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Suspension Culture of an Antibacterial Peptide Producing Cell Line from *Bombina orientalis*

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Abstract The suspension culture of an anchorage-dependent cell line (Bok-1) from *Bombina orientalis* was successful in respects of cost and efficiency. The amount of cells obtained from the suspension culture was almost equivalent to that from the anchorage-dependent culture. This result shows the possibility of suspension culture for scale-up. The cells in suspension produced an antibacterial peptide as much as anchorage-dependent cells did. The cell growth (6.0×10^6 cells/ml) and viability (>80%) at 10 rpm were higher than that at 0 rpm (1.9×10^6 cells/ml, 65~80%) and 30 rpm (1.8×10^6 cells/ml, 40~76%). The size of cells became smaller at the agitation rate of 30 rpm. The antibacterial activities of cell extracts from suspension cultured cells were confirmed against gram-negative and gram-positive bacteria by the inhibition zone assay and the liquid growth inhibition assay.

Key words: Suspension culture, Bok-1, antibacterial peptide, agitation rate, shear stress

An animal cell culture has become an important aspect ofbiotechnology. Molecules are being cloned and expressed in a few cultured animal cell lines for *in vitro* and *in vivo* applications. If the products of the cell lines are valuable, the cells should be introduced to the large-scale animal cell culture system. The industrial-scale cell culture systems for manufacturing of human and animal health care products have been developed over the past few decades. Providing sufficient surface areas for cell growth, however, still remains the limitation of scale-up. Therefore, the development of suspension culture systems for anchorage-dependent cells has been one of major topics in studies of the animal cell culture.

Antibacterial peptides comprise a diverse class of molecules used in host defense by plants, insects, and animals [4, 13]. Many kinds of antibacterial peptides are already known to researchers. For example, frog skin is an extraordinary source of biologically active peptides. These antibacterial peptides have useful characteristics that show a broad antibacterial spectrum including gram-negative and gram-positive bacteria, cause little damage to human cells like red blood cells, and have little resistance compared with antibiotics. Of particular interest are those that may also have antiviral [7, 8] or antitumor [6] activities. The potential applications for antibacterial peptides have led to an increased effort to find new structures, and more than 70 have so far been identified [2]. Functional and biochemical characterizations have revealed that they comprise a heterogeneous collection of molecules, although most can be defined as low molecular mass proteins (<10 kDa) that act in a stoichiometric manner [2].

Bok-1 cells were originally anchorage-dependent cells, which were originated from embryo cells of a species of Korean frog, *Bombina orientalis* [18]. In this study, the applicability of suspension culture of Bok-1 cell was studied to reduce the cost of culture and make handling easier. This will be beneficial for the large-scale production of the antibacterial peptide.

MATERIALS AND METHODS

Anchorage-dependent Animal Cell Culture

The medium (pH 7.6) consisted of 5:4:1 ratio of L-15 medium Leibovitz, TNM-FH insect medium, BME (basal medium eagle, Sigma Chemical Co., St. Louis, U.S.A.) mixture, 5% FBS (fetal bovine serum, Gibco BRL, Gaithersburg, MD, U.S.A.) and antibiotics (100 μg/ml streptomycin and 10 U/ml penicillin, Sigma Chemical Co.).

Bok-1 cells were cultured in 75 or 175 cm^2 T-flask (Falcon, Becton Dickinson Labware, Franklin Lakes, U.S.A.) in a humidified 5% CO_2 incubator (Forma

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Scientific, Marietta, U.S.A.) at 25°C. During the culture period, the medium was not exchanged and the number of cells was counted with a haemocytometer every two days. Each time in counting the cultured cells in a T-flask, all the cells were detached from the vessel.

Suspension Animal Cell Culture

The cells of *Bombina orientalis* (Bok-1 cell line) were cultured in suspension using a 100 ml spinner flask (Bellco Biotech., Vineland, U.S.A.). The medium composition was the same as above. The spinner flask was placed in a humidified 5% CO_2 incubator at 25°C (Forma Scientific). The cells in the spinner flask on the magnetic stirrer (Wheaton Sci., Millville, U.S.A.) were suspended by agitation. The agitation rate was fixed during the culture period at 0, 10, and 30 rpm. The morphological change of the cells was investigated by the comparison of photographs taken with an inverted microscope (Olympus CK2-TR, Tokyo, Japan). To protect cell adhesion to the wall of a spinner flask, Sigmacote (Sigma Chemical Co.) was coated on the vessel in advance.

