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Production of Anti-Infectious Hematopoietic Necrosis Virus (IHNV) Substances by Immobilized Whole Living Cells of Aquatic Bacteria in a Bioreactor System

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A simple, low-cost and efficient bioreactor system for production of anti-infectious hematopoietic necrosis virus (IHNV) substances by bacteria isolated from aquatic environments was devised on a laboratory scale. Whole living cells of anti-IHNV substances producing bacteria, *Pseudomonas* sp. 51BBW-29 and *Alteromonas* sp. 48HS-27, were immobilized in calcium alginate beads prepared by 1% sodium alginate, 0.45 M $CaCl_2$, and C_2Y or MC_2Y broth. Eighteen g of the beads were packed into a 25 m*l* reaction column and incubated at 15°C for 5 days in a circulating broth medium at a flow rate of 0.35 m*l*/h. After activation of the immobilized bacteria, the beads were incubated in a continuous flow of fresh broth with intermittent exposure to air. In the beads incubated under these conditions, the immobilized bacteria grew well at the gel surface and high viability of approximately 10^{10} CFU/bead was retained for at least 6 days from day 8 to 14. During 31 days of the experiment with the bioreactor system, the highest production was 69.3 ΔIHD_{50} for *Pseudomonas* sp. 51BBW-29 (at 14–17 days incubation) and 48.0 ΔIHD_{50} for *Alteromonas* sp. 48HS-27 (at 8–11 days incubation), which were 2.5 and 2.7 times higher than those of conventional batch culture (27.3 and 18.0 ΔIHD_{50} for the two strains at 2–5 days incubation). The immobilized bacteria continuously produced high levels of anti-IHNV substances for 15 days (day 5–20).

Key words: antiviral substance, alginate beads, bioreactor, IHNV, *Pseudomonas*, *Alteromonas*

A large number of bacteria isolated from aquatic environments showed anti-infectious hematopoietic necrosis virus (IHNV) activity[1,2] and produced extracellular substances which could be divided into three types (I–III) according to their antiviral properties.[3] Of these bacteria, a representative strain, *Pseudomonas* sp. 51BBW-29 produced a heat-stable and comparatively low molecular weight substance, having the property of Type III which inhibits viral replication in the host cell. Another representative strain, *Alteromonas* sp. 48HS-27 produced a heat-labile and high molecular weight antiviral substance with the property of Type II that inactivates the virus directly or inhibits viral invasion of the host cells.[3]

Production of antiviral agents through the use of a bioreactor system has been reported by Yokozeki *et al.*[4] They produced adenine arabinoside (Ara-A) from uracil arabinoside and adenine by a transglycosylase with whole cells of *Enterobacter aerogenes*. The multistep reaction system using the metabolisms of *Bacillus* sp. to produce an antibiotic, bacitracin, was reported by Morikawa *et al.*[5,6] However, to our knowledge there has been no previous report concerning the production of antiviral substances by the multistep reaction system.

In this study, we attempted to produce anti-IHNV substances by using immobilized whole living cells of two bacterial strains in calcium alginate beads and to design a simple, low-cost and efficient production system on a laboratory scale.

Materials and Methods

Bacterial Cultures

Two strains of bacteria, *Pseudomonas* sp. 51BBW-29 and *Alteromonas* sp. 48HS-27[3] were used for this study. *Pseudomonas* sp. 51BBW-29 and *Alteromonas* sp. 48HS-27 were inoculated into 50 m*l* of CYG broth composed of 0.5% casamino acids (Difco), 0.05% yeast extract (Difco), 0.1% glucose (Wako), 0.68% NaCl, 0.04% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2$ (anhydrous), pH 7.2[2] and MCYG broth composed of 0.5% casamino acids (Difco), 0.05% yeast extract (Difco), 0.1% glucose (Wako) and 50% Herbst's artificial seawater, pH 7.8,[1] respectively. The inoculated broths were incubated at 25°C for 2 days with shaking (160 rpm).

A conventional batch culture of CYG and MCYG broths was made as described previously[1–3] for comparison with a bioreactor. This culture was incubated at 15°C for 14 days with shaking.

