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Evaluation of Optimal Culture Conditions for Recombinant Ghost Bacteria Vaccine Production with the Antigen of *Streptococcus iniae* GAPDH

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For the production of ghost bacteria vaccine to prevent the streptococcal disease in aquaculture fish species, a double cassettes vector was constructed and cloned in *Escherichia coli* DH5α. Ghost bacteria vaccine production from *Escherichia coli* DH5α/pHCE-InaN-GAPDH–Ghost 37 SDM (SIG) was maximized at a glucose concentration of 1 g/l, agitation of 300 rpm, and aeration of 1 vvm. The maximal efficiency of ghost bacteria formation was obtained at the mid-exponential phase (OD_{600}=2.0) with the concentration of 0.77 g/l for SIG. The molecular mass of GAPDH was detected at 67 kDa with the insoluble fraction, by SDS–PAGE and Western blot. The protective efficacy of ghost bacteria vaccine was evaluated by challenge test using olive flounder. The cumulative mortalities of the positive control, formalin-killed cell (FKC) vaccine, and SIG vaccine immunized groups were 91%, 74%, and 57%, respectively. These results suggest that SIG vaccine showed efficacy as a vaccine and had a higher potential to induce protective antibodies than did FKC vaccine.

Keywords: Ghost bacteria vaccine, *S. iniae*, GAPDH, challenge test

Streptococcus iniae (*S. iniae*) is a hemolytic, Gram-positive pathogen in wild and cultured fish species worldwide. The estimated annual impact of infection by *S. iniae* on the aquaculture industry reached US$100 million globally [1]. *S. iniae* has also been reported to cause the fulminant soft tissue infection in human [20, 21]. Therefore, the development of a vaccine against *S. iniae* is essential to reduce economic losses in the aquaculture industry and to protect people involved in the aquaculture facilities and raw fish restaurant industries. The control of streptococcal diseases using synthetic chemicals and antibiotics has caused problems including the emergence of antibiotic-resistant microbes, consumer's concern over drug residues, and environmental impacts.

Thus, recombinant ghost bacteria have been given increasing attention as a new approach for a vaccine delivery vehicle. For the production of ghost bacteria vaccine, a plasmid harboring double cassettes with pHCE-InaN-GAPDH and a ghost 37 SDM cassette was constructed and cloned in *E. coli* DH5α (Fig. 1). A constitutive expression system with HCE promoter facilitates high-level expression of antigen proteins without induction by a chemical inducer like IPTG. The signal sequence of InaN (N-terminus of the ice nucleation protein gene from *Pseudomonas syringe*) was capable of displaying bacterial antigens on the surface of *Escherichia coli* [11, 19]. 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 [3, 16, 17, 22]. The lysis E gene expression in *E. coli* with plasmid pλPR-cI-lysis E 37 SDM was carried out with temperature increase from 37°C to 42°C. The lysis of *E. coli* for the ghost bacteria formation occurred within 1 h after temperature increase, and the lysis process was completed after 2 h post-induction [6, 8].

GAPDH of bacteria was reported as multiple binding sites to various mammalian proteins, such as fibronectin, lysozyme, and cytoskeletal proteins, myosin and actin, of the host cells [2, 3, 13, 15]. These properties suggest that GAPDH may play a key role in adherence and attachment to the host cell. Therefore, a ghost bacterial strain of *E. coli* DH5 containing surface-expressed GAPDH was constructed as *E. coli* DH5α/pHCE-InaN-GAPDH-ghost 37 SDM.

The objective of this study was to determine the optimum fermentation condition for ghost bacteria vaccine production using *E. coli* DH5α/pHCE-InaN-GAPDH-ghost 37 SDM. Challenge tests using olive flounder were carried out to evaluate the efficacy of the ghost bacteria vaccine.

