Studies on Production of Bifidogenic Growth Stimulator by Propionibacterial Strains

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Tomoaki Kouya

Graduate School of Science and Technology
Niigata University

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Tomoaki Kouya

高屋　朋彰

Graduate School of Science and Technology
Niigata University

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Chapter 1

General Introduction

1-1. Usefulness of probiotics and prebiotics

Metchnikoff (1) pointed out one hundred years ago that intestinal microflora and human health are closely related. Several hundred species (*Bifidobacteria*, *Lactobacillus*, *Escherichia coli*, *Bacteroides*, and *Clostridium*, etc.) of bacteria exist in intestinal microflora (2). Disruption of the intestinal microflora, due to pathogenic bacteria (3-5), antigens (6), or other unfavorable factors (7-9), causes intestinal dysfunction. *Lactobacillus*, *Bifidobacterium,* and *Propionibacterium*, which have been used for a long time in the manufacture of dairy products, have beneficial effects on the hosts: immunological enhancement, improved intestinal functions and protection against infections (10-12). There are two approaches to increasing the number of these beneficial bacteria in the gastrointestinal tract. One is the oral administration of living beneficial microorganisms to improve the intestinal microflora, terms "probiotics" (13, 14). Another approach is to supply the beneficial microorganisms already present in the intestine with specific carbon and energy sources, that is, to improve the composition of intestinal biota using dietary supplements, being named "prebiotics" (15). Recently, combination of probiotics and prebiotics, called "synbiotics", were performed (16, 17). Table 1-1 summarizes several beneficial components and their positive effects on the host (10, 18-27).

1-2. Historical background of propionibacteria

Propionibacteria was discovered by Freudenreich and Orla-Jensen in 1906 (28). Many propionibacteria were isolated from dairy products such as cheese and milk. C. B. Van Niel has investigated the characterization of propionibacteria, such as morphology, physiology, and biochemistry of fermentation in 1928 (29). Propionibacterial species are characterized as Gram-positive, non-spore-forming, nonmotile, facultatively anaerobic or aerotolerant, and

Table 1-1. Examples of studies on the effect of probiotics, prebiotics, and synbiotics on the host.

	Beneficial strain and/or component	Effects	Reference
Probiotics	*Bifidobacterium longum*	Improvement of lactose utilization	18
	Bifidobacterium bifidum	Inhibition of enterohemorrhagic *E. coli*	19
	Lactobacillus casei	Deconjugation of bile salt	20
	Lactobacillus rhamnosus	Stimulation of the immune system in animal hosts	10
	Propionibacterium thoenii	Bacteriocin produced	21
	Propionibacterium shermanii	Production of vitamin B_{12}	22
Prebiotics	Chitosan oligosaccharide	Growth stimulator for *Lactobacillus* and *Bifidobacterium*	23
	Fructo-oligosaccharide	Leads to rapid growth of bifidobacteria	24
	Propionibacterium freudenreichii	Growth stimulator for *Bifidobacterium*	25
Synbiotics	*Bifidobacterium longum* / *Bifidobacterium animalis* and fructo-oligosaccharide / inulin	High bifidogenic effectiveness	26
	Bifidobacteria and fructo-oligosaccharide	Reduction of colon cancer risk	27

rod-shaped bacteria (30, 31). Propionibacteria were characterized and classified into various species. These species were divided into two groups: “dairy propionibacteria” and “cutaneous propionibacteria”.

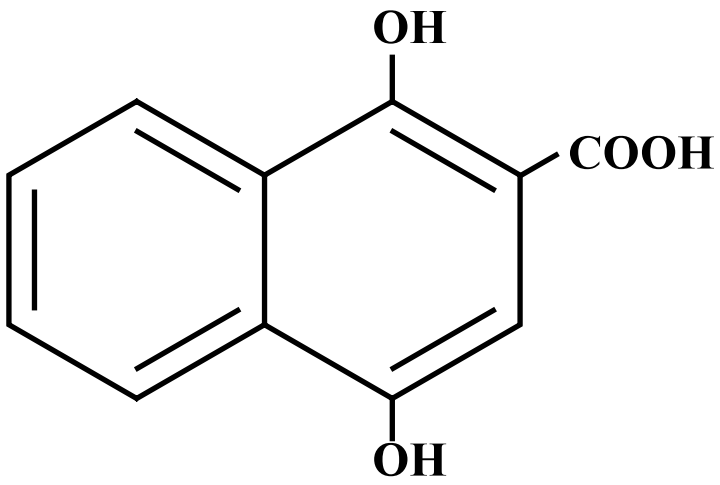
Cutaneous propionibacteria, which live on the surface of skin, were extended originally by transferring four species of anaerobic corynebacteria in 1974 by Moore *et al.* (32). Other species were transferred to *Propionibacterium* from *Actinomyces* by Charfreitag *et al.* (33). For example, *Propionibacterium acnes*, *Propionimicrobium lymphophilum*, and *Propionibacterium propionicum* (formerly *Arachnia propionica*) belong to this category (34).

