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Production of Extracellular Bifidogenic Growth Stimulator (BGS) from *Propionibacterium shermanii* Using a Bioreactor System with a Microfiltration Module and an On-line Controller for Lactic Acid Concentration

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Production of a bifidogenic growth stimulator (BGS) by *Propionibacterium freudenreichii* subsp. *shermanii* (*Propionibacterium shermanii*) using lactic acid as a carbon source was investigated using different cultivation methods. When a continuous bioreactor system with a filtration device was used at a dilution rate of 0.075 h^{-1}, the average BGS concentration was 2.4 mg/*l*, which corresponds to a BGS productivity per cultivation time of 1.8×10^{-1} mg·l^{-1}·h^{-1}. The BGS productivity per cultivation time in continuous cultivation with filtration was 1.9-fold that (9.4×10^{-2} mg·l^{-1}·h^{-1}) in a conventional batch cultivation. In fed-batch cultivation with feed-back control using an on-line lactic acid controller with a lactic acid biosensor, it was possible to prevent substrate inhibition by maintaining the lactic acid concentration in culture broth low at 3.3 g/*l*, and an enhanced BGS production (31 mg/*l*) was successfully attained. The BGS productivity per cultivation time (2.1×10^{-1} mg·l^{-1}·h^{-1}) in the fed-batch cultivation with feed-back control was 2.2-fold that in the conventional batch cultivation. A new bioreactor system was developed by coupling a continuous bioreactor system with a filtration device to an on-line lactic acid controller. Using the new bioreactor system, we produced BGS continuously at a high level of 47 mg/*l*. The BGS productivities per cultivation time (3.5 mg·l^{-1}·h^{-1}) and the total volume of medium used (1.7×10^{-1} mg·l^{-1}·h^{-1}) obtained in the new bioreactor system were 37-fold and 2.1-fold those in the conventional batch cultivation, respectively. These results described above clearly demonstrate the positive effects of both the continuous filtration for removal of metabolites (propionic and acetic acids) inhibitory to cell growth and feed-back control of lactic acid concentration in the culture broth on BGS production by *P. shermanii*. This paper is the first report on BGS production by the propionic acid bacterium using lactic acid as a carbon source.

[**Key words:** *Propionibacterium shermanii*, bifidogenic growth stimulator, lactic acid, membrane bioreactor, feed-back control]

Propionibacterial species are characterized as gram-positive, non-spore-forming, nonmotile, facultatively anaerobic or aerotolerant, and rod-shaped bacteria (1, 2). They are divided into two principal groups: the dairy and cutaneous propionibacteria. The former propionibacteria are important starter organisms in dairy fermentation and are best known to produce the characteristic eyes and flavor of Swiss-type cheeses (1–3). They also may contribute to natural fermentations of silage and olives (4) and can produce a variety of industrially important products such as propionic acid, vitamin B_{12}, and bacteriocins (5–7). Recently, propionic acid bacteria have received much attention as both probiotics beneficial for human health (8, 9) and producers of prebiotics stimulating selectively the growth of beneficial intestinal bacteria such as bifidobacterial species (10, 11).

In 1994, a novel bifidogenic growth stimulator (BGS) produced by *Propionibacterium freudenreichii* was found by Kaneko *et al.* (12), who showed that BGS is not a non-digestible sugar but a mixture of 1,4-dihydroxy-2-naphthoic acid (DHNA), 2-amino-3-carboxy-1,4-naphthoquinone, and unknown compounds. We have recently reported that BGS, mainly DHNA, is produced by propionibacterial strains such as *P. freudenreichii* subsp. *shermanii* (*Propionibacterium shermanii*), *P. acidipropionici*, and *P. jensenii* except for *P. freudenreichii* (13). The stimulation of the specific growth of bifidobacteria by BGS produced by the propionic acid bacteria is considered to be due to a mechanism quite different from that induced by oligosaccharides (14). A combination of BGS produced by a propionic acid bacterium and conventional oligosaccharides as prebiotics is expected to result in an additional and/or synergistic effect on the main-

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tenance of desirable intestinal microflora. BGS produced by propionic acid bacteria is recognized to be one of the most important nonsugar prebiotics that play an increasingly important role in improving human health. However, there are no reports on the enhanced production of BGS from propionibacterial strains by developing a sophisticated bioreactor system. We previously developed bioreactor systems with a microfiltration module, which enables the continuous removal of metabolites inhibitory to cell growth and production of a target product, and the complete recycling of cells to the bioreactor (15–20). Although continuous bioreactor systems with membrane separation have disadvantages inherent in practical applications such as scale-up and membrane maintenance (21), we successfully applied a bioreactor system to the production of useful materials, such as starter cultures (15, 16), vitamin B_{12} (17), intracellular enzyme (18, 19), and bacteriocin (20), by several anaerobic bacteria that themselves excrete growth inhibitors. On the other hand, it was found by many researchers that unlike many other bacteria, propionic acid bacteria prefer lactic acid to lactose and glucose (22). We also found that *P. freudenreichii* preferentially assimilates lactic acid prior to glucose, independent of the pH of the medium when the medium containing a mixture of lactate and glucose was used (23). Recently, we have reported that the preferential substrate utilization of *P. shermanii* could be applied to the production of high optical purity of lactic acid by eliminating L-lactate and D-lactate initially contaminating in starting materials while preserving glucose as the substrate for the subsequent fermentation of lactic acid (24).