Growth Inhibition Zone Assay

Cultured Bok-1 cells were lyzed in the extraction buffer [0.2 M sodium acetate (pH 4.0), 0.2% Triton X-100, 3 mM phenylmethylsulfonyl fluoride (PMSF)] with a tissue teasor (Biospec Products Inc., Racine, U.S.A.) at 30,000 rpm for 30 sec. The cell lysate was centrifuged at 20,000×g for 20 min at 4°C to remove cell debris. Prepared cell extracts were used for an inhibition zone assay for the antibacterial activity against gram-negative bacterium (*E. coli*, D21) and gram-positive bacterium (*Bacillus megaterium*, Bm11).

The liquid medium for bacterial culture was LB (Luria-Bertani) broth which was composed of 1% bactotrypton, 0.5% yeast extract (Difco, Detroit, U.S.A.) and 1% NaCl (pH 7.0). The solid medium for bacterial lawn was 1.5% agar/LB broth (Difco).

A 9×3 cm plate received 15 ml of melted 1.5% agar/LB broth (pH 7.2). One hundred ml of 1.5% agar/LB broth was mixed at 55°C with 2.5 ml LB broth which contained bacteria cultured to approximately 0.5~0.6 OD_{600}. Five ml of the mixed broth was poured on the solidified agar/LB medium.

The cell extracts were added into the cylinder (diameter: 7 mm; thickness of wall: 1 mm; height: 10 mm) on the lawn of both gram-negative and gram-positive bacteria and incubated for 24 h at 37°C for *E. coli* and at 30°C for *B. megaterium*. Each sample was a cell extract derived from 5.0×10^6 cells (1×) cultured either in T-flasks or in suspension. The antibacterial activities were estimated by measuring diameters of the clear zones after subtraction of the cylinder wells.

Liquid Growth Inhibition Assay

The antibacterial activities from both samples were also compared by liquid growth inhibition assay [2]. Ten μl aliquots equivalent to 5×10^6 cell extract were added into each well of a microtiter plate which contained 100 μl of a midlogarithmic phase culture of bacteria (*E. coli*, D21, and *B. megaterium*, Bm11). The starting absorbance of *E. coli* and *B. megaterium* was 0.025 and 0.03 at 570 nm, respectively. The microtiter plate was incubated for 24 h at 25°C. The growth inhibition was determined by measuring the turbidity of the medium at 570 nm.

RESULTS AND DISCUSSION

Anchorage-dependent Cell Culture

Bok-1 cells, which were originally an anchorage dependent cell line, were cultured in 75 or 175 cm^2 T-flasks. The number of cells was increased nearly 200 times from 5.6×10^4 cells/ml to 1.1×10^7 cells/ml for 10 days (Fig. 1). The medium was not exchanged during the culture period. The cells did not grow well after 10 days culture. The nutritional demand of Bok-1 cells was very low compared with other cell lines like human cells. When the cells were cultured at the inoculating concentration of 3.0×10^5 cells/ml in a 175 cm^2 T-flask for 10 days, 2.7~3.5×10^9 cells were obtained with 1 l of medium.

Suspension Cell Culture

The Bok-1 cells were cultured in a 100 ml spinner flask for 10 days at 10 rpm without medium exchange. The concentration of cells increased 100 times from 3.0×10^4 cells/ml to 2.9×10^6 cells/ml (Fig. 2). This result demonstrated that the Bok-1 cells could be cultured in a suspension culture system. The yield of cells was 2.9×10^9

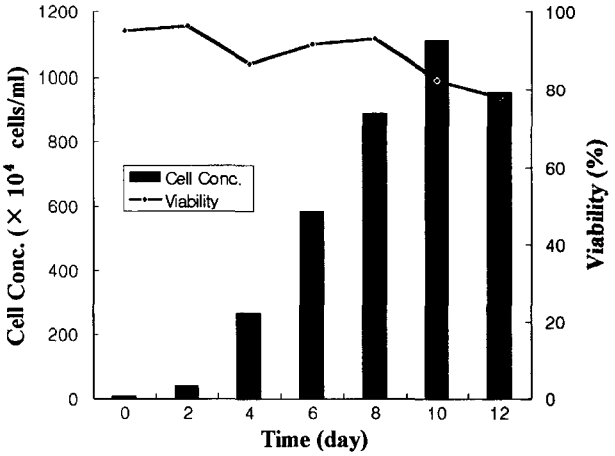

Fig. 1. Cell growth curve of Bok-1 cells cultured in a 75 cm^2 T-flask.