Virus and Cell Culture

The infectious hematopoietic necrosis virus (IHNV) strain ChAb[7] and CHSE-214 cells were used for the plaque assay to determine the production of anti-IHNV substance.[3]

Immobilization of Bacterial Cells

After incubation, the culture fluid of *Pseudomonas* sp. 51BBW-29 (50 m*l*) was centrifuged at 4,000 rpm for 20 min at 4°C. The cells were resuspended into a 100 m*l* mixture consisting of 50 m*l* of intermittently sterilized 2% sodium

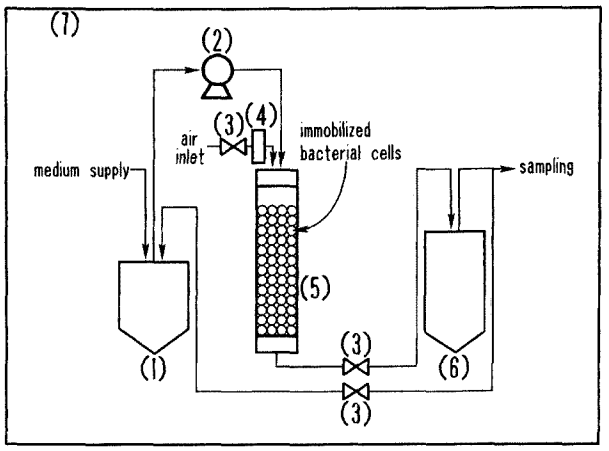


Fig. 1. Schematic diagram of the bioreactor system employed for the production of antiviral substances.
(1) medium reservoir; (2) pump; (3) valve; (4) air filter; (5) reaction column; (6) products reservoir; (7) incubator.

alginate (100°C, 10 min × 2) and 50 m*l* of two-fold concentrated C_2Y broth medium. The C_2Y broth was made up of 1% casamino acids (Difco), 0.05% yeast extract (Difco), 0.68% NaCl, 0.04% KCl, and 0.02% $MgSO_4 \cdot 7H_2O$ (pH 7.2). MC_2Y broth, a modified C_2Y broth containing 50% Herbst's artificial seawater without $CaSO_4$ (pH 7.8) was used for the suspension of *Alteromonas* sp. 48HS-27. The 100 m*l* cell suspension was aseptically dropped into a cooled 0.45 M $CaCl_2$ solution while stirring and the aliginate beads (approximate diameter: 3 mm) which formed in the $CaCl_2$ solution were hardened in the refrigerator for at least 2 h before use.

Bioreactor System and Operating Conditions

A schematic diagram of the bioreactor system is shown in Fig. 1. All operations were performed at 15°C in the incubator (7). The alginate beads (18 g wet weight, *ca.* 1,200 beads) with the immobilized bacterial cells were packed into a 25 m*l* reaction column (inside diameter: 15 × 141 mm) (5) and immersed in C_2Y or MC_2Y broth. Twenty-five m*l* of C_2Y or MC_2Y broth was supplied from the medium reservoir (1) to the reaction column through a silicone tube (inside diameter: 1 mm) at a flow rate of 0.35 m*l*/h *via* a pump (2). In the circulating system, the outflow medium from the reaction column to the products reservoir (6) was recirculated to the medium reservoir. In the continuous system, fresh medium was readily supplied to the reaction column without recirculating. For aeration of the system, the medium in the column was allowed to discharge to the products reservoir (6) every 3 days and the beads were exposed to air supplied through an air filter (pore-size 0.2 μm, Millipore) (4) for a few minutes. One m*l* of sample was taken out from the products reservior on certain days (day 0, 2, 5, 8, 11, 14, 17, 20, 23, 26, and 31), and the antiviral activity was determined.

Scanning Electron Micrograph

The immobilized cells of *Pseudomonas* sp. 51BBW-29 on the surface and inner portion (about 1 mm from the surface) of the beads incubated under various conditions were observed by a scanning electron microscope. The beads were immersed in 2% glutaraldehyde solution for 48 h at 4°C, and dehydrated in a graduated ethanol series (50–100%) and substituted by amyl acetate. The preparations were dried to the critical point in CO_2, fixed on a specimen mount, and sputter-coated with gold. The specimens were observed in a JEOL JSM-T330A SEM at 20 kV. The bacterial numbers per 100 μm^2 were counted from microphotographs.

Viable Counts of Bacteria in Alginate Bead and Culture Medium

Five alginate beads from each sample were homogenized by a mortar and pestle in PBS or 50% Herbst's artificial seawater, and viable counts were determined by a standard plate count using FWA medium[8] for *Pseudomonas* sp. 51BBW-29 and SA medium[9] for *Alteromonas* sp. 48HS-27. Viable counts in the culture medium were also determined with FWA or SA medium.

Determination of Anti-IHNV Substance Production

Each culture fluid taken from the conventional batch culture and bioreactor was filtrated using a Millex-HA filter (pore-size 0.45 μm, Millipore). Serial two-fold dilutions of the filtrate were prepared by using fresh medium and the anti-IHNV activity of each dilution was determined by plaque assay according to the method of Kamei *et al.*[10,11] Then the dilution rate to show 50% inhibition was calculated from the dilution rate response curves according to the method of Hayden *et al.*[12] and daily production per m*l* (ΔIHD_{50}) was estimated from the disparity in the values of 50% inhibitory dilution rate on two consecutive sampling days.