In the present work, we evaluated the positive effect of utilization of a continuous bioreactor system on the production of BGS by *P. shermanii* using lactic acid as a carbon source. To alleviate growth inhibition by lactic acid used as a substrate and consequently to obtain a high productivity of BGS, we used an on-line lactic acid controller for maintaining the concentration of lactic acid suitably low in fed-batch cultivation. Furthermore, we investigated the efficient production of BGS by the propionibacterial strain using the continuous bioreactor system coupled to an on-line lactic acid controller.

MATERIALS AND METHODS

Microorganisms and culture medium *P. freudenreichii* subsp. *shermanii* PZ-3 (*Propionibacterium shermanii*) kindly provided by Prof. N. Nishio, Hiroshima University was used as the BGS-producing strain in this study. *Bifidobacterium longum* OLB 6001 obtained from Meiji Dairies Corporation (Odawara) was used as a test microorganism for BGS activity assay. The microorganisms were anaerobically cultured at 37°C in TPY medium containing 10 g of lactic acid, 8 g of trypticase peptone (Becton Dickinson, Franklin Lakes, NJ, USA), 3 g of phytone peptone (Becton Dickinson), 5 g of yeast extract (Becton Dickinson), 2 g of K_2HPO_4, 3 g of KH_2PO_4, 0.5 g of $MgCl_2 \cdot 6H_2O$, 0.5 g of L-cysteine, and 0.01 g of $FeSO_4 \cdot 7H_2O$ per liter (pH 6.5).

Evaluation of tolerance to organic acids *P. shermanii* was cultivated in test tubes to examine the effects of initial concentrations of carbon source (lactic acid) and metabolites (propionic and acetic acids) on cell growth and metabolic activities. The bacterium was precultivated statically in test tubes containing 10 ml of TPY medium. The precultured cells were separated from the culture broth containing metabolites by centrifugation at 25,700×*g* for 10 min and then inoculated into fresh medium with one of the organic acids at an initial turbidity in the 0.05–0.10 range at 660 nm. Lactic acid and a mixture of propionic and acetic acids at various initial concentrations were initially added to fresh TPY medium. The initial pH of the medium containing one of the organic acids or a mixture of propionic and acetic acids was adjusted to 6.5 by adding 4 N NaOH. The effect of each organic acid or a mixture of propionic and acetic acids was evaluated on the basis of initial specific growth rate (μ), turbidity at 660 nm, lactic acid consumption, and BGS production for 48 h.

Batch cultivation Batch cultivation was carried out in a bioreactor with a working volume of 0.7 *l* (TBR-1; Chiyoda Seisakusho, Nagano). The initial pH of the medium containing 10 g/*l* lactic acid as a carbon source was adjusted to 6.5 by adding 4 N NaOH and the pH was maintained at 6.5 throughout the cultivation by adding 4 N NaOH using a peristaltic pump coupled to a pH controller. A mixture of N_2 and CO_2 at a volume ratio of 9:1 was sparged at 0.3 vvm throughout the cultivation to maintain anaerobic conditions.

Continuous cultivation with a filtration device The bioreactor system described previously (16–20) was used for continuous cultivation with a filtration unit in this study. It consisted of a microfiltration module (Microza PSP 103; Asahi Kasei Co., Tokyo) with an effective filtration area of 0.2 m^2 for the continuous cross-flow filtration of the culture broth. Prior to use, the membrane module was sterilized with a sodium hypochlorite as described previously (17). The rates of filtration of the culture broth and supply of fresh medium containing 10 g/*l* lactic acid were both usually maintained constant at 52.5 ml/h, which corresponds to a dilution rate (D) of 0.075 h^{-1}.

Fed-batch cultivation using a lactic acid controller In fed-batch cultivation, an on-line lactic acid controller (BF-400; Able & Biott Co., Tokyo), in which the lactic acid concentration in the sample was enzymatically determined with a biosensor using lactate oxidase (EC 1.13.12.4.), was coupled to the bioreactor for batch cultivation described above. The system allowed not only the automatic measurement of the concentration of lactic acid in culture broth every 20 min but also the control of the concentration of lactic acid in culture broth at a desired level by supplying the fresh TPY medium containing 50 g/*l* lactic acid on the basis of the lactic acid concentration determined during cultivation. In the controller, the culture broth used for the measurement was diluted to a suitable concentration of lactic acid by a membrane dialysis device; therefore, the culture broth once used was discarded without returning to the bioreactor. The volume of culture broth required for a cycle of measurement of lactic acid concentration in culture broth was 5 ml. The measurement and control using the controller were carried out at 2-h intervals and the lactic acid concentration in the culture broth was usually maintained at about 3 g/*l*. The other culture conditions were identical to those in batch cultivation described above.