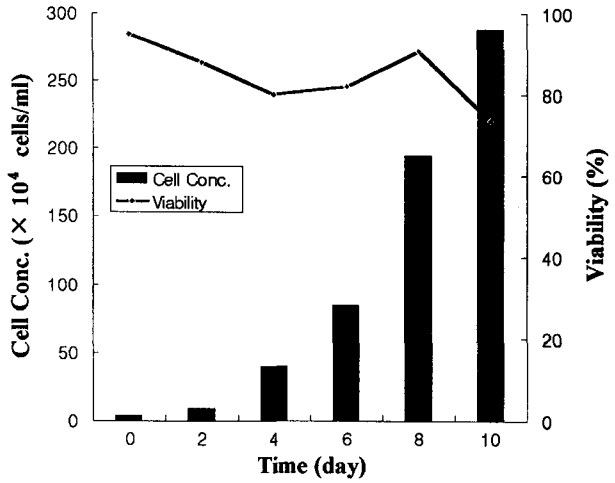

Fig. 2. Cell growth curve of Bok-1 cells cultured in a 100 ml spinner flask at 10 rpm.

with 1 l of medium, which is nearly equivalent to that of the 175 cm^2 T-flask culture. When the cells were cultured at 10 rpm in the inoculating concentration of 3.0×10^5 cells/ml, we could obtain 6.0×10^9 cells with 1 l of medium. The concentration was higher than that cultured in 175 cm^2 T-flasks. The same results were also obtained with 250 and 500 ml spinner flasks (data not shown). These results showed the possibility of suspension culture for scale-up. In the suspension culture, Bok-1 cells could be cultured for more than 10 days without medium exchange.

In order to study the effects of agitation rate on the cell growth, the cells were cultured at 0, 10, and 30 rpm in a 100 ml spinner flask. The cells were also cultured in a T-flask as a control. As shown in Fig. 3, the cell concentration was 6.0×10^6 cells/ml at 10 rpm after 10 days culture. The cells cultured at 0 or 30 rpm did not

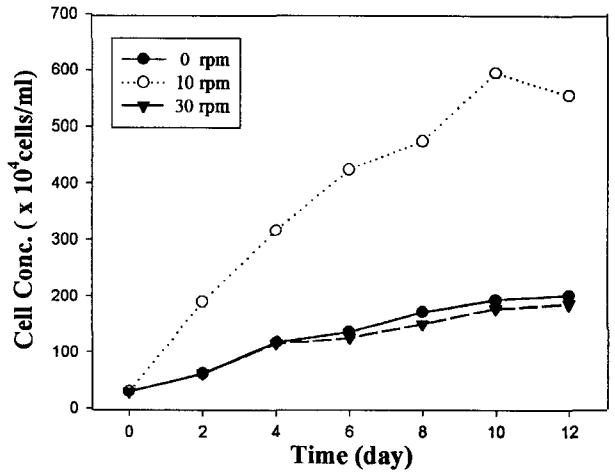

Fig. 3. Effect of the agitation rate on the growth of Bok-1 cells cultured in a 100 ml spinner flask.

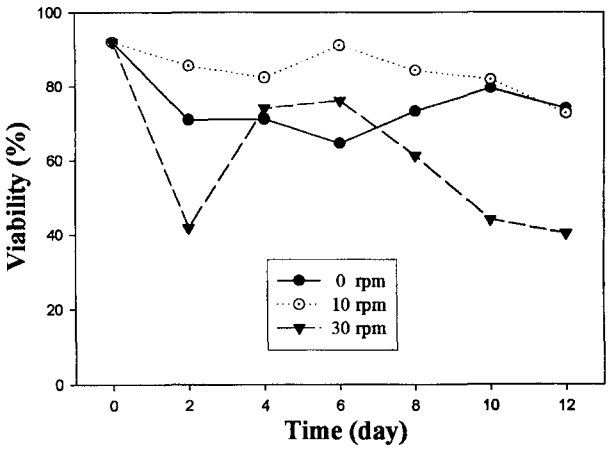

Fig. 4. Effect of the agitation rate on the viability of Bok-1 cells cultured in a 100 ml spinner flask.

