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Efficient production of recombinant protein in immobilized insect cell culture using serum-free basal media after baculovirus infection

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Abstract

To develop an efficient biological production process using the baculovirus–insect cell system, recombinant protein production was investigated in an immobilized cell culture with medium replacement. Sf9 insect cells were naturally entrapped within reticulated polyvinyl formal resin biomass support particles (BSPs; 2 x 2 x 2 mm cubes; pore diameter 30–50 μm) in a 2.5-l stirred-tank bioreactor. The immobilized cells were grown to a density of over 10^7 cells/cm^3-BSP, with the medium of 10% fetal bovine serum (FBS)-supplemented TNM-FH replaced at appropriate intervals, before infection with a recombinant baculovirus carrying the β-galactosidase gene. When serum-free TNM-FH was used instead of 10% FBS-supplemented TNM-FH for medium replenishment on and after post-infection day 1, the immobilized cells showed a high β-galactosidase yield comparable to that obtained in a culture using the serum-supplemented medium throughout. This finding indicates that immobilized insect cell culture allows not only intensification of cell culture but also recombinant protein production in protein-free basal media.

Keywords: Insect cell culture; Baculovirus; Immobilization; Biomass support particles; Bioreactors; Recombinant protein production

1. Introduction

The baculovirus–insect cell system has been extensively used not only in the expression of recombinant proteins for basic research applications but also in the production of bioinsecticides, vaccines, and diagnostic and therapeutic proteins [1–4]. On infection with a recombinant nucleopolyhedrovirus encoding the foreign gene of interest under the control of the very strong polyhedron promoter, insect cells in culture often express extremely large quantities of recombinant proteins through post-translational processing and modifications of higher eukaryotes during the very late stage of infection. Because of the lytic nature of the viral infection process, batch culture is commonly used for recombinant protein production using the baculovirus–insect cell system. Especially when infection is performed at high cell densities, however, protein production in batch culture is often limited by nutrient depletion in the culture medium [5–7], and fed-batch or perfusion cultures have been examined as attractive alternatives to batch culture to address this problem [8–12]. In a previous study [13], we applied the "passive" immobilization technique using porous biomass support particles (BSPs) [14–17] to the baculovirus–insect cell system in shake-flask culture. Sf9 insect cells were naturally retained within reticulated polyvinyl formal (PVF) resin BSPs (2 x 2 x 2 mm cubes) in situ in shake flasks and the entrapped cells grown to the high cell density of over 5×10^7 cells/cm^3-BSP with regular replacement of the culture medium. After infection with a recombinant baculovirus, the immobilized cells showed a high specific productivity when the medium was replaced at appropriate intervals to avoid nutrient depletion.

Insect cell culture has traditionally been performed in basal media supplemented

with around 10% vertebrate serum, most usually fetal bovine serum (FBS), to support cell growth, baculovirus infection, and recombinant protein production. The use of serum, however, suffers a number of disadvantages, including high cost, variable lot-to-lot performance, difficulties in downstream processing and purification of target products, and potential contamination by pathogenic agents such as mycoplasmas, viruses, and proteinaceous infectious particles (prions). Much effort has therefore gone into the development of serum-free media for use in insect cell culture [18]. Although such media are currently available commercially, more cost-effective serum-free culture technologies are still required for industrial-scale production of valuable biologicals. In a previous study [19], we examined the effect of medium cross-replacement with serum-supplemented and serum-free TNM-FH on recombinant β-galactosidase production in shake-flask cultures of non-immobilized Sf9 cells. When FBS was eliminated by replacing serum-supplemented TNM-FH with serum-free TNM-FH on post-infection day 1, virus-infected cells produced β-galactosidase with a yield nearly equal to that obtained in a culture where serum-supplemented TNM-FH was replaced with fresh serum-supplemented TNM-FH on day 1. When serum-supplemented TNM-FH was replaced with serum-free TNM-FH at less than 24 h post-infection, the β-galactosidase yield became lower the earlier the medium was replaced. This result shows that serum exerts a promoting effect on recombinant protein production up to post-infection day 1, but is not essential thereafter.

The above findings suggest a promising method of recombinant protein production: a two-step culture, in which cells are first grown and virus-infected in a serum-containing medium and recombinant protein is then produced in a protein-free basal medium after medium replacement on post-infection day 1. In the two-step culture,

almost all the recombinant protein is produced in the protein-free medium, which facilitates downstream processing and purification of target products and thereby reduces production costs. This is also the case for the production of proteins such as β-galactosidase expressed intracellularly by the baculovirus–insect cell system, since the proteins can be released into the culture medium due to the loss of cell membrane integrity caused by the lytic nature of the virus infection process [13, 20]. On the other hand, the two-step culture requires medium replacement involving separation of the medium from the cells in the course of the culture, which may be considered difficult on a large scale. The cell-medium separation can however be carried out simply and easily in immobilized cell culture as reported previously [13, 16]. In the present study, we investigated two-step culture of cells immobilized within BSPs in shake flasks and a 2.5-l stirred-tank bioreactor. Our results show that the immobilized cell culture allows not only intensification of cell culture but also recombinant protein production in protein-free basal media.