Continuous cultivation with a filtration device and a lactic acid controller The bioreactor system with continuous filtration and feed-back control of lactic acid concentration was constructed by combining the continuous cultivation system with a microfiltration module and the on-line lactic acid controller described above. In the continuous cultivation, the rate of filtration of culture broth was usually maintained constant at 52.5 ml/h (D = 0.075 h^{-1}), which is similar to that for the continuous cultivation with a filtration device described above. After the start of filtration of culture broth, TPY medium containing 10 g/*l* lactic acid was intermittently supplied to the bioreactor with a peristaltic pump coupled to a level controller. When the concentration of lactic acid decreased to below 3 g/*l*, the control of lactic acid concentration was started by supplying TPY medium containing 50 g/*l* lactic acid with another

peristaltic pump coupled to the on-line controller. That is, a feed pump connected to a level controller and another pump coupled to the on-line controller were used to supply fresh medium to keep both the working volume in the bioreactor at 0.7 *l* and the lactic acid concentration in the culture broth at approximately 3 g/*l*.

Analytical methods Cell concentration was determined by measuring turbidity at 590 or 660 nm. The number of viable propionibacterial cells was counted by the plate culture method. Viable cell number was expressed as colony forming units per milliliter (cfu/ml). The supernatant obtained by centrifugation (25,700×*g*, 10 min) of culture broth was analyzed for BGS, lactic acid, acetic acid, and propionic acid concentrations. BGS concentration was measured by a modified agar diffusion plate method, as reported previously (13). The concentrations of lactic, acetic, and propionic acids were simultaneously measured using an HPLC system (Shimadzu, Kyoto) for analysis of organic acids, as reported previously (25).

RESULTS AND DISCUSSION

Inhibitory effect of lactic acid on cell growth Propionic acid bacteria can utilize lactic acid more readily than lactose and glucose (21–23). However, the high concentration of lactic acid is considered to be inhibitory to cell growth and BGS production by the bacteria. *P. shermanii* was grown in test tubes containing fresh medium with lactic acid at various initial concentrations, and cell growth (specific growth rate and turbidity) and metabolic activities (lactic acid consumption and BGS production) were compared. Figure 1 shows the effect of initial lactic acid concentration on cell growth and metabolic activities. The specific growth rate and turbidity at 48 h were significantly affected by the initial concentration of lactic acid. When the bacterium was cultivated in the culture broth containing 20 g/*l* lactic acid, the specific growth rate and turbidity were lower than one-fifth those in the culture broth containing 5 g/*l* lactic acid. As the initial concentration of lactic acid increased, the concentration of lactic acid consumed at 48 h gradually decreased similarly to specific growth rate and turbidity. BGS production by the bacterium was very sensitive to the initial concentration of lactic acid, and BGS concentration in the culture broth with 20 g/*l* lactic acid decreased more than two orders of magnitude of the value in the culture broth with 5 g/*l* lactic acid. The results show that it is necessary to maintain lactic acid concentration in culture broth lower than 10 g/*l* in order to keep BGS production and cell growth as high as possible.

Batch cultivation Figure 2 shows the results of the batch cultivation of *P. shermanii* using 10 g/*l* lactic acid as a carbon source. The pH of the medium was maintained at 6.5 throughout the cultivation. The bacterium was observed to grow logarithmically in the initial 16 h. Although lactic acid remained in the broth, the growth of *P. shermanii* stopped after 32 h. This decrease in growth rate at the late stage of the cultivation seems to be not caused by the depletion of nutrients in the medium, but by the accumulation of metabolites inhibitory to cell growth, similarly to other organic acid-producing bacteria reported previously (15–20). Propionic acid (6.4 g/*l*) and acetic acid (2.5 g/*l*) accumulated as the main metabolites, while 10 g/*l* lactic acid was consumed completely. The final turbidity obtained at 48 h was 3.8, at which the viable cell concentration was 5.5×10^9 cells/ml.