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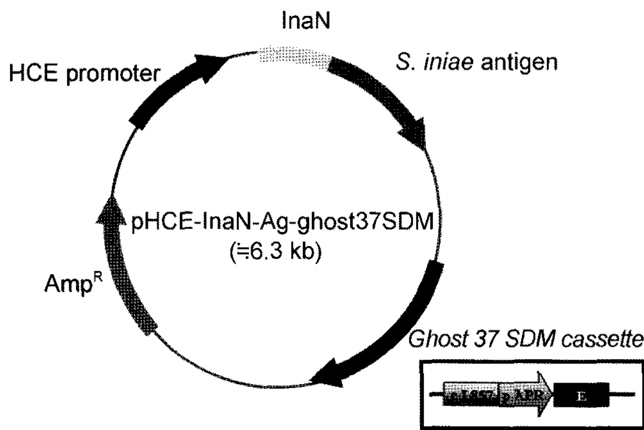

Fig. 1. Recombinant plasmid map of pHCE-InaN-GAPDH-ghost 37 SDM [8].

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

E. coli DH5α/pHCE-InaN-GAPDH ghost 37 SDM (named *E. coli/S. iniae* GAPDH ghost: SIG) [8] was 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. A single colony of *E. coli/S. iniae* GAPDH ghost was inoculated into 30 ml of medium in a 100-ml Erlenmeyer flask. The culture was incubated for 12 h at 37°C, 200 rpm on a rotary shaker and used as a seed culture. The subculture was carried out using 150 ml of fresh medium in a 500-ml baffled Erlenmeyer flask with 5% inoculums size, of 37°C and 200 rpm on a rotary shaker for 6 h. Batch cultivations were carried out in a 5-l jar fermenter (Model KF-5; Korea Fermenter Company Ltd., Inchon, Korea) containing 3 l of initial medium. The initial temperature, agitation speed, and aeration rate during the culture were set of 37°C, 300 rpm, and 1.0 vvm.

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 [20]. 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 an absorbance of 1.0. The residual glucose concentration was measured according to the procedure described by Dubois *et al.* [4] with modification. The expression of antigen GAPDH was determined by SDS-PAGE and Western blot. The samples were prepared by boiling washed cells for 5 min in sample buffer. Proteins from equal amounts of cells measured as optical densities were loaded on the gel. The part of electrophoresed gel was transferred onto a 0.45-μm pore nitrocellulose membrane (BioTrace, U.S.A.) at 100 V for 1 h in a Bio-Rad mini Trans-Blot electrophoretic transfer cell for Western blot analysis [9, 12].

Induction of Heterologous Protein Expression

The lysis of recombinant *E. coli* for the ghost bacteria formation decreases the turbidity of the culture broth, which could be detected by the measurement of optical density at 600 nm. When the batch cultures reached the 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 increase of temperature from 37°C to 42°C. The optical density was measured until no further decrease of optical density was detected. After the expression of lysis E gene, 10 μl of fermentation samples were taken at 1 h intervals and spread onto LB agar plates containing 50 μg/ml ampicillin for the determination of the efficiency of ghost bacteria formation. The plates were incubated in 37°C for 12 h and the efficiency of ghost bacteria formation was analyzed by the colony counting method [3].

Vaccination and Challenge Tests

Healthy olive flounders with an average body weight of 18 g±0.5 g and an average length of 15 cm±0.5 cm were obtained from a local commercial fish farm in Korea. Fishes were divided into 4 groups of 25 individuals and were acclimated in 200-l tanks for 1 week at 20–24°C. For the immunization, group 1 (positive control) and 2 (negative control) were fed with a mixture of 100 μl of PBS per 1 g of commercial feed ratio. Groups 3 and 4 were fed with the mixture of 100 μl (mg/ml) of formalin-killed cell vaccine (FKC) or *E. coli/S. iniae* GAPDH ghost (SIG) per gram of commercial feed. At 8 days after immunization, all fishes from immunized and non-immunized groups were challenged intraperitoneally (IP) by injection of 0.1 ml of living *S. iniae* suspension containing approximately 2×10^7 cells/ml. All fishes were maintained under identical conditions and clinical signs were observed. The cumulative mortality was detected for 7 days after the challenge tests. Dead fishes were collected daily and necropsied. Kidney samples of those were streaked on tryptic soy agar (TSA) containing 1.5% NaCl to confirm the presence of *S. iniae*.