2. Materials and methods

2.1. Cell line, baculovirus, and media

The insect cell line used was Sf9 (Invitrogen, Carlsbad, CA, USA) derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda*. Cell density was determined by microscopically counting the number of cells with a Bürker-Türk hemocytometer, while cell viability was judged by trypan blue dye exclusion [7]. The

recombinant baculovirus used was *Autographa californica* nucleopolyhedrovirus (AcNPV) containing the *Escherichia coli* β-galactosidase gene inserted downstream of the polyhedrin promoter in place of the polyhedrin gene (Invitrogen). β-Galactosidase is reported to be relatively stable against proteases derived from the baculovirus–insect cell system [21], and was therefore employed as the model recombinant protein to evaluate the actual level of protein production without interference due to degradation. The virus stock solution titer was determined by plaque assay as described previously [7].

The Sf9 cells were maintained at 27°C in T-flasks in a non-humidified incubator. The culture medium used for routine maintenance was TNM-FH, consisting of 51.19 g/l TNM-FH (Sigma Chemical, St. Louis, MO, USA) and 0.35 g/l $NaHCO_3$, with addition of 10 mg/l gentamicin sulfate (Invitrogen) and 1 g/l Pluronic F-68 [block co-polymer glycol of poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene), Sigma], supplemented with 10% FBS (ICN Biomedicals, Aurora, OH, USA). TNM-FH is a modification of a basal synthetic medium, Grace's medium, by supplementation with 3.33 g/l lactalbumin hydrolysate and 3.33 g/l yeast extract. In the immobilized cell culture, serum-free TNM-FH and Grace's medium [44.5 g/l Grace's medium (Sigma), 0.35 g/l $NaHCO_3$, 10 mg/l gentamicin sulfate, and 1 g/l Pluronic F-68] were employed in addition to 10% FBS-supplemented TNM-FH. The serum-containing medium used in the bioreactor culture was supplemented with antifoam (Antifoam C Emulsion; Sigma), 0.2 ml/l.

2.2. Shake-flask culture of immobilized cells

A reticulated PVF resin sponge sheet consisting of filter material (Aion, Osaka, Japan; pore diameter 30–50 μm; porosity 0.88; apparent density 0.15 g/cm^3) was cut into 2 x 2 x 2 mm cubes for use as BSPs. Two-hundred and fifty BSPs were autoclaved with phosphate-buffered saline (PBS) in 100-ml screw-capped Erlenmeyer flasks. The Sf9 cells were dislodged from the T-flasks by streaming medium over the adherent cells with a pipette. After suspension in fresh serum-supplemented medium, cells were grown for a few days at 27°C in a spinner flask with constant stirring at around 90 rpm. Cells in the exponential growth phase were collected by centrifugation and resuspended in fresh serum-containing medium. After removal of PBS from the Erlenmeyer flasks and addition to each of 15 ml of cell suspension, the cells and the BSPs were incubated for 2 d at 27°C on a reciprocal shaker (90 oscillations/min; amplitude 30 mm) for inoculation. Following removal of the cell suspension, 15 ml of fresh serum-supplemented medium was added to each flask and incubation on a reciprocal shaker resumed, with the culture medium being replaced completely with fresh serum-supplemented medium every 2 days. At the time of each medium replacement, five BSPs were sampled from each flask for assessment of the density of viable cells entrapped within them by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [13].

After growth of the immobilized cells to a density of over 10^7 cells/cm^3-BSP and medium replacement with fresh serum-supplemented or serum-free medium, high-titer viral stock solution was added to each flask to give a multiplicity of infection (MOI) of 2 plaque-forming units per cell (pfu/cell). The time of viral solution addition was designated as post-infection time zero. Around post-infection day 1, the culture medium was replaced with 15 ml of fresh serum-containing or serum-free medium and

shake-flask culture of the immobilized cells was resumed, with the culture medium being replaced with 15 ml of the respective fresh medium every 2 days. At the time of each medium replacement, ten BSPs were removed from each flask to measure the immobilized cell density and the β-galactosidase activity. Prior to β-galactosidase activity assay, five BSPs and the removed culture broth were stored at –20°C.