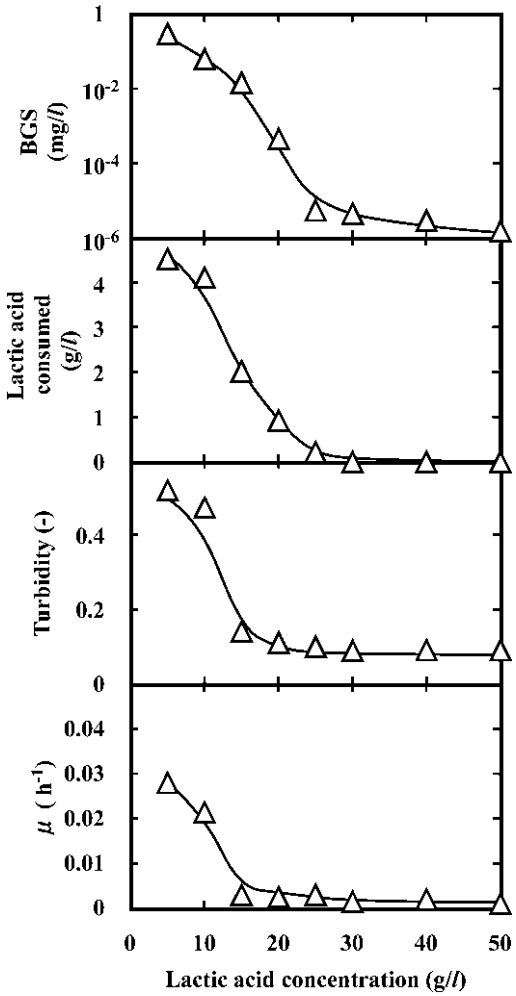

FIG. 1. Effect of initial lactic acid concentration on cell growth, lactic acid consumption, and BGS production by *P. shermanii*. The cultivation was carried out in test tubes for 48 h. The specific growth rates were examined on the basis of the initial increase in turbidity at 590 nm.

BGS concentration increased gradually as the cells grew and then remained nearly constant at 3.8 mg/*l* after 40 h.

Table 1 shows the inhibitory effect of the concentrations of propionic and acetic acids on cell growth (specific growth rate and turbidity) and metabolic activities (lactic acid consumption and BGS production). All of the four measured parameters were significantly affected by propionic acid even at low concentrations. When the initial concentration of propionic acid was 5 g/*l*, the specific growth rate and the concentration of BGS produced were about one-third and one-fourth, respectively, as compared with those in fresh medium without both organic acids. Cell growth rate and metabolic activities gradually decreased as the initial concentration of acetic acid increased although the degree of inhibition by acetic acid was lower than that by propionic acid. Moreover, when a mixture of propionic and acetic acids at a ratio of 2:1, which was observed usually in anaerobic cultivation of *P. shermanii*, was initially added, cell growth rate and metabolic activities significantly decreased with increasing initial total concentrations of propionic and

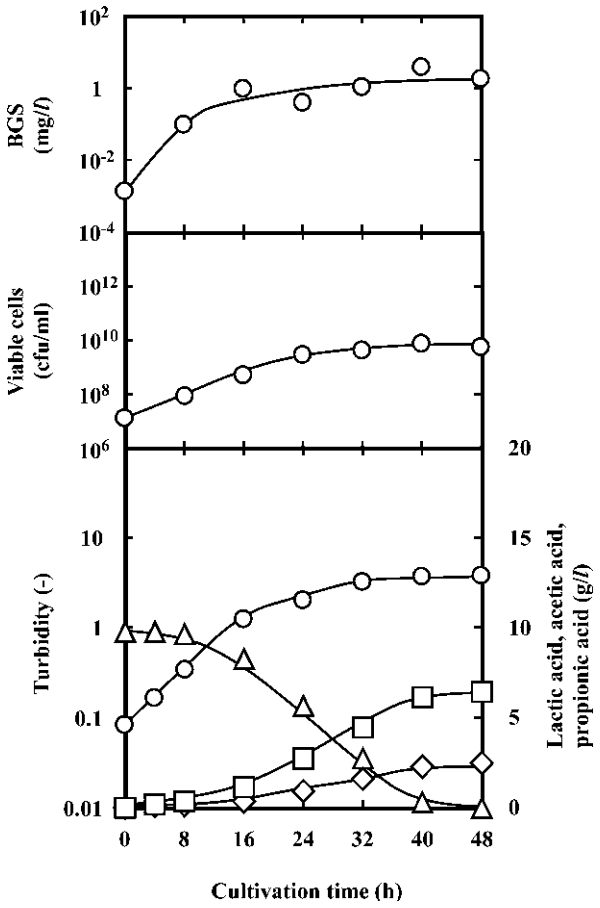

FIG. 2. Batch cultivation of *P. shermanii*. Symbols: open circles, BGS, viable cells, and turbidity at 660 nm; open triangles, lactic acid; open diamonds, acetic acid; open squares, propionic acid.

acetic acids. The results suggest that removal of propionic and acetic acids, the metabolites inhibitory to cell growth and metabolic activities, is expected to prolong logarithmic growth phase and to achieve a high BGS productivity.

Continuous cultivation with a filtration device We applied a bioreactor system with a microfiltration module to remove propionic and acetic acids continuously and attain a high cell concentration for continuous production of BGS. Figure 3 shows the results of continuous cultivation with filtration at a constant rate of 52.5 ml/h. The filtration rate was decided upon tentatively to evaluate the effect of the removal of metabolites on cell growth and BGS production. Continuous production of BGS and filtration were started at 24 h of cultivation when the growth rate began to decrease. The concentrations of propionic and acetic acids in the culture broth were kept lower than those in batch cultivation (Fig. 2); consequently, the high growth rate continued until 60 h. Thereafter, the cell concentration was maintained high and the apparent concentration of lactic acid was nearly zero after 72 h although lactic acid was supplied at a constant rate. The number of viable cells reached more than 4×10^{10} cfu/ml at 36 h. However, the viable cell concentration decreased to $1–5\times10^{9}$ cfu/ml after the depletion of lactic acid, as shown in Fig. 3. The concentrations of propionic and acetic acids increased gradually up to 72 h, subsequently remaininig constant at 6.4 g/*l* and 3.1 g/*l*, respectively. The BGS concentration gradually increased during the continuous operation by the complete recycling of cells and subsequently remained nearly constant after 120 h. BGS was produced continuously for 120–216 h and its average concentration was about 2.4 mg/*l*.

