production by group A streptococcus. *Infect Immun.* **68,** 4245-4254.
13. Pancholi, V. and V. A. Fischetti. 1998. Alpha-enolase, a novel strong plasmin (ogen) binding protein on the surface of pathogenic streptococci. *J. Biol. Chem.* **273,** 14503-14515.
14. Ronchel, M. C., A. Molina, W. Witte, S. Lubitz, J. L. Molin, and C. Ramos. 1998. Characterization of cell lysis in *Pseudomonas putida* induced upon expression of heterologous killing genes. *Appl. Environ. Microbiol.* **64,** 4904-4911.
15. Szostak, M. P., Hensel, A., Eko, F. O., Klein, T., Mader, and H., Haselberger. 1996. Bacterial ghosts: non-living candidate vaccine. *J. Biotechnol.* **44,** 161-170.
16. Shiloach, J. and R. Fass. 2005. Growing *E. coli* to high cell density- a historical perspective on method development. *Biotechnol. Adv.* **23,** 345-357.
17. Sutcliffe, I. C. and R. R. Russell. 1995. Lipoproteins of gram-positive bacteria. *J. Bacteriol.* **177,** 1123-1128.
18. Swiatlo, E. and D. Ware. 2003. Novel vaccine strategies with protein antigens of *Streptococcus pneumoniae*. *FEMS Immunol. Med. Microbiol.* **38,** 1-7.
19. Thune, R. L., L. A. Stanley, and R. K. Cooper. 1993. Pathogenesis of Gram negative bacterial infections in warm fish. *Annu. Rev. Fish Dis.* **3,** 37-68.
20. Weinstein, M. R., D. E. Low, Mc A. Geer, B. Willey, D. Rose, M. Coulter, P. Wyper, A. Borczyk, and M. Lovgren. 1996. Invasive infection due to *Streptococcus iniae*: a new or previously unrecognized disease. *Can. Commun. Dis. Rep.* **22,** 129-132.
21. Witte, A., G. Wanner, and W. Lubitz. 1992. Dynamics of PhiX174 protein E mediated lysis of *Escherichia coli*. *Arch. Microbiol.* **157,** 381-388.

초록 : 연쇄구균증 항원- enolase, GAPDH, sagA, piaA 에 대한 재조합 고스트 박테리아 백신의 생산 최적화

라채훈 · 김영진 · 손창우[1] · 정대영[1] · 김성구*
(부경대학교 생물공학과, [1](주) KB solutions)

본 연구는 5-L 발효기를 이용하여 재조합 고스트 박테리아(*E. coli* DH5α / pHCE-InaN-(enolase, GAPDH, sagA or piaA)-ghost 37 SDM) 백신의 산업화를 위해 탄소원 공급조건, 교반속도, 산소공급 조건 등의 최적 배양조건과 고스트 박테리아 발현 유도를 위한 온도조절 시점과 그에 따른 발현효율 최적화를 조사하기 위해 수행하였다. 각각 다른 4종의 항원 유전자를 보유한 고스트 박테리아를 LB 배지를 이용하여 배양한 결과 모두 1 g/l glucose, 300 rpm, 1 vvm에서 최대 균주 성장을 나타내었다. 고스트 박테리아 생성 효율의 경우 초기 대수증식기(OD_{600}=1.0)에서 고스트 발현을 유도했을 때 각각 최대효율인 99.99%를 나타내었으나 중기 대수증식기(OD_{600}=2.0)와 말기 대수증식기(OD_{600}=3.0)에서는 고스트 박테리아 생성이 낮은 효율을 나타내었다. 또한 SDS-PAGE 와 western blot를 이용하여 각각 다른 4종의 항원 단백질 발현 여부를 확인한 결과 enolase (78 kDa), GAPDH (67 kDa), sagA (26 kDa), piaA (26 kDa) 에서 항원 단백질 band를 확인할 수 있었다. 따라서 본 연구결과 확립된 배양 조건과 발현효율 최적화 조건은 연쇄구균증 질병에 대해 E. coli를 이용한 고스트 박테리아 백신이 양식 산업에 있어 상업적으로 유용한 백신의 최적생산을 위해 사용 될 수 있을 것으로 사료된다.