2.3. Immobilized cell culture in bioreactor

Fig. 1 ➔ Fig. 1 shows a schematic diagram of the bioreactor system used (Tokyo Rikakikai, Tokyo, Japan). The glass bioreactor vessel consisted of a cylindrical body and a rounded bottom. Mixing in the reactor was performed by an impeller with four blades rotating at 90 rpm. A mixed gas of O_2 and N_2 was introduced from a nozzle at a constant rate set manually according to oxygen demand. The dissolved oxygen (DO) concentration in the culture medium was controlled at 20% oxygen saturation by automatically changing the composition of the supplied gas based on the reading of a DO sensor. The temperature was kept constant by recycling water from a water bath at 27°C through the jacket. The pH was monitored using a pH sensor, but, even without active adjustment, did not change substantially in the course of the cultures.

The bioreactor, containing 20,000 reticulated PVF resin BSPs [approximately 2 x 2 x 2 mm cubes (6.7 mm^3); pore diameter 30–50 μm; Aion] and water, was sterilized by autoclaving. After removal of water and addition of 1 l of cell suspension in fresh serum-supplemented medium to the bioreactor, the cells and the BSPs were agitated intermittently at 10-min intervals for an initial 3 h, after which the bioreactor was stirred continuously. The cell suspension was removed four days later and the cells retained

within the BSPs were cultured further in medium replaced completely with 1 l of fresh serum-supplemented medium every 2 days. Following growth of the immobilized cells to a density of over 10^7 cells/cm^3-BSP and removal of the conditioned medium, a high-titer viral stock solution was added together with 1 l of fresh serum-supplemented medium to give an MOI of 2 pfu/cell. The time of viral solution addition was designated as post-infection time zero. After incubation of the immobilized cells with serum-supplemented medium for about 1 d, the culture medium was replaced completely with fresh serum-containing or serum-free medium and immobilized cell culture was continued in the bioreactor, with the culture medium replaced with 1 l of the respective fresh medium every 2 days. The BSPs and the culture broth were sampled every day to measure the immobilized cell density and the *β*-galactosidase activity.

2.4. β-Galactosidase assay

To release intracellular *β*-galactosidase, 450 μl of 0.556% Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether) in Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, and 0.05 M 2-mercaptoethanol, pH 7.0) was added to 50-μl samples of the culture broth, while 1 ml of 0.5% Triton X-100 in Z buffer was added to five or ten BSPs. The preparations were then subjected to the appropriate shaking and dilution in Z buffer. *β*-Galactosidase activity was determined by enzymatic hydrolysis of *o*-nitrophenyl-*β*-D-galactoside (ONPG) to *o*-nitrophenol and galactose as previously described [7]. One unit (U) of *β*-galactosidase activity is defined as the amount of enzyme required to hydrolyze 1 μmol of ONPG to *o*-nitrophenol per minute at 28°C and pH 7.0.

3. Results

3.1. Two-step culture of immobilized cells in shake flasks

In light of the previous finding [19] with non-immobilized Sf9 cells that FBS is not essential to recombinant protein production in baculovirus-infected cells on and after post-infection day 1, an investigation was undertaken using two step culture of immobilized cells in shake flasks; the immobilized cells are first grown and virus-infected in serum-containing medium and recombinant protein is then produced in serum-free basal medium after medium replacement on post-infection day 1. Fig. 2 shows the time course of change in cell density and β-galactosidase production in shake-flask cultures of cells immobilized within BSPs and infected with the baculovirus at a density of around 2.9×10^7 cells/cm^3-BSP. No significant difference was observed in the time profile of immobilized cell density under any of the culture conditions tested (Fig. 2a). Since cell growth was not observed, essentially all cells appear to have been synchronously infected with the baculovirus at a low MOI of 2 pfu/cell. This may reflect underestimation of infectious baculovirus titers by conventional plaque assay [22]. In Fig. 2b, the β-galactosidase yield (total β-galactosidase produced per flask) was calculated from the β-galactosidase activity of the BSPs and the cumulative value of the β-galactosidase activities in the removed culture broth [13]. When serum-supplemented medium was used for replenishment throughout the culture period, the β-galactosidase yield reached over 7,000 U on day 7. In contrast, when serum-free

Fig. 2 ➔

TNM-FH was employed throughout, the β-galactosidase yield was approximately 5,000 U, around 2/3 of that obtained with the serum-supplemented medium. When serum-supplemented TNM-FH was replaced with serum-free TNM-FH on post-infection day 1, the β-galactosidase yield remained as high as in the culture using serum-supplemented TNM-FH throughout. These results, obtained in cells immobilized within BSPs at the high density of over 10^7 cells/cm^3-BSP, agree with those obtained with non-immobilized cells [19].