grow well. The concentrations of the cells were 1.9×10^6 cells/ml without agitation and 1.8×10^6 cells/ml at 30 rpm after 10 days culture, which were about 68~70% lower than concentration of that cultured at 10 rpm. The viability of cells was higher than 80% at 10 rpm through 10 days (Fig. 4). Without agitation the viability decreased to 65~80%. When the agitation rate increased to 30 rpm, the viability decreased to 40~76%. Therefore, 10 rpm was more optimal than 0 or 30 rpm for both cell growth

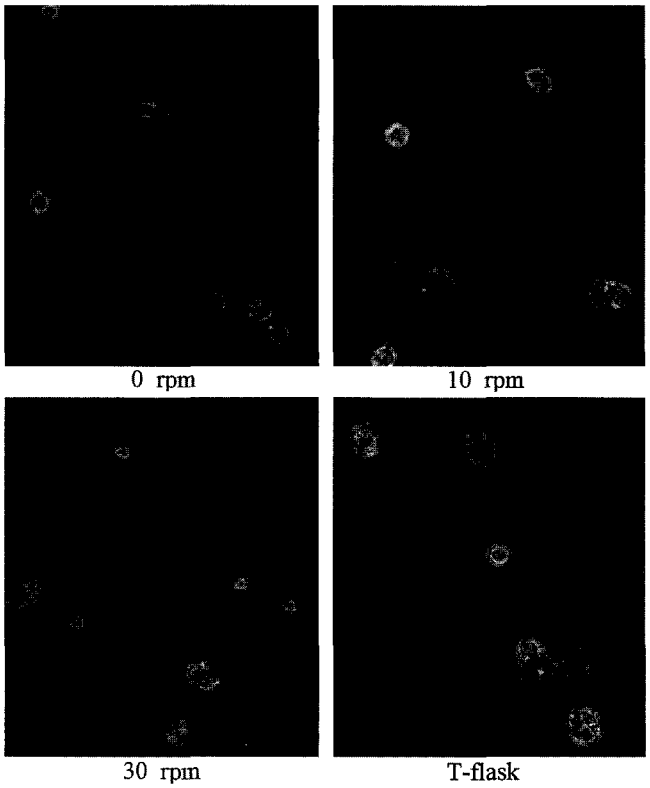

Fig. 5. Photographs of Bok-1 cells cultured at various agitation rates for 10 days (×200).

and viability in suspension culture of Bok-1 cells. The morphological change of the cells was also observed at 30 rpm (Fig. 5). Over-agitation was not pertinent to the growth and viability of Bok-1 cells due to shear stress [21]. When the cells were cultured without agitation or at 10 rpm, the morphological change of the cells was not found. As expected, the cell culture without agitation was not effective due to insufficient oxygen supply and poor medium mixing [21]. One of the characteristics in the Bok-1 cell line was that over-agitation caused cell lysis or size-reduction.

Growth Inhibition Zone Assay

In order to study the effect of the suspension culture on the antibacterial activity of Bok-1 cells cultured, the growth inhibition zone assay [10] was performed using a gram-negative bacterium (*E. coli*) and gram-positive bacterium (*B. megaterium*). As indicated by the diameters of the clear zones, the antibacterial activities of the cell extract from the suspension cultured cells were about the same as that of the cell extract from the T-flask cultured cells (Fig. 6). Therefore, the suspension culture of the Bok-1 cell produced the antibacterial peptide as much as the anchorage-dependent culture did. This result implies that cells in suspension still produce the antibacterial peptide and the Bok-1 cell line is pertinent to suspension culture for scale-up.

The diameters against gram-positive bacterium are a little smaller than those against gram-negative bacterium. The zones of gram-positive bacterium, however, were more clear than those of gram-negative bacterium.

Liquid Growth Inhibition Assay

The antibacterial activity of cell extracts obtained from both T-flask culture and suspension culture was also confirmed by the liquid growth inhibition assay [2]. In this study, the growths of *E. coli* and *B. megaterium* were compared with growth in the presence of cell extracts. As shown in Figs. 7 and 8, the growth of both gram-negative and gram-positive bacterium was inhibited by addition of the cell extract. The cell extract from cells cultured in suspension was as effective as that from cells cultured in a T-flask.