Results

Electron Microscopic Observation of Pseudomonas sp. 51BBW-29 Immobilized in Alginate Beads

Cells of *Pseudomonas* sp. 51BBW-29 on the surface and inner portion of the beads were observed under an electron microscope before and after incubation under the conditions determined in the experiments as described above (Fig. 2). Before incubation, the number of bacterial cells in the beads were 2–5 cells/100 μm^2 on the surface and inner portion. After incubation for 14 days, cell numbers of 17–338 cells/100 μm^2 were observed on the surface of the beads, while the numbers inside the beads did not increase and remained at between 2–5 cells/100 μm^2.

Viable Bacterial Numbers and Anti-IHNV Substances Production in Conventional Batch Culture and Bioreactor System

Viable counts of *Pseudomonas* sp. 51BBW-29 and *Alteromonas* sp. 48HS-27 in medium incubated in the conventional batch culture and in a bead of the bioreactor system incubated for 14 days are shown in Fig. 3. The cell count of the two strains in the culture media reached maximums of 2.5×10^9 and 1.3×10^8 CFU/m*l* after 5 days of incubation, respectively, but thereafter the viable counts decreased for the next 9 days. On the other hand, the counts of both strains in a bead of the bioreactor system were maintained at a high count of 10^9 to 10^{10} CFU for at least 6 days from day 8 to day 14.

Production of anti-IHNV substances is shown in Fig. 3.

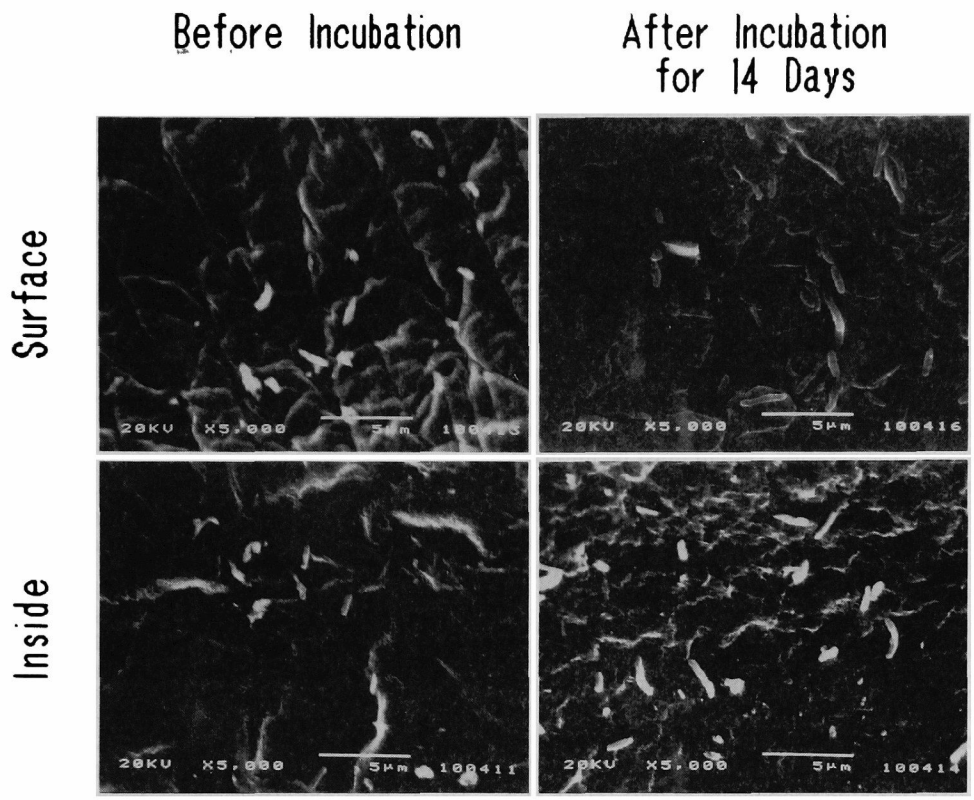


Fig. 2. Electron micrographs of *Pseudomonas* sp. 51BBW-29 immobilized in 1% calcium alginate beads.

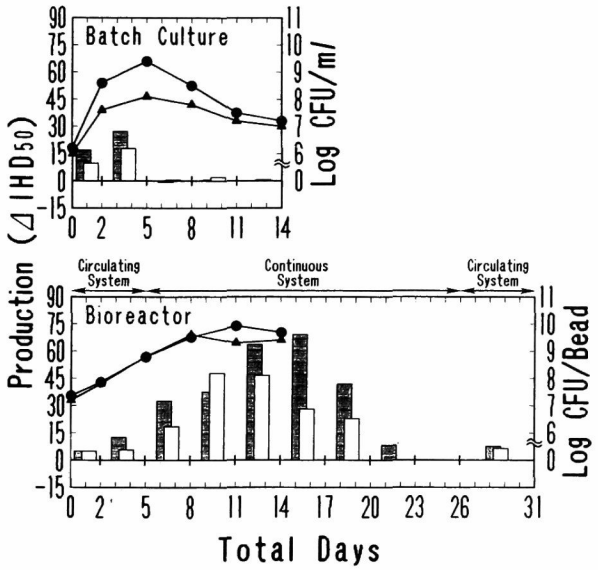


Fig. 3. Viable count of bacteria in culture medium and in an alginate bead, and production of anti-IHNV substances after incubation at 15°C by the conventional batch culture with agitation and the bioreactor system.