Dairy propionibacteria, such as *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici*, are traditionally used as starter cultures in cheese manufacture. They improve the cheese flavor to obtain the specific quality of Swiss-type cheeses such as Emmental and Gruyere. And what is more, they have been used for their positive effects in many fields due to their abilities to produce propionic acid, vitamin B_{12} (22), vitamin K (35), and bacteriocins (36), to inhibit the growth of putrid bacteria in gut (36), to suppress the carcinogenic β-glucuronidase, azoreductase and nitroreductase of mice (37), to improve lactose intolerance (38, 39). Furthermore they are able to stimulate the growth of bifidobacteria by producing bifidogenic growth stimulator (BGS) (25). For these reason, propionibacteria have a potential of probiotics and prebiotics.

1-3. Positive function of bifidogenic growth stimulator (BGS)

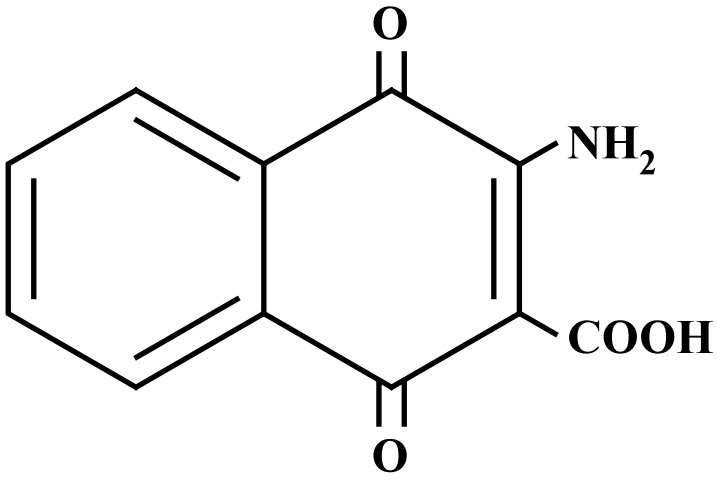
In 1994, a novel bifidogenic growth stimulator (BGS) produced by *Propionibacterium freudenreichii* was found by Kaneko *et al.* (25), who showed that BGS is not a nondigestible sugar but a mixture of 1,4-dihydroxy-2-naphthoic acid (DHNA), and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) as shown in Figure 1-1. Isawa *et al.* (40) reported *P. freudenreichii* ET-3 produced 10 mg/*l* DHNA and 0.2 mg/*l* ACNQ. The specific

A



$C_{11}H_8O_4$ **MW : 204**

B



$C_{11}H_7NO_4$ **MW : 217**

Fig. 1-1. Structural formula of BGS. (A) 1,4-dihydroxy-2-naphthoic acid (DHNA) (B) 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ)

stimulation of the growth of bifidobacteria by these BGS from the propionic acid bacteria is considered to be based on a mechanism quite different from that by oligosaccharides. Figure 1-2 denotes the glucose metabolism of bifidobacteria and the positive action of ACNQ proposed by Yamazaki *et al.* (41, 42). In glucose metabolism of bifidobacteria in the presence of ACNQ and $Fe(CN)_6^{3-}$, NAD(P)H in the cells is oxidized by ACNQ with the aid of diaphorase activity, and reduced ACNQ donates the electron to $Fe(CN)_6^{3-}$. The exogenous oxidation of NADH by the ACNQ/ $Fe(CN)_6^{3-}$ system results in the remarkable generation of pyruvate and a decrease in lactate production. DHNA is known that a precursor of menaquinone (vitamin K_2) (40, 43). DHNA is a more strong growth stimulator for bifidobacteria than ACNQ. It is interesting to note that DHNA has positive effects for not only bifidobacteria but also human. Okada *et al.* (44) reported that DHNA attenuates colonic inflammation not only by balancing intestinal microflora but also by suppressing lymphocyte infiltrarion through reduction of mucosal addressin cell adhesion molecule 1 (MAdCAM-1).

1-4. Construction of this thesis

The purpose of this thesis is to optimize the BGS production by propionibacterial strains because BGS has a high potential for improvement of human intestinal environment. Before going into details, I address that I studied this issue from two viewpoints, fermentation engineering and proteomic approach.

This thesis consists of six chapters and conclusions.

In Chapter 1 (this chapter), I introduce the usefulness of probiotics and prebiotics, history of propionibacteria, and positive functions of bifidogenic growth stimulator. In addition, the purpose of this study and construction of this thesis are described.