Fed-batch cultivation using a lactic acid controller In the continuous cultivation with filtration at a constant rate, lactic acid supplied as a carbon source was consumed completely by cells during the continuous operation as described above. The lack of lactic acid in the culture broth

TABLE 1. Inhibitory effects of the initial concentrations of metabolites on cell growth, lactic acid consumption, and BGS production by *P. shermanii*

Initial conditions[a]		μ[b] (10^{-2} h^{-1})	Turbidity[c] (660 nm) (−)	Lactic acid consumed[c] (g/*l*)	BGS[c] (10^{-2} mg/*l*)
Propionic acid (g/*l*)	Acetic acid (g/*l*)				
0	0	2.15	0.49	4.18	18.1
5	0	0.80	0.26	3.32	4.94
10	0	0.60	0.23	1.41	2.99
15	0	0.30	0.18	1.31	2.93
20	0	0.20	0.17	0.98	2.79
30	0	0.05	0.13	0.39	0.39
0	2.5	2.10	0.42	3.88	10.5
0	5	1.85	0.39	3.21	7.41
0	10	1.25	0.28	3.14	4.73
0	15	1.00	0.23	2.42	3.46
0	20	0.90	0.22	1.16	3.00
3.4	1.6	1.73	0.35	3.51	7.06
5	2.5	0.74	0.26	3.12	3.55
10	5	0.45	0.20	0.84	2.84
20	10	0.19	0.17	0.13	0.91
26.7	13.3	0.05	0.13	0.02	0.30

[a] The initial lactic acid concentration was 10 g/*l*.
[b] The specific growth rates were examined on the basis of the initial increase in turbidity at 590 nm.
[c] Data were obtained at 48 h of cultivation.

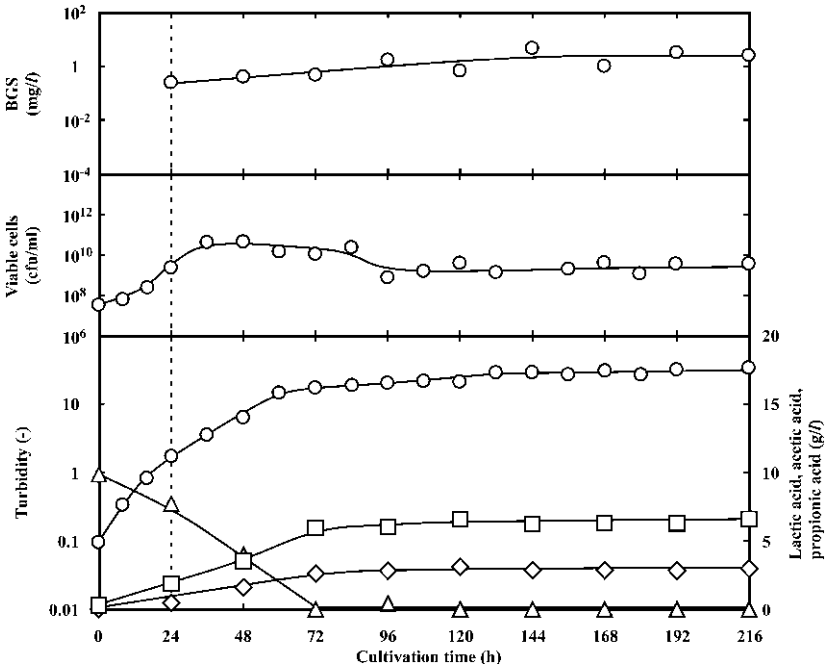

FIG. 3. Continuous production of BGS using a bioreactor with a microfiltration module. Filtration was started at 24 h, as indicated by the dotted line. D was maintained at 0.075 h^{-1}. The same volume of fresh medium with 10 g/*l* lactic acid as that of the filtrate was supplied. Symbols are the same as those shown in Fig. 2.