RESULTS AND DISCUSSION

Optimization of Culture Conditions

The effect of various glucose concentrations on the growth by *E. coli/S. iniae* GAPDH ghost was carried out using a 5-l fermenter. The glucose concentration was adjusted to 0, 1, 2, and 3 g/l for carbon source optimization. As shown in Fig. 2A, 1 g/l of glucose concentration showed high cell mass production. Further increase of the glucose concentration decreased the cell growth, due to the inhibitory effect of substrate [7, 18]. The specific growth rate was also highest at the culture with 1 g/l of glucose. One g/l of glucose in LB broth was selected for the further production of *E. coli/ S. iniae* GAPDH ghost.

Agitation provides proper mixing of the fermentation broth and has a significant effect on the productivity of the fermentation system. Fig. 2B shows the effect of agitation speeds ranging from 100 to 400 rpm on the production of ghost bacteria using *E. coli/S. iniae* GAPDH ghost. Cell density was low because of the low concentration of dissolved oxygen by the insufficient mixing at 100 rpm. The maximum production of cell density was obtained at the agitation speed of 300 rpm with the highest specific growth rate and the cell density of 1.8 g/l *E. coli/S. iniae* GAPDH ghost. Further increase in agitation speed adversely

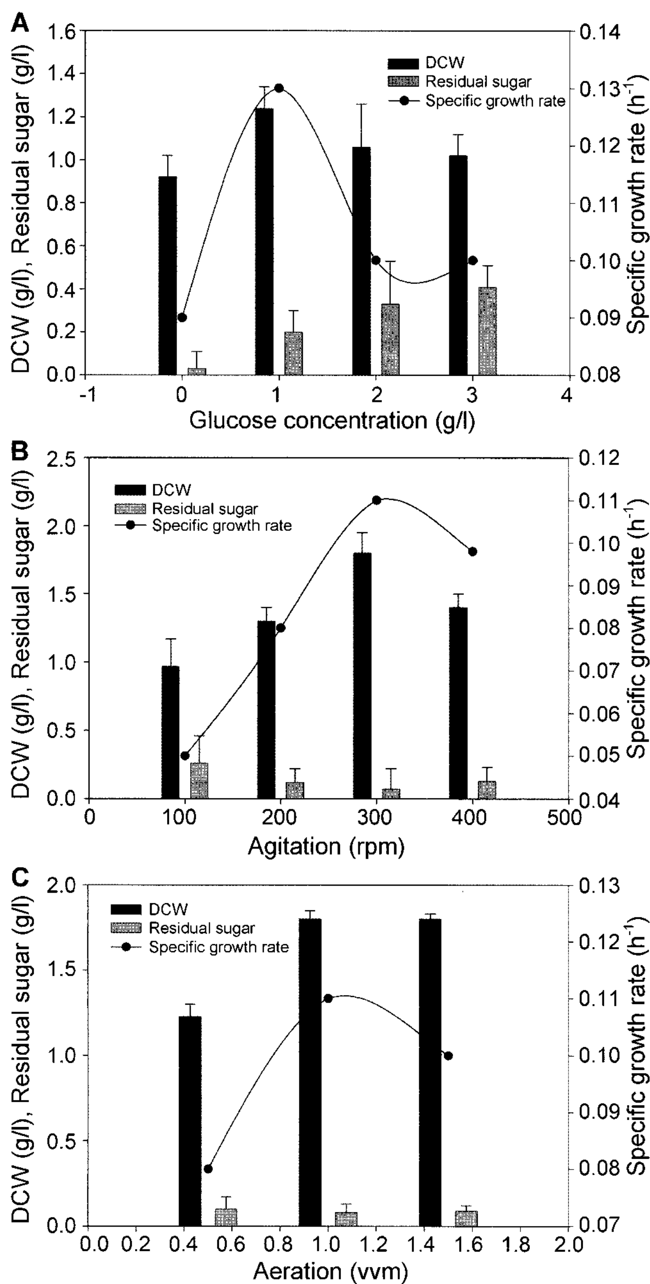

Fig. 2. Optimization of culture conditions of *E. coli/S. iniae* GAPDH ghost using a 5-l fermenter.

A. Glucose concentration. **B**. Agitation. **C**. Aeration.

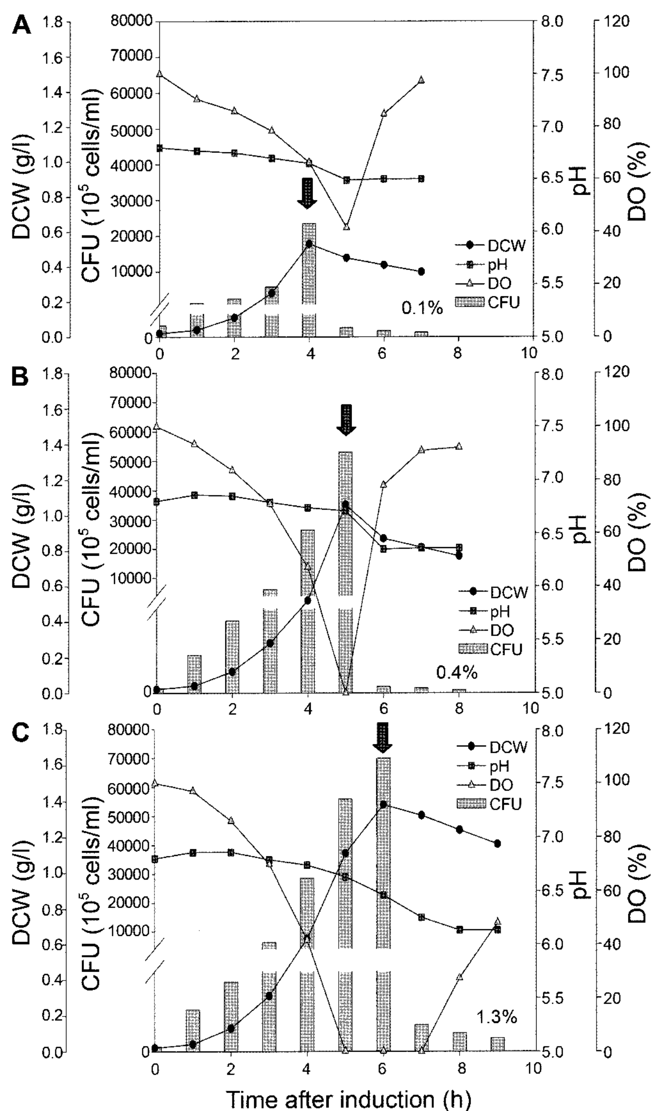

Fig. 3. Efficiency of the ghost bacteria formation and growth of *E. coli/S. iniae* GAPDH ghost by temperature induction of the lysis E gene.

A. Temperature induction point: early log phase (OD_{600}=1.0). **B**. Temperature induction point: mid-log phase (OD_{600}=2.0). **C**. Temperature induction point: late log phase (OD_{600}=3.0). The arrow indicates temperature shift from 37°C to 42°C.

affected the cell density as well as specific growth rate. Therefore, the agitation speed of 300 rpm was chosen as the optimal agitation speed.