In Chapter 2, I deal with the production of extracellular BGS by anaerobic and aerobic

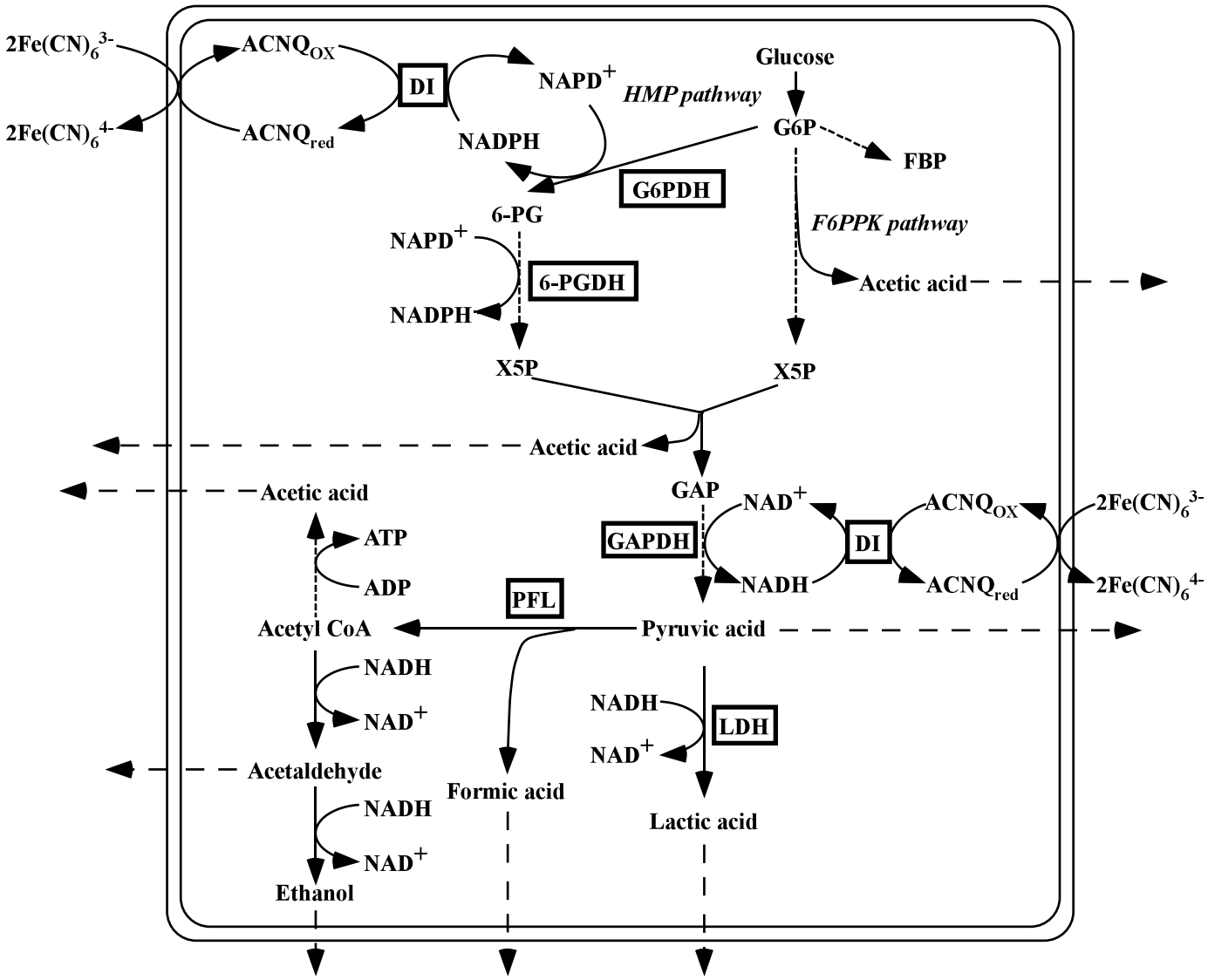


Fig. 1-2. Glycolytic pathways of bifidobacteria and proposed reactions for 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ)-mediated $Fe(CN)_6^{3-}$ reduction (14, 42). The bold line indicates the step catalyzed by one enzyme, and the dotted line denotes the step catalyzed by more than two enzymes.

FBP: Fructose-1,6-bisphosphate
X5P: xylulose-5-phosphate
GAP: glyceraldehyde-3-phosphate
6-PG: 6-phosphogluconate
DI: diaphorase
G6PDH: glucose-6-phosphate dehydrogenase
6-PGDH: 6-phosphogluconate dehydrogenase
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
PFL: pyruvate-formate-lyase

conditions of several propionibacterial strains. By developing the modified BGS bioassay method, the BGS activity could be determined more sensitively than the conventional method. To investigate BGS production by six dairy propionic acid bacterial strains (*Propionibacterium freudenreichii* ET-3, *P. shermanii* PZ-3, *P. acidipropionici* JCM 6428, *P. acidipropionici* JCM 6432, *P. jensenii* JCM 6433, and *P. jensenii* JCM 6438), they were cultivated under anaerobic and aerobic conditions.