In a previous study [19], we found in shake-flask culture of non-immobilized Sf9 cells that both lactalbumin hydrolysate and yeast extract promote β-galactosidase production in baculovirus-infected cells. Lactalbumin hydrolysate and yeast extract also stimulated β-galactosidase production in the case of immobilized cells (Fig. 2b). In TNM-FH (Grace's medium supplemented with lactalbumin hydrolysate and yeast extract), a 3-fold higher β-galactosidase yield was obtained than in basal synthetic Grace's medium. When lactalbumin hydrolysate and yeast extract were removed by changing TNM-FH to Grace's medium on day 1, the β-galactosidase yield achieved was comparable to that in the culture using TNM-FH throughout. A similar result was obtained in a previous study with non-immobilized cells [19]. More interestingly, when serum-supplemented TNM-FH was replaced with Grace's medium on post-infection day 1, the virus-infected immobilized cells went on to produce β-galactosidase in a similar manner to those in the culture using serum-supplemented TNM-FH throughout, and with nearly equal product yield. A similar pattern was observed in shake-flask culture of non-immobilized cells (data not shown). These results clearly show that, in a basal synthetic medium such as Grace's medium, recombinant protein production can be adequately achieved using baculovirus-infected cells on and after

post-infection day 1.

3.2. Scaled-up culture of immobilized cells in bioreactor

Next, immobilized cell culture in the 2.5-l stirred-tank bioreactor was investigated. Sf9 cells were entrapped within BSPs at a cell density of more than 10^6 cells/cm^3-BSP by stirring empty BSPs with cell suspension in the bioreactor. The cells inoculated into the BSPs were then grown to the high cell density of over 10^7 cells/cm^3-BSP by replenishing the medium with serum-containing TNM-FH at appropriate intervals. Fig. 3 shows the time course of changes in immobilized cell density and β-galactosidase production after baculovirus infection in a bioreactor culture using 10% FBS-supplemented TNM-FH throughout. The β-galactosidase yield (total β-galactosidase produced in the bioreactor) reached over 4×10^5 U on post-infection day 6 (Fig. 3b). The specific productivity, given here by (total β-galactosidase produced)/(total number of immobilized cells on post-infection day 0), was calculated to be 178 U/(10^6 cells), which was slightly higher than that obtained in the shake-flask culture of immobilized cells with serum-supplemented TNM-FH [126 U/(10^6 cells)].

Fig. 3 ➔

Fig. 4 shows the time course of β-galactosidase production in a bioreactor culture where serum-free TNM-FH instead of serum-supplemented TNM-FH was used for replenishment on and after post-infection day 1. The β-galactosidase yield reached 4.6×10^5 U with specific productivity of 158 U/(10^6 cells), comparable to that obtained in the bioreactor culture using serum-containing TNM-FH throughout.

Fig. 4 ➔

4. Discussion

In the present study, two-step culture of Sf9 cells immobilized within porous PVF resin BSPs was carried out in a 2.5-l stirred-tank bioreactor as well as in 100-ml shake flasks. When incubated in serum-supplemented TNM-FH up to post-infection day 1, cells immobilized at the high density of over 10^7 cells/cm^3-BSP showed equal β-galactosidase production in TNM-FH regardless of whether FBS was present on and after post-infection day 1. During the baculovirus infection cycle, polyhedrin is expressed in the very late stage, after the polyhedrin promoter is switched on about 24 h post-infection [1]. The above finding therefore indicates that serum is required to stimulate recombinant protein production until the polyhedrin promoter is activated, but is not essential when recombinant proteins are abundantly produced instead of polyhedrin. Comparable-level production of β-galactosidase was also achieved in shake-flask culture of immobilized cells where serum-supplemented TNM-FH was changed to basal synthetic Grace's medium on post-infection day 1 (Fig. 2). Recombinant protein production by densely immobilized cells in a protein-free basal medium such as Grace's medium as well as TNM-FH would simplify separation and purification processes and reduce the manufacturing costs of cellular products. While the mechanism by which serum exerts a promoting effect on recombinant protein production in baculovirus-infected cells remains unknown, serum-free media for insect cells have been developed [18]. In the present study, 10% FBS-supplemented TNM-FH was used up to post-infection day 1, but serum-free media that support good cell growth and recombinant protein production appear to be usable as well. Unfortunately, however, commercially available serum-free media are often very expensive. Hence,

where a proprietary serum-free medium is employed, the use of a protein-free basal medium after about post-infection day 1 is still cost-effective especially for large-scale production of valuable biologicals.