Aeration provides dissolved oxygen transfer as well as mixing effect to the fermentation broth. During the fermentation, the transfer of oxygen occurs from air bubbles into the medium and then to the cell. Thus, the oxygen transfer from the air bubble to microbial cells through the liquid medium is essential for cell growth and product formation [10]. As shown in Fig. 2C, the effect of aeration rate on cell density was evaluated with the aeration rates of 0.5, 1.0, 1.5 vvm at the agitation speed of 300 rpm. At the aeration rate of 1.0 and 1.5 vvm, maximum cell density was obtained with 1.8 g/l for *E. coli/S. iniae* GAPDH ghost. One and 1.5 vvm aeration rates showed a similar growth trend. However, the specific growth rate of 1.0 vvm showed higher than that of 1.5 vvm. Therefore, 1 vvm was chosen as the optimal aeration rate.

Efficiency of Ghost Bacteria Formation

The efficiency of ghost bacteria formation was evaluated using a 5-l fermenter. As shown in Fig. 3, the efficiency of

the ghost bacteria formation in non-lyophilized ghost *E. coli*/*S. iniae* antigen GAPDH was 99.9%, 99.6%, and 98.7% with the inductions at OD_{600}=1.0 (Fig. 3A: early logarithmic phase), 2.0 (Fig. 3B: mid-logarithmic phase), and 3.0 (Fig. 3C: late logarithmic phase), respectively. Early logarithmic phase (OD_{600}=1.0) induction showed high efficiency of ghost bacteria formation. This result confirmed that the action of protein E to make holes is related to the cell divisions [5]. This result indicates that 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 protein E or reduced power of the λP_R promoter in *E. coli* DH5α. With the consideration of ghost cell mass production, the mid-exponential phase (OD_{600}=2.0, Fig. 3B) was selected as the optimal ghost bacteria formation with the concentration of 0.77 g/l for *E. coli*/*S. iniae* GAPDH ghost.

Three hours after the induction at 42°C, ghost bacteria were harvested and the antigen expression was analyzed by SDS–PAGE and Western blot. Fig. 4A shows an approximately 67 kDa GAPDH band was observed in the insoluble fraction; however, no band was detected in the soluble fraction in SDS–PAGE analysis. A 67 kDa GAPDH band was also shown in the insoluble fraction; however, no band was detected in the soluble fraction in Western blot analysis (Fig. 4B). This indicates that *E. coli*/*S. iniae* GAPDH ghost expressed on the membrane surface of *E. coli*.

Efficacy of *E. coli*/*S. iniae* Antigen GAPDH Ghost

Recombinant ghost bacteria attract increasing attention as a new approach for a vaccine delivery vehicle. The potential application of this technology has been reported in mammalian pathogenic Gram-negative bacteria [5, 14].

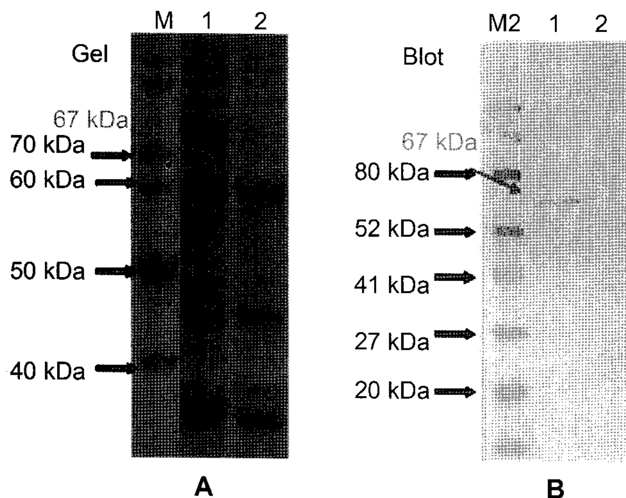

Fig. 4. Analysis of antigen GAPDH expression by SDS–PAGE (**A**) and Western blot (**B**).
M1: Protein ladder marker; lane 1: Insoluble fraction; lane 2: Soluble fraction; M2: Pro Sieve-Color Protein Markers (Cambrex, Rockland, U.S.A.).