In Chapter 3, I mention the production of BGS using waste materials from food industry such as glycerol and lactose. In addition, I carried out the batch cultivations using L-lactic acid as a carbon source because propionibacteria is able to utilize lactic acid more preferentially than lactose. Furthermore, I investigated BGS production by the co-cultivation of *P. shermanii* PZ-3 and *Lactobacillus* strains using lactose as a carbon source.

In Chapter 4, I establish the bioreactor system of high performance for BGS production using lactic acid as a carbon source. First of all, the tolerance to organic acids (lactic acid, propionic acid, and acetic acid) of *Propionibacterium shermanii* was evaluated. Secondly, I performed a continuous fermentation with a microfiltration module to remove by-products (propionic acid and acetic acid) for reduction of growth suppression. Furthermore, to alleviate growth inhibition by lactic acid, I used an on-line lactic acid controller for maintaining a lactic acid concentration at an optimal level in a fed batch cultivation. Finally, I investigated the efficient production of BGS by the propionibacterial strain using the continuous bioreactor system coupled to the on-line lactic acid controller.

In Chapter 5, I analyze the effects of oxygen supply on metabolisms of propionibacteria from the viewpoint of the enzymatic activity and the expression of proteins. Firstly, three different batch cultivations (anaerobic, aerobic, and dissolved oxygen-controlled conditions) were performed and the cell growth, the concentration of metabolites, and the amount of BGS produced were compared among the different cultivations. The cell free extracts were

prepared by homogenization and sonication since the cell wall of propionibacteria is so hard. Enzymatic activities that relate to glycolysis (Embden-Meyerhof-Parnas pathway), tricarboxylic acid cycle (Krebs cycle), propionic acid biosynthesis, and menaquinone biosynthesis were measured by colorimetric determination methods using the cell free extracts of *P. jensenii* cells. Two-dimensional electrophoresis and N-terminal amino acid sequencing were carried out in order to detect and identify up-regulate or down-regulate proteins under aerobic condition as compared with the amount of each protein expressed under anaerobic condition.

In Chapter 6, I describe the concluding remarks obtained from the results in the foregoing chapters. Moreover, I present a future plan of the research.

Chapter 2

Production of Bifidogenic Growth Stimulator by Anaerobic and Aerobic Cultivations of Several Propionibacterial Strains

Summary

Production of a bifidogenic growth stimulator (BGS) by propionic acid bacteria was investigated under anaerobic and aerobic culture conditions. To measure the concentration of extracellular BGS produced by propionic acid bacteria, I evaluated the effects of bioassay conditions using *Bifidobacterium longum* as a test microorganism on the formation of a growth-stimulation zone. The diameter of the growth-stimulation zone was significantly affected by both the component concentrations and the pH of a bioassay medium. The optimum component concentrations and pH of a bioassay medium were one-half of the normal values and 8.5, respectively. The bioassay method enables the measurements of the concentration of BGS produced by propionic acid bacteria ranging in concentrations from 0.1 μg/*l* to 1 mg/*l* using 1,4-dihydroxy-2-naphthoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) as standards. Of the six dairy propionic acid bacterial strains tested, four strains (*Propionibacterium freudenreichii* ET-3, *P. shermanii* PZ-3, *P. acidipropionici* JCM 6432, and *P. jensenii* JCM 6433) produced BGS in a concentration range of 4-23 mg/*l* under the anaerobic culture conditions. Analysis by high performance liquid chromatography (HPLC) showed that more than 70% of total BGS produced in supernatant samples was DHNA and no ACNQ was produced by the strains. The effect of oxygen supply on BGS production was investigated for the four BGS-producing strains. The aerobic conditions exerted positive effects on BGS production by only *P. acidipropionici* JCM 6432. The concentration of BGS obtained in the aerobic cultivation of *P. acidipropionici* JCM 6432 was 1.3-fold than that in anaerobic cultivation. Different properties (BGS production as well as cell growth and glucose metabolism) occurring in response to the aerobic conditions were observed, depending on the propionic acid bacterial strain used. This chapter is the first report on BGS production by propionibacterial strains except for *P. freudenreichii.*

2-1. Introduction

The human gastrointestinal tract constitutes a complex microbial ecosystem comprising several hundred different species of bacteria (45, 46). Because most of these bacteria are either beneficial or harmful to the human health, it is of great importance to promote beneficial bacteria such as bifidobacteria and lactobacilli and suppress potentially deleterious bacteria such as clostridia and bacteroidaceae among the intestinal microflora. There are two separate approaches to increasing the number of health-promoting bacteria in the gastrointestinal tract. One is the oral administration of live, beneficial microorganisms, termed probiotics, and another is to supply those already present in the intestine with a specific carbon and energy source, that is, to selectively modify the composition of the microflora using dietary supplements. These specific dietary components were named prebiotics in 1995 by Gibson and Roberfroid (15). They defined them as nondigestible but fermentable food ingredients that beneficially affect the host by selectively stimulating the growth of the beneficial bacteria and /or reducing the number of harmful bacteria in the colon. Although both probiotics and prebiotics are considered to have the potential to improve human health, the approach using the latter to increasing beneficial bacteria in the colon potentially provides some advantages over the probiotic strategy (47). Probiotic bacteria must survive transit through hostile conditions in the stomach and then adapt quickly to their new environment. Moreover, they must compete for nutrients and colonization sites against an established microflora with species that already occupy the available physical and metabolic niches. In contrast to probiotics, prebiotics target commensal bacteria with specificity to the host and with effective colonization sites. These endogenous bacteria are also unlikely to cause immunological problems associated with the intake of foreign antigens.