The specific β-galactosidase productivity obtained in bioreactor culture was slightly higher than in shake-flask culture. Recombinant protein production by the baculovirus–insect cell system has been reported to be significantly depressed at low DO concentrations of near zero [5, 23]. In the present study, the DO concentration was maintained at 20% oxygen saturation in the bioreactor. Although the DO concentration was not measured during shake-flask culture, oxygen limitation in the inner part of BSPs may be the reason for the lower β-galactosidase productivity in this method. Since the immobilized cell bioreactor allows sufficient supply of oxygen, it should be useful for efficient large-scale manufacture of biologicals at high productivity. As cells were entrapped within 2-mm cubed BSPs, medium replacement was effected simply and easily in the bioreactor as well as in the shake flasks. The results obtained in the bioreactor culture were under suboptimal conditions, but more efficient protein production could be achieved by optimizing both particle number density and feeding strategies of medium and oxygen.

In other cell cultures for production of biologicals, the use of different media in accordance with the culture characteristics would realize efficient production under the optimal medium conditions, taking account of cases where the medium formulation appropriate for product formation is different from that for cell growth. For example, hybridoma cells grown in DF medium supplemented with insulin and transferrin are reported to produce sufficient amounts of monoclonal antibody in DF medium without the proteins [24]. High-level expression of human soluble thrombomodulin in serum-

free medium has also been demonstrated in recombinant CHO-K1 cells grown in serum-containing medium [25]. Since immobilization techniques facilitate the separation of cells from medium, immobilized cell culture allows not only intensification of cell culture by maintaining favorable conditions in the culture environment, but also efficient production of biologicals through use of different culture media according to the culture characteristics.

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Figure captions

Fig. 1. Schematic diagram of immobilized cell bioreactor system. M: motor; MFC: mass flow controller; P: peristaltic pump.

Fig. 2. Two-step culture of Sf9 cells immobilized within reticulated polyvinyl formal (PVF) resin biomass support particles (BSPs) in shake flasks. (a) Density of immobilized cells; (b) β-galactosidase yield. Mean of initial immobilized cell densities: 2.9×10^7 cells/cm^3-BSP. Cells were infected at a multiplicity of infection (MOI) of 2 plaque-forming units per cell (pfu/cell). Symbols indicate change from medium 1 to medium 2 at 22 h post-infection: TNM-FH supplemented with 10% fetal bovine serum (FBS) changed to FBS-supplemented TNM-FH (○), serum-free TNM-FH (□), or basal synthetic Grace's medium (Δ); serum-free TNM-FH changed to serum-free TNM-FH (◊) or Grace's medium (x); Grace's medium changed to Grace's medium (∇). Arrows indicate times of medium replacement.

Fig. 3. Production of β-galactosidase by BSP-immobilized cells in 2.5-l bioreactor culture using TNM-FH supplemented with 10% FBS throughout. Initial density of immobilized cells: 1.7×10^7 cells/cm^3-BSP. Cells were infected at an MOI of 2 pfu/cell. Symbols: ○, density of immobilized cells; □, β-galactosidase yield; Δ, β-galactosidase in the BSPs; ◊, β-galactosidase in the culture broth. Arrows indicate times of medium replacement.

Fig. 4. β-Galactosidase production in two-step culture of immobilized cells in

bioreactor. TNM-FH supplemented with 10% FBS was switched to serum-free TNM-FH at 20 h post-infection. Initial density of immobilized cells: 2.2×10^7 cells/cm^3-BSP. Cells were infected at an MOI of 2 pfu/cell. Arrows indicate times of medium replacement. Symbols are the same as in Fig. 3.