As shown in Fig. 5, the efficacy of *E. coli*/*S. iniae* GAPDH ghost as a vaccine was evaluated by challenge test using olive flounder for 7 days. The negative control group showed no clinical signs or mortality during the period. In contrast, initial cumulative mortalities of the positive control, formalin-killed *S. iniae* (FKC) vaccine, and ghost bacteria vaccine groups injected with *S. iniae* appeared at 2 days post-infection. The finial cumulative mortalities were detected at 7 days post-infection. The cumulative mortalities of positive control, FKC vaccine, and ghost bacteria vaccine immunized groups were 91%, 74%, and 57%, respectively. Darkening of the skin, lethargy, and hemorrhages on the ventral side of the body as the clinical signs of dead olive flounder could be observed. It was found that incompletely immunized fishes with FKC and SIG vaccines became moribund and died from 2 to 5 days post-infection. In contrast, the successfully immunized fishes with FKC and SIG vaccines could survive against the challenge with *S. iniae* after 5 days post-infection. This result suggests that the adaptive humoral immune responses of olive flounder were successfully induced by oral SIG vaccine, and the immune system could protect the fishes from the challenge of *S. iniae*. The ghost bacteria SIG vaccine showed higher potential to induce protective antibodies than FKC vaccine.

In conclusion, aquaculture is one of the fast growing industries in the world. As global wild fish stocks continue to be depleted, it will be even more important to provide safe, efficient production of food for human consumption. The implementation of effective, long-term vaccination programs is the most likely target for the control of the disease caused by *S. iniae*. In this study, production of ghost bacteria as vaccine from *E. coli*/*S. iniae* GAPDH ghost was optimized by controlling the fermentation conditions such as glucose concentration of 1 g/l, agitation speed of

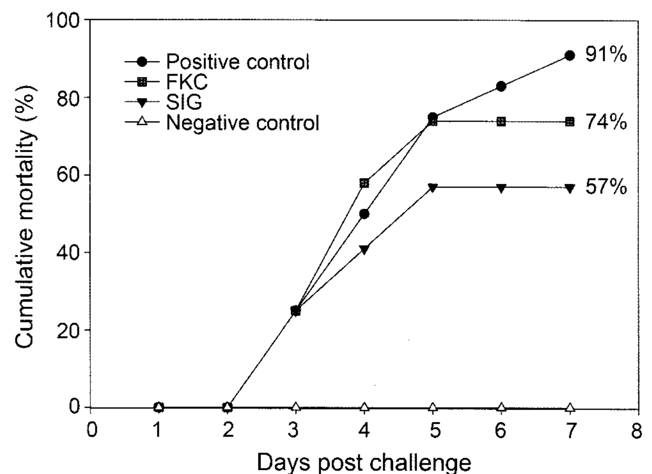

Fig. 5. Cumulative mortality of olive flounder challenged with *Streptococcus iniae* after immunization by *E. coli*/*S. iniae* GAPDH ghost (SIG) vaccine, formalin-killed *S. iniae* (FKC) vaccine, or PBS (positive controls).

300 rpm, and aeration of 1 vvm. In addition, the maximum efficiency of ghost bacteria production was obtained at OD_{600}=2.0 with the concentration of 0.77 g/l for *E. coli/S. iniae* antigen GAPDH.

The protective efficacy of *E. coli/S. iniae* antigen GAPDH was evaluated by challenge test using olive flounder. The cumulative mortalities of the positive control, FKC vaccine, and ghost bacteria vaccine immunized groups were 91%, 74%, and 57%, respectively. Fishes immunized with ghost vaccine showed significantly higher survival rates than FKC vaccine. The ghost bacteria vaccine could play a key role in protection against *S. iniae* [7].

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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