Candidate prebiotics are nondigestible and fermentable oligosaccharides such as lactulose, fructo-oligosaccharide, soybean oligosaccharide, galacto-oligosaccharide, and

xylo-oligosaccharide (48-50). The oligosaccharides entering the colon are fermented by the microflora predominantly to short-chain fatty acids (SCFAs), mainly butyrate, propionate, lactate, and acetate and consequently enhance the proliferation of beneficial intestinal bacteria. SCFAs also serve as an energy source for epithelial tissues of the host. These beneficial bacteria and their fermentation products affect positively the physiology and immunology of the host. A number of beneficial effects of oral administration of prebiotics seem to be due to their favorable effect on the small intestine via improved sugar digestion and absorption, glucose and lipid metabolism, and protection against known risk factors for cardiovascular disease (16, 51).

In 1994, Kaneko *et al.* (25) found a novel bifidogenic growth stimulator (BGS) produced by *Propionibacterium freudenreichii*. The BGS is not a nondigestible sugar but probably a mixture of 1,4-dihydroxy-2-naphthoic acid (DHNA), 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ), and unknown compounds. DHNA is a precursor of menaquinone (vitamin K) (40, 43). Yamazaki *et al*. proposed that ACNQ functions as a mediator of electron transfer from NAD(P)H to O_2 and H_2O_2 in bifidobacterial cells (41). They also speculated that the exogenous oxidation of NADH is an efficient way for bifidobacteria to store pyruvate and to generate ATP (42). A very interesting finding was reported by Satomi *et al.* (52), that is, a significant increase in the number as well as in the frequency of occurrence of bifidobacterial cells in fecal samples of healthy human adults was observed during the intake period of the culture powder of *P. freudenreichii* containing the BGS. The effect of the BGS on stool frequency and quantity was confirmed by Hojo *et al.* (53) who showed that the ingestion of tablets containing the culture of *P. freudenreichii* increased the number of defecations of constipated females by improving intestinal microflora. A recent preliminary report has shown that 4 weeks of ingesting BGS tablets resulted in clinical and endoscopic improvements in patients with active ulcerative colitis with no side effects (54).

The promotion of the specific growth of bifidobacteria by BGS produced by the

propionic acid bacterium and thereby its health-promoting effects were considered to be due to a mechanism quite different from that by oligosaccharides. A combination of BGS produced by a propionic acid bacterium and conventional oligosaccharides as prebiotics is expected to result in additional and/or synergistic effect on the maintenance of desirable intestinal microflora. BGS produced by propionic acid bacteria is considered to be one of the important nonsugar prebiotics that play an increasingly important role in improving human health. However, there are no reports on BGS production using propionibacterial strains except for *P. freudenreichii* (25, 35).

In this study, I investigated the optimal bioassay conditions for sensitively measuring BGS concentration in culture supernatants obtained from cultures of propionic acid bacteria. To clarify the effect of oxygen supply on BGS production, I compared the amount of BGS produced among six propionibacterial strains that were cultivated under anaerobic and aerobic culture conditions using a bioassay method under the optimal conditions.

2-2. Materials and Methods

2-2-1. Microorganisms

P. freudenreichii ET-3（7025）and *Bifidobacterium longum* OLB 6001 obtained from the Food Functionality Institute, Meiji Dairies Corporation (Odawara) were used throughout this study. *B. longum* OLB 6001 was used as a test microorganism for BGS activity assay. *Propionibacterium freudenreichii* subsp. *shermanii* (*Propionibacterium shermanii*) PZ-3 was kindly provided by Prof. N. Nishio of Hiroshima University. *Propionibacterium acidipropionici* JCM 6427, *P. acidipropionici* JCM 6432, *P. jensenii* JCM 6433, and *P. jensenii* JCM 6438 were purchased from Japan Collection of Microorganisms.

2-2-2. Fermentation

Propionibacterial strains were cultivated in TPY medium containing 30 g of glucose,

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). The cultures were incubated at 37ºC for *P. freudenreichii* and *P. shermanii*, and 30ºC for *P. acidipropionici* and *P. jensenii*. The microorganisms were precultured statically in test tubes containing TPY medium for 36-48 h. The precultured cells were inoculated into TPY medium at an initial turbidity of about 0.1 at 660 nm. Cultivations were carried out in a fermentor (TBR-1, Chiyoda Seisakusho, Nagano) with a working volume of 700 ml. The pH was maintained at 6.5 by adding 4 N NaOH using a peristaltic pump coupled to a pH controller. In anaerobic cultivations, a mixed gas of N_2 and CO_2 at a volume ratio of 9:1 was sparged at 0.3 vvm throughout the fermentation to maintain anaerobic conditions. In aerobic cultivations, air was sparged at 0.3 vvm throughout the fermentation.