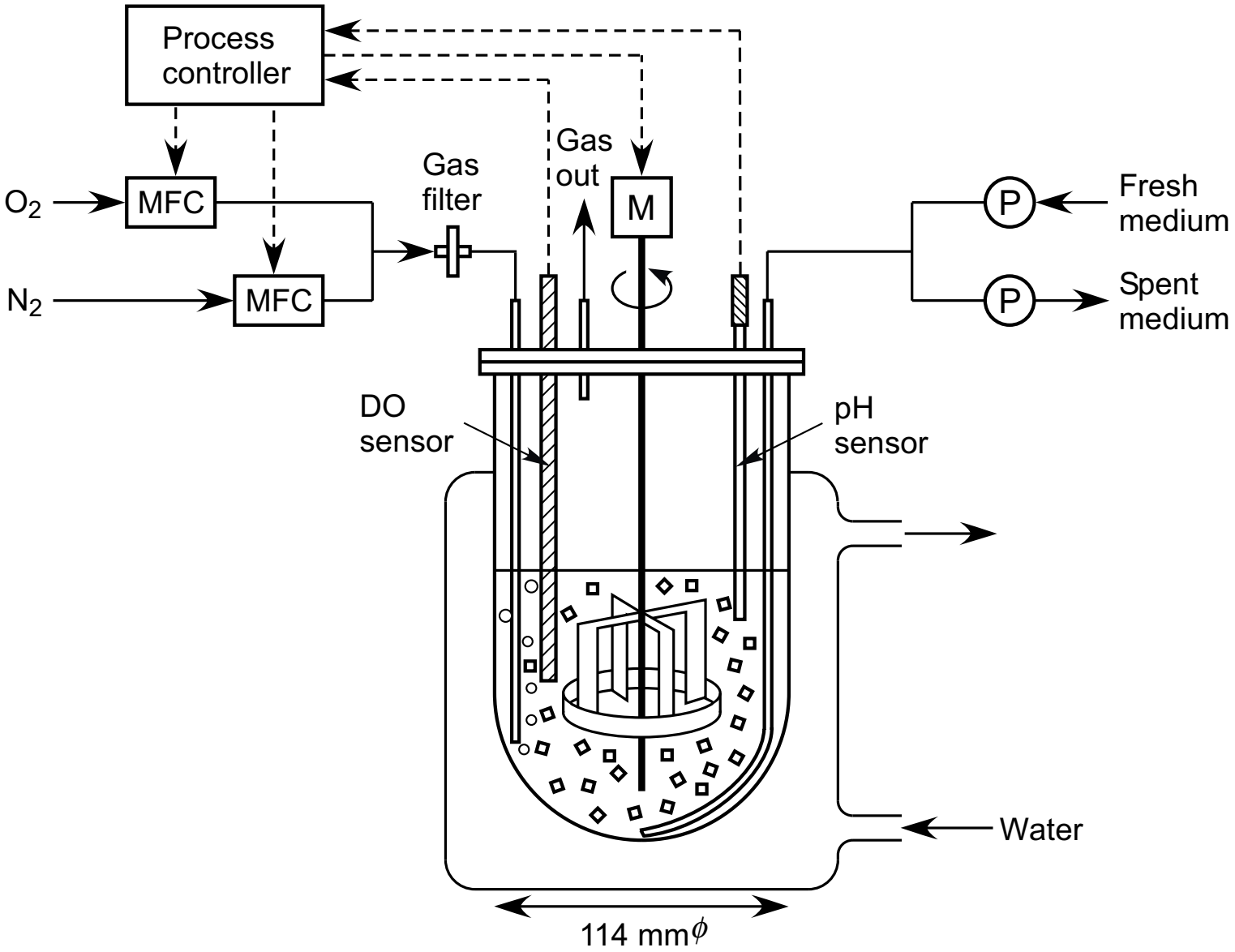

Fig. 1. Yamaji et al.