Closed symbol: Viable bacteria count. Bar: Average production of anti-IHNV substance. ●, ■: *Pseudomonas* sp. 51BBW-29. ▲, □: *Alteromonas* sp. 48HS-27.

The production of *Pseudomonas* sp. 51BBW-29 and *Alteromonas* sp. 48HS-27 in the conventional batch culture increased until day 2–5 and showed a maximum of 27.3 and 18.0 ΔIHD_{50}, respectively. The production of the two strains in the bioreactor system however, increased until day 14–17 or day 8—11 and showed a maximum of 69.3 or 46.7 ΔIHD_{50}, respectively. In both strains, higher productions than the maximums in the batch culture were maintained from day 5–8 to day 17–20, but the productions then decreased to zero by day 5 in the batch culture and day 20–26 in the bioreactor system. However, by simply converting the continuous culture system to the circulating system, production in the bioreactor system recovered by day 26–31 to the levels of the initial 5 days.

Discussion

Recently, many kinds of useful products have been produced by various bioreactor systems. Many of these systems have been described and some of them have been scaled up to an industrial level.[13] The entrapment technique of microbial cells in calcium alginate gels is one of the most important immobilizing methods introduced by Hackel *et al.*[14] and has been widely used since the gel is mild, inexpensive, non-toxic and the technique is easy to perform.[15–18]

The alginate beads prepared by 1% sodium alginate, 0.45 M $CaCl_2$ and broth were physically stable enough for our system and rupturing of the beads was not seen during the 7 week observation period. A curing time of longer than 2 h after bead formation in 0.45 M $CaCl_2$ was also regarded as enough time to complete gelation.[19]

It has been noted that alginate gels have a large number of pores[20] and the pores are related to the diffusion of nutrients and products.[17] In our system using 1% sodium alginate, our data showed that although the immobilized

bacteria were maintained in the beads, the antiviral substances produced were released efficiently into the medium. However, electron microscopic observation of *Pseudomonas* sp. 51BBW-29 cells immobilized in the beads revealed that bacterial growth in the beads was limited to near the gel surface (Fig. 2). The reason for this may be that *Pseudomonas* is an aerobic bacterium, thus grows only in the presence of free oxygen. This suggests that the ability of the beads to produce antiviral substance is influenced by the amount of oxygen supplied into the beads.

The sufficient supply of oxygen is considered the biggest problem in using aerobic microorganisms during an operation[21] and the limited diffusion ability of the matrix makes oxygen supply more difficult with entrapped aerobic cells.[22] Therefore, to achieve a higher and more uniform bacterial density in the beads, the aeration system must be improved through such methods as the use of catalase and hydrogen peroxide as internal bead aeration.[22,23] Attempts to form beads with smaller diameters or to develop another matrix capable of supplying oxygen more proficiently might help to solve of this problem.

In our bioreactor system, *Pseudomonas* sp. 51BBW-29 and *Alteromonas* sp. 48HS-27 could propagate to approximately 10^{10} CFU per bead and the production of anti-IHNV substances in C_2Y or MC_2Y broth reached 2.5 and 2.7-fold respectively that of conventional batch culture (Fig. 3). The C_2Y and MC_2Y broth were devised from CYG and MCYG broths for high production of anti-IHNV substances in our bioreactor system. The initial 5 days activation of the entrapped bacteria in the circulating system was essential to maintain high production in the continuous system. A production greater than the maximum of the batch culture was continued for 15 days from day 5 to 20 (Fig. 3). This means that *Pseudomonas* sp. 51BBW-29 and *Alteromonas* sp. 48HS-27 continuously produced a large amount of anti-IHNV substances for a longer period by the use of this simple and low-cost system.

Although the production of antiviral substances by the two strains in this bioreactor system was down to zero at day 23–26 (*Pseudomonas* sp. 51BBW-29) or 20–26 (*Alteromonas* sp. 48HS-27), production was recovered by simply changing the operation system from continuous to circulating. We assume that this phenomenon is related to the growth phase of the bacteria in the beads, because the immobilized strains produce the antiviral substances better during their stationary growth phase or death phase, and production does not always correspond to bacterial growth (data not shown). To accomplish a continuous production over a longer term and at a higher production rate, further investigations related to the bacterial growth phases and improvements in the aeration system will be necessary.

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