2-2-3. Assay of BGS activity

BGS concentration was measured by a modified agar diffusion plate method (43). *B. longum* OLB 6001 was used as a test strain, cultivated anaerobically in TPY medium at 37ºC for 15 h. The turbidity at 660 nm (T_{660}) of the test strain at late logarithmic growth phase was about 3. The culture broth was diluted 100 times and then 0.1 ml of the diluted culture broth was inoculated into 10 ml of melted TPY agar medium (usually pH 8.5) at 55ºC. The agar medium inoculated was poured into a petri dish of 90 mm diameter and allowed to solidify. A sterile paper disc of 8 mm in diameter was placed on the surface of the agar medium. Fifty microliters of the sample was slowly and gradually injected into the disc and the plates were incubated anaerobically in a jar with a deoxidizing reagent (AnaeroPak A-03; Mitsubishi Gas Chemicals, Tokyo) at 37ºC. The growth zone around the disc was measured directly after incubation. To establish suitable assay conditions for BGS activity, the concentrations of constituents of TPY medium and the pH of TPY medium were evaluated. The concentrations of the constituents except glucose, K_2HPO_4, and KH_2PO_4, were adjusted

to 1/3-, 1/2-, 1- and 2-fold those of the standard medium. The pH of the medium was adjusted from 5.5 to 9.5. Commercial DHNA (Wako Pure Chemical Industries, Osaka) and synthetic ACNQ, which is a gift from the Food Functionality Institute, Meiji Dairies Corporation, were used as standards for measuring BGS concentration. Data are averages of triplicate determinations.

2-2-4. Other analytical methods

Cell concentration was determined by measuring T_{660}. 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, DHNA, ACNQ, glucose, lactic acid, acetic acid, and propionic acid concentrations. BGS concentration was determined by the agar diffusion plate assay as described above. In the assay, a modified TPY medium with its constituent concentrations decreased to 1/2 those of the standard medium was usually used as described above and the pH of the medium was usually adjusted to 8.5. To determine the concentrations of DHNA and ACNQ, supernatant samples obtained by centrifugation (25,700×*g*, 10 min) of culture broth were mixed with 0.5% (w/v) L-ascorbic acid solution and methanol (1:4:5, vol/vol/vol). The amounts of DHNA and ACNQ were simultaneously measured at 254 nm using a high performance liquid chromatography (HPLC) system according to the method of Furuichi *et al*. (35). 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 (55). Glucose concentration was measured using a glucose oxidase-peroxidase kit (Glucose C-II Test Kit Wako; Wako Pure Chemical Industries).

2-3. Results and Discussion

2-3-1. Effects of bioassay conditions on the formation of growth-stimulation zones

DHNA and ACNQ were used as representative BGSs and diluted with 0.2% (w/v) L-ascorbic acid solution as an antioxidizing reagent. The diameter of the growth-stimulation zone of *B. longum* was measured by an agar diffusion plate assay as described in Materials and Methods. The measurements were made using BGS solutions ranging in concentrations from 0.1 μg/*l* to 1 mg/*l*. Figure 2-1 shows a photograph of the growth-stimulation zone of *B. longum* obtained in the bioassay using 0.1 μg/*l* DHNA solution as a standard BGS. The growth-stimulation zone of *B. longum* was observed around a paper disc containing a solution of DHNA even at the very low concentration. Figure 2-2 shows the effects of the concentrations of components in TPY medium and the pH of the medium on the formation of the growth-stimulation zone by DHNA. The diameter of the growth-stimulation zone was dependent on the concentration of DHNA, when media with adjusted concentrations of components were used (Fig. 2-2A). When the concentrations of components in the medium were adjusted to 1/3 those of the standard medium, no formation of the growth-stimulation zone was observed. The maximum diameters of the growth-stimulation zone were obtained when the concentrations of components were adjusted to 1/2 those of the standard medium (1/2 TPY medium). The optimum concentrations of components in the medium for BGS assay were found to be 1/2. The effect of the pH of the medium on the formation of the growth-stimulation zone was examined when 1/2 TPY medium was used. At pHs of 5.5 and 9.5, no formation of the growth-stimulation zone was observed (Fig. 2-2B). When the pH of the medium was 8.5, the diameter of the growth-stimulation zone was larger than when the pH were 6.5 and 7.5, irrespective of the concentration of DHNA. Consequently, the optimum pH of the medium for BGS assay was found to be 8.5.