might cause the decrease in viable cell concentration and consequent decrease in BGS productivity. To examine the effect of the control of lactic acid concentration in the culture broth on cell growth and BGS production, we combined an on-line controller of lactic acid concentration with the bioreactor for batch cultivation. Apart from feed-back control based on the indirect measurement of lactic acid in culture broth such as pH change (26), to our knowledge, no other study on fed-batch cultivation with feed-back control by direct measurement of lactic acid in culture broth has been reported. Figure 4 shows the results of fed-batch cultivation with control of lactic acid concentration at 3 g/*l* using the on-line controller. The control of lactic acid concentration was started at 42 h of cultivation when the concentration decreased to below 3 g/*l*. Using the on-line controller, the concentration of lactic acid remained fairly constant throughout the fed-batch cultivation. Indeed, the average concentration of lactic acid determined by HPLC was 3.3 g/*l* during the fed-batch cultivation, as shown in Fig. 4. After 48 h, turbidity was maintained although lactic acid was intermittently supplied. The number of viable cells reached more than 1×10^{10} cfu/ml during 48–96 h. However, the number of viable cells gradually decreased after 96 h and then remained at $1–4\times10^{9}$ cfu/ml during 144–216 h. The decrease in cell concentration after 96 h was probably due to propionic acid accumulated at high concentrations (about 20 g/*l*). BGS concentration gradually increased during the feeding operation, reaching the maximum of 30.7 mg/*l* at 144 h. After 144 h, BGS concentration decreased very slowly. The maximum concentration of BGS obtained in the fed-batch cultivation is approximately 8-fold that in the batch cultivation. The control of lactic acid concentration in the culture broth using the on-line controller resulted in the significant increase in BGS concentration, although the cultivation time required for reaching the maximum BGS concentration was longer than that for the batch cultivation.

Continuous cultivation with a filtration device and a lactic acid controller To clarify the combined effect of continuous filtration and the feed-back control of lactic acid concentration described above on cell growth and BGS production, we used the continuous cultivation system with a filtration device and a lactic acid controller for BGS production by *P. shermanii*. Figure 5 shows the results of the continuous cultivation with not only filtration at a constant rate of 52.5 ml/h ($D=0.075$ h^{-1}) but also the control of lactic acid concentration at 3 g/*l* using the on-line controller. To maintain the lactic acid concentration in the culture broth at 3 g/*l*, TPY medium containing 10 g/*l* lactic acid was supplied with a peristaltic pump coupled to a level controller in a filtration device and TPY medium containing 50 g/*l* lactic acid was also fed with another peristaltic pump coupled to the on-line controller as described in Materials and Methods. As shown in Fig. 5, both the filtration of culture broth and the supply of fresh medium were started at 24 h and the control of lactic acid concentration in the culture broth was started at 46 h. The concentration of lactic acid in the culture broth remained nearly constant during 108–216 h of the cultivation. The average concentration of lactic acid in culture broth determined by HPLC was 2.1 g/*l* during 144–216 h of the cultivation. The difference between the set lactic acid concentration and the measured lactic acid concentration was probably attributable to the rapid consumption of lactic acid supplied by viable cells at a high concentration as described below. To control accurately the lactic acid concentration in culture broth at a set level, the concentration of lactic acid in fresh medium and/or the feeding speed of fresh medium

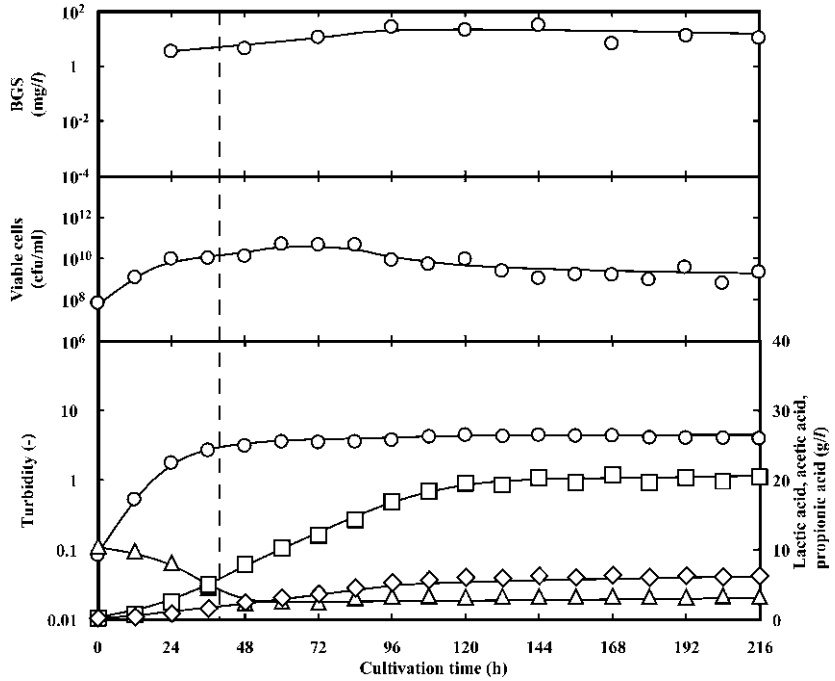

FIG. 4. Fed-batch production of BGS using a bioreactor with an on-line lactic acid controller. Control of lactic acid concentration in the culture broth was started at 42 h, as indicated by the broken line. The concentration of lactic acid was maintained at about 3 g/*l* by feeding fresh medium with 50 g/*l* lactic acid. Symbols are the same as those shown in Fig. 2.