There was little difference between the antibacterial activity of 5.0×10^6 cells ($1\times$) and that of 1.0×10^7 cells ($2\times$). This shows that the antibacterial activity from 5.0×10^6 cells of Bok-1 was enough to inhibit the growth of *E. coli* and *B. megaterium* in the concentration of OD_{570} = 0.025 and 0.03, respectively.

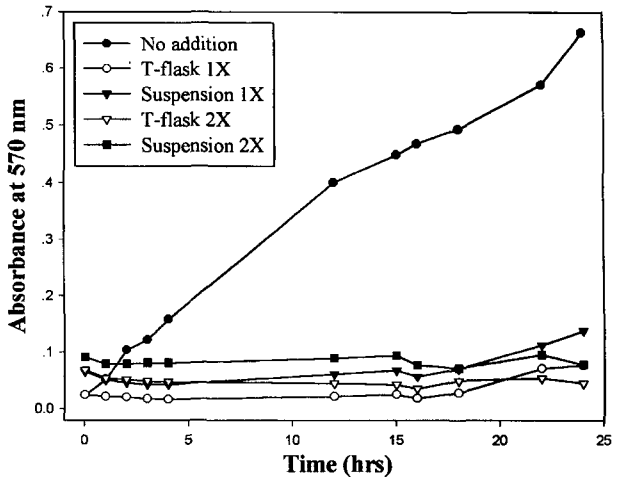

Fig. 7. Growth inhibition of *E. coli* with cell extracts of Bok-1 cells cultured in a T-flask and a 100 ml spinner flask.
1X, 5×10^6 cells; 2X, 1.0×10^7 cells.

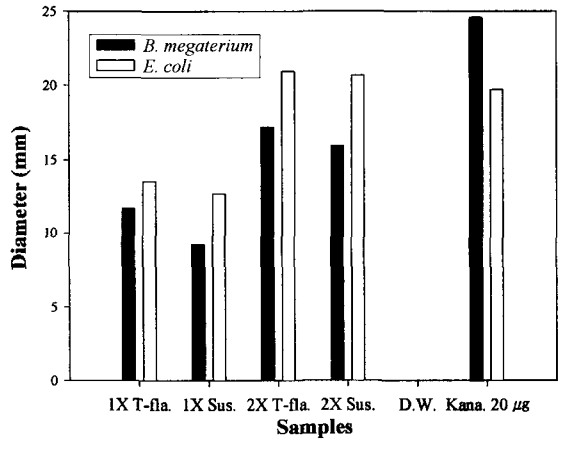

Fig. 6. Antibacterial activities of Bok-1 cells cultured in a spinner flask and a T-flask.
1X, 2.5×10^7 cells; 2X, 5.0×10^7 cells; T-fla., T-flask culture; Sus., suspension culture; D.W., distilled water; kana., kanamycin.

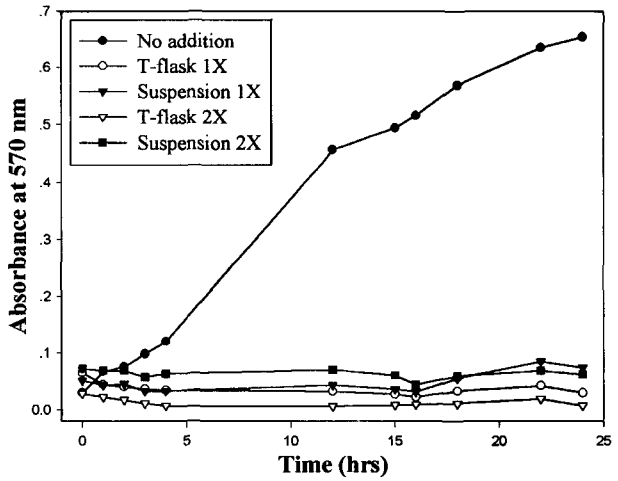

Fig. 8. Growth inhibition of *B. megaterium* with cell extracts of Bok-1 cells cultured in a T-flask and a 100 ml spinner flask.
1X, 5×10^6 cells; 2X, 1.0×10^7 cells.

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