Under the optimum conditions described above, bifidobacterial cells were cultured in the presence of DHNA and ACNQ and the diameter of their growth zone was measured. Figure 2-3 shows the determination of DHNA and ACNQ by HPLC and the diameter of

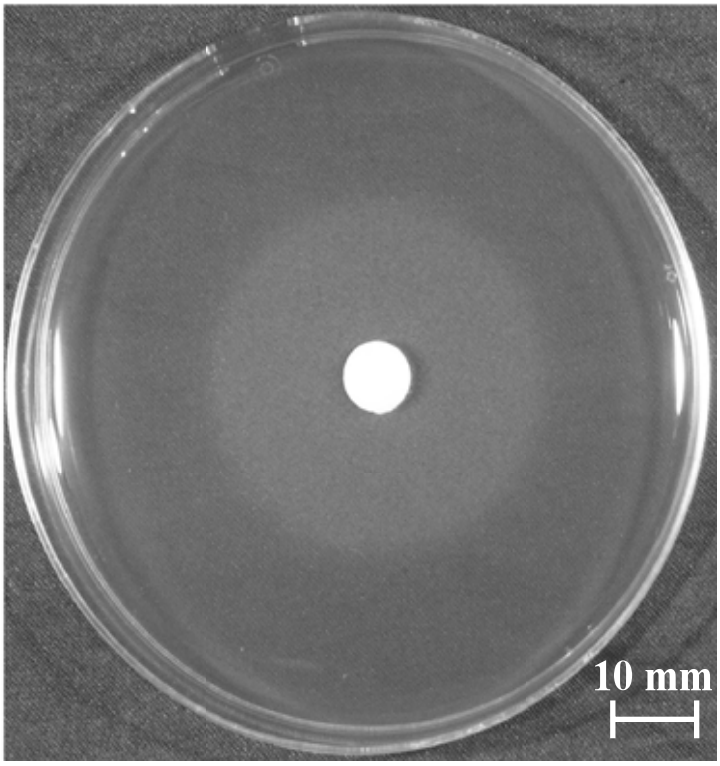


Fig. 2-1. Photograph of growth-stimulation zone formed by 0.1 μ g/*l* DHNA solution *B. longum* OLB 6001 was used as the test strain and 1/2 TPY medium at pH 8.5 was used in the bioassay.

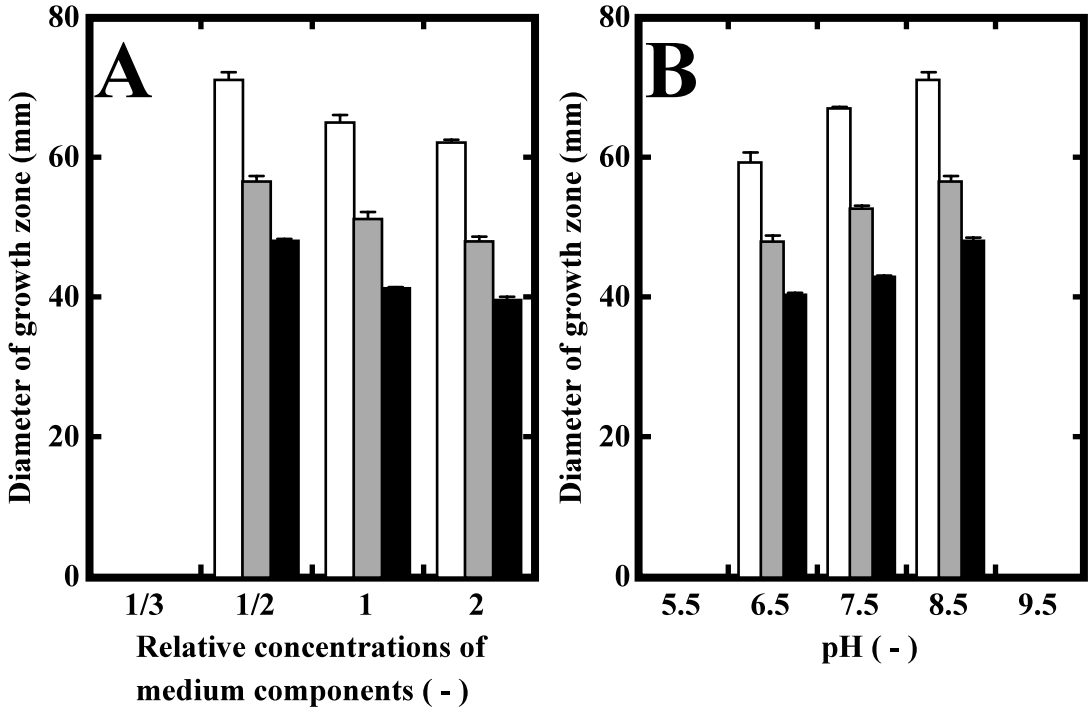


Fig. 2-2. Effect of assay conditions on formation of growth-stimulation zone. The concentrations of components (A) and the pH (B) of the assay medium were adjusted. DHNA concentrations were adjusted at 1 mg/*l* (open bars), 10 μ g/*l* (hatched bars), and 0.1 μ g/*l* (solid bars). The data represent the mean values (n = 3). The error bars indicate the standard deviations.