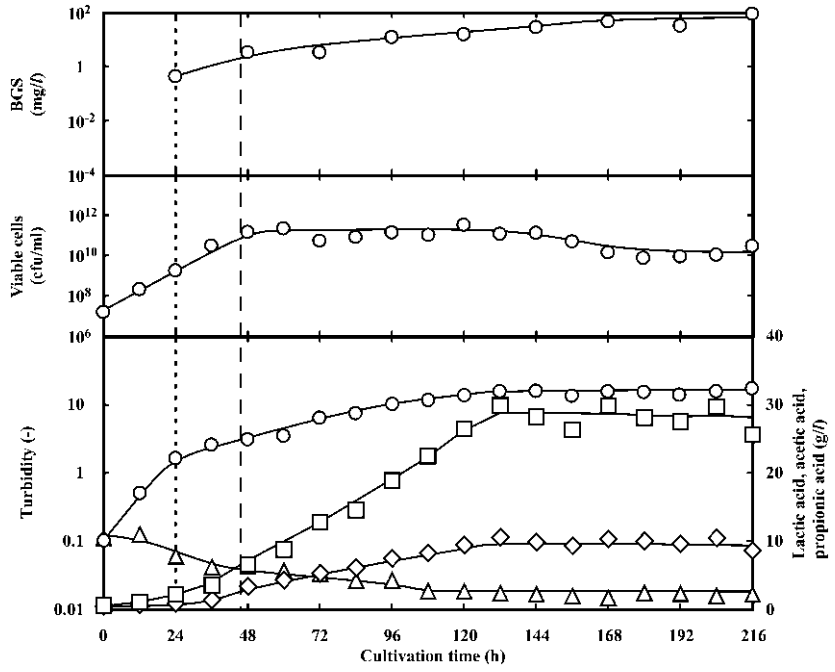

FIG. 5. Continuous production of BGS using a bioreactor with a microfiltration module and an on-line lactic acid controller. Filtration was started at 24 h, as indicated by the dotted line. D was maintained at 0.075 h^{-1}. The same volume of fresh medium with 10 g/*l* lactic acid as that of the filtrate was supplied. Control of lactic acid concentration in the culture broth was started at 46 h, as indicated by the broken line. The concentration of lactic acid was maintained at about 3 g/*l* mainly by feeding fresh medium with 50 g/*l* lactic acid. Symbols are the same as those shown in Fig. 2.

need to be regulated according to its consumption rate. Using the continuous cultivation system with a lactic acid controller and a filtration unit, the turbidity at 660 nm and the number of viable cells significantly increased to 15 at 120 h and more than 1×10^{11} cells/ml at 48 h, respectively, which were higher than those in fed-batch cultivation with an on-

TABLE 2. Comparison of BGS productivity among different cultivation methods

Run no.	Cultivation method	Total cultivation time T (h)	Total amount of lactic acid S_t (g)	Total volume of medium used V_t (l)	Turbidity (660 nm) (–)	Viable cells (10^9 cfu/ml)	Propionic acid (g/l)	Acetic acid (g/l)	BGS B_{av} (mg/l)	Total amount of BGS B_t (mg)	BGS productivity			Remarks
											$B_{av}D$ (10^{-2} mg·l^{-1}·h^{-1})	$B_t/(S_t \cdot T)$ (10^{-3} mg·l^{-1}·h^{-1})	$B_t/(V_t \cdot T)$ (10^{-2} mg·l^{-1}·h^{-1})	
1	Batch cultivation	48	7.0	0.7	3.8	5.5	6.4	2.5	3.8[a]	2.7	9.4[a]	8.0	8.0	Fig. 2
2	Continuous cultivation with a filtration device	216	108	10.8	28.9[b]	3.0[b]	6.4[b]	3.1[b]	2.4[b]	17.5	18.0[b]	0.75	0.75	Fig. 3
3	Fed-batch cultivation with a lactic acid controller	216	26.0	1.1	4.4	1.1	20.5	6.3	30.7[a]	33.2	21.4[a]	5.9	14.0	Fig. 4
4	Continuous cultivation with a filtration device and a lactic acid controller	216	–[c]	10.8	15.2[b]	35[b]	27.7[b]	9.8[b]	46.8[b]	391	351[b]	–	16.8	Fig. 5

[a] Data were obtained at 40 h of batch cultivation (run 1) and at 144 h of fed-batch cultivation with a lactic acid controller (run 3).
[b] Data are the mean values during stationary phase of 120–216 h for run 2 and 144–216 h for run 4.
[c] The total amount of lactic acid used in run 4 was not precisely determined.

line lactic acid controller (Fig. 4). This is probably due to continuous supply of other fresh nutrients as well as lactic acid by a feed pump connected to a level controller. However, the high cell concentration caused the accumulation of metabolites inhibitory to cell growth at high concentrations despite the continuous removal of the culture broth containing the metabolites by filtration. After 168 h, the cell concentration remained nearly constant and the average concentrations of propionic and acetic acids were about 28 g/l and 9.8 g/l, respectively. The BGS concentration increased gradually up to 144 h, and then remained at the maximum of 47 mg/l. The maximum BGS concentration obtained in the continuous cultivation (Fig. 5) was 12–20-fold those in both the batch cultivation (Fig. 2) and continuous cultivation with filtration (Fig. 3), and 1.5-fold that in the fed-batch cultivation with the lactic acid controller (Fig. 4), respectively.