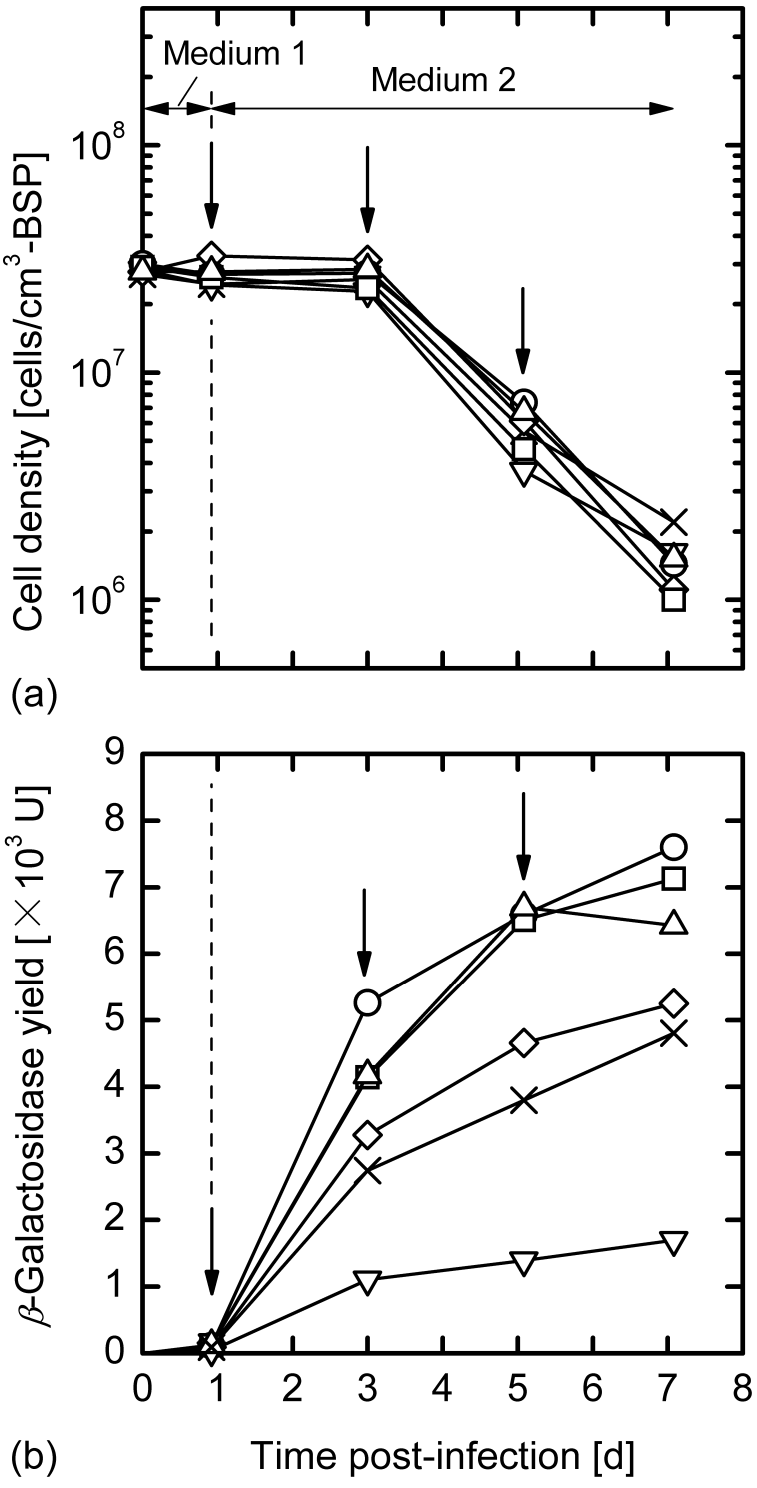

Fig. 2. Yamaji et al.