Comparison of BGS productivity among different cultivations Table 2 shows a comparison of BGS productivities in terms of the total amount (S_t) of lactic acid added as a carbon source and the total volume (V_t) of medium used as well as cultivation time (T) among different cultivation methods. That is, BGS productivity is expressed by not only $B_{av}D$ but also $B_t/(S_t \cdot T)$ and $B_t/(V_t \cdot T)$, where B_{av}, B_t, and D denote average BGS concentration in the cultivation, total amount of BGS produced, and dilution rate, respectively. The turbidity remained high in the continuous cultivation with a filtration device (run 2 in Table 2), but the mean concentration of viable cells for continuous operation was lower than the final concentration in the conventional batch cultivation (run 1 in Table 2). The reason seems to be the high ratio of dead cells to total ones. The continuous removal of culture broth containing metabolites inhibitory to cell growth is considered to be favorable mainly for the maintenance of high growth rate and high cell concentration, as shown in Fig. 3. The mean concentration of BGS obtained in run 2 was lower than that in run 1, but the BGS productivity per cultivation time, $B_{av}D$, in run 2 was 1.9-fold that in run 1. In the fed-batch cultivation with feed-back control of lactic acid concentration (run 3 in Table 2), lactic acid concentration in the culture broth was maintained at about 3 g/l and the enhanced BGS concentration (30.7 mg/l) was observed in spite of the pronounced increase in the concentrations of propionic and acetic acids. The maximum concentration of BGS obtained at 144 h in run 3 was 8.1-fold that in run 1 and 12.8-fold that in run 2. $B_{av}D$ obtained in run 3 was 2.3-fold that in run 1. Thus, the feed-back control of lactic acid concentration using the on-line controller probably resulted in the increase in BGS productivity per cultivation time. The results described above show that BGS was successfully produced using the continuous cultivation system with a filtration device and a lactic acid controller (run 4 in Table 2). By supplying fresh medium with lactic acid using the on-line controller corresponding to the consumption of lactic acid, the number of viable cells was maintained high during the cultivation. The average number of viable cells in run 4 was 3.5×10^{10} cfu/ml during 144–216 h which was much higher than those in runs 2 and 3. B_{av} obtained in run 4 was 12–20-fold those in runs 1 and 2 and 1.5-fold that in run 3. Consequently, B_t and $B_{av}D$ obtained in run 4 were 145-fold and 37-fold those in run 1, respectively. The high $B_{av}D$ clearly demonstrated the combined effect of both continuous filtration for removing inhibitory metabolites and the feed-back control of lactic acid concentration in the culture broth on BGS production by *P. shermanii*.

To compare precisely the performances of different cultivation techniques, BGS productivities in terms of S_t and V_t should be used, as reported previously (16, 18, 20). In runs 2 and 4, a large volume of fresh medium with lactic acid was supplied. This decreases the efficiency for utilization of nutrients in the medium. BGS productivity per total volume of medium used, $B_t/(V_t \cdot T)$, in run 2 was approximately one-tenth that in the case of batch cultivation (run 1), but $B_t/(V_t \cdot T)$ values obtained in runs 3 and 4 were 1.8-fold and 2.1-fold that in run 1, respectively. The feed-back control of lactic acid concentration in culture broth using the on-line controller enabled the increase in $B_t/(V_t \cdot T)$. However, BGS productivity per total amount of lactic acid used, $B_t/(S_t \cdot T)$, obtained in run 3 was lower than that in run 1 mainly because of the long time required to reach the maximum BGS concentration in run 3. In runs 2 and 4, a large volume of fresh medium containing lactic acid was necessary to maintain the concentrations of metabolites inhibitory to cell growth and BGS production low. This resulted in the very low $B_t/(S_t \cdot T)$ in runs 2 and 4, since $B_t/(S_t \cdot T)$ in run 4 was estimated to be lower than 5.0×10^{-4} mg·g^{-1}·h^{-1} although the precise value was not obtained. The highest $B_t/(S_t \cdot T)$ was obtained in run 1 among different cultivation methods. Thus, the low productivity in run 2, when the volume of the medium used is considered, can be overcome to some extent using an on-line lactic acid controller, as shown in runs 3 and 4. However, improvement of $B_t/(S_t \cdot T)$ cannot be achieved in run 4 because a large volume of the medium

with lactic acid was required to maintain the concentrations of metabolites inhibitory to cell growth and formation of the target product low. To improve further $B_t/(S_t \cdot T)$ as well as $B_t/(V_t \cdot T)$, the rates of filtration of culture broth and supply of the fresh medium, and the lactic acid concentrations in culture broth and fresh medium for feeding will be optimized in future studies. Moreover, the dynamic metabolic changes caused by the removal of inhibitory metabolites and maintenance of the concentration of a carbon source suitably low are of great interest in relation to BGS production. We are now investigating the effect of the concentrations of some carbon sources on the activities of enzymes involved in BGS biosynthesis. The results obtained will be reported elsewhere.

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