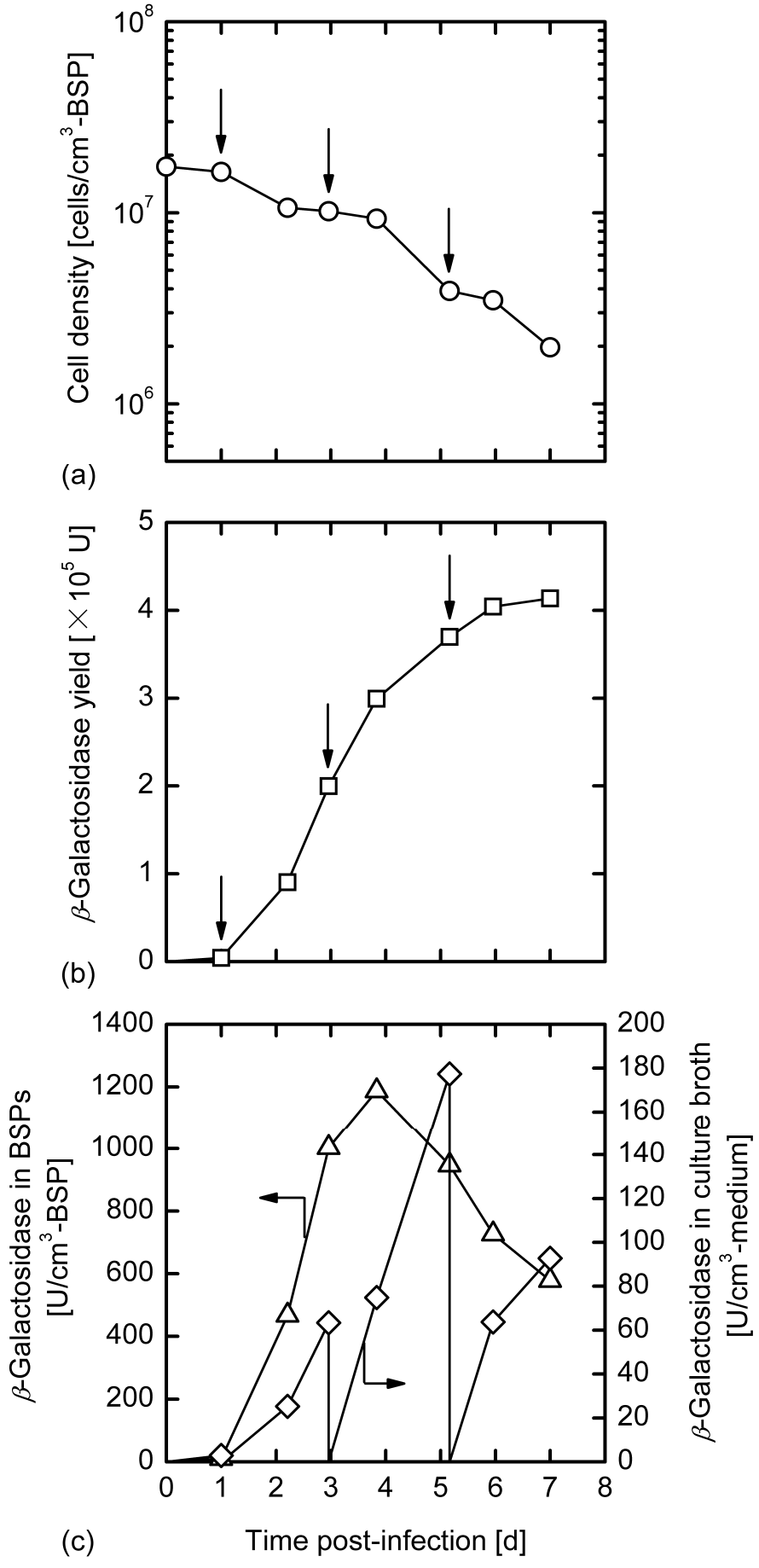

Fig. 3. Yamaji et al.

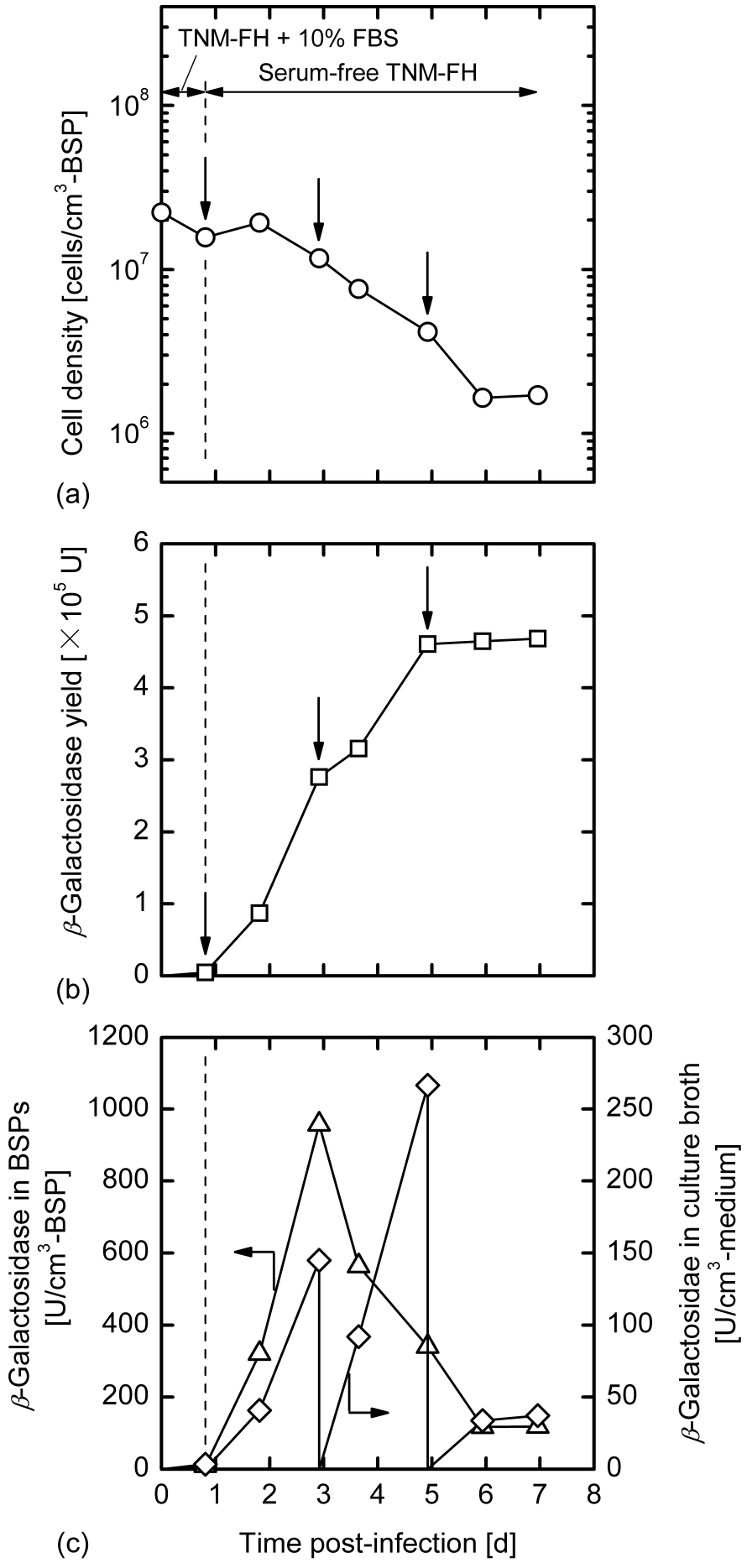

Fig. 4. Yamaji et al.