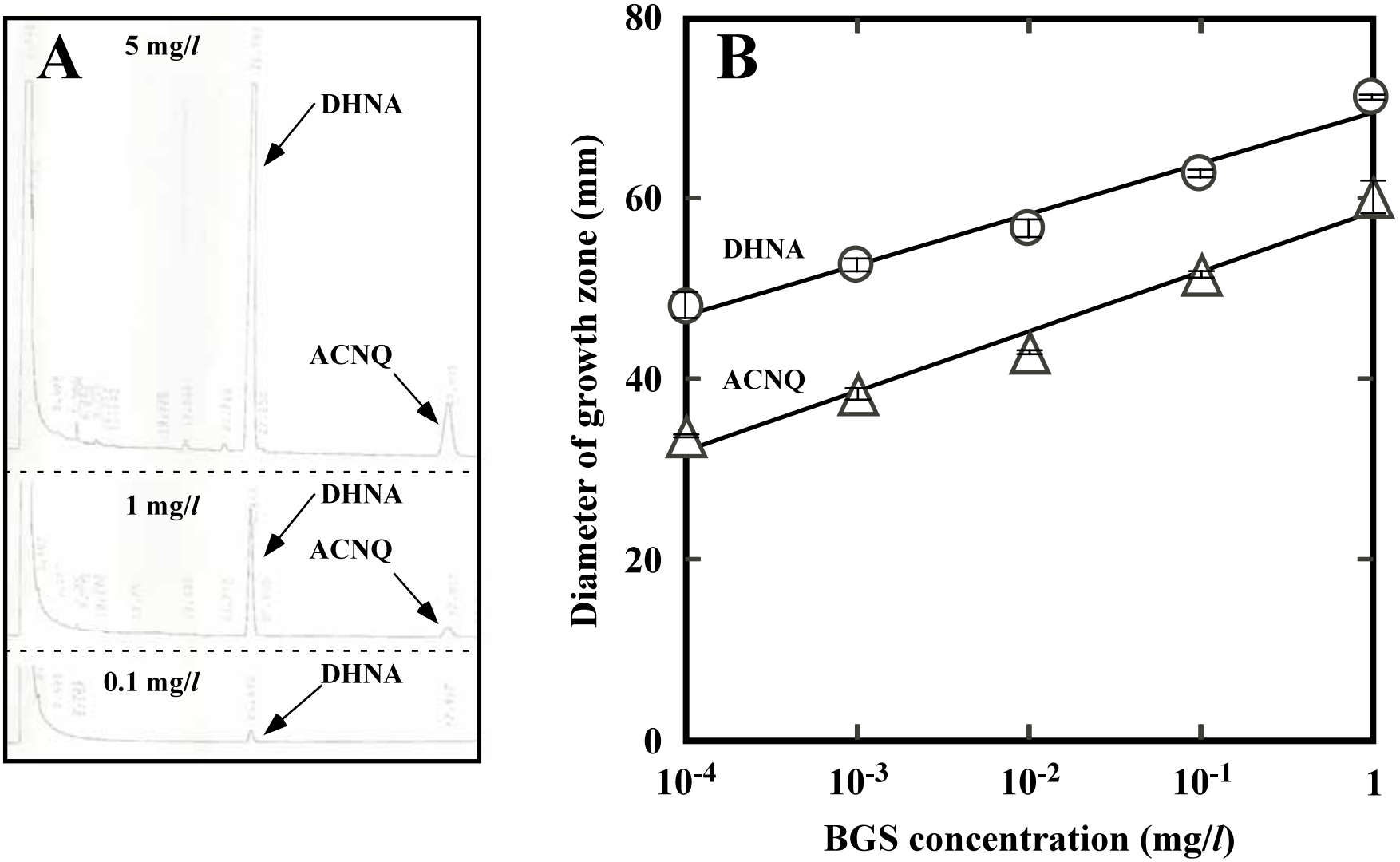


Fig. 2-3. Determination of DHNA and ACNQ by HPLC (A) and growth-stimulatory activity of DHNA (open circles) and ACNQ (open triangles) as a BGS (B). The data represent mean values (n = 3). The error bars indicate the standard deviations.

growth-stimulation zone as a function of BGS concentrations. Detection limits of DHNA and ACNQ concentrations by HPLC analysis were 0.1 mg/*l* and 1 mg/*l*, respectively (Fig. 2-3 (A)). On the other hand, by using BGS bioassay method, both DHNA and ACNQ were detected at a concentration of 0.1 μg/*l*. Therefore, the sensitivity of the bioassay method is much higher than that of HPLC analysis. When the diameters of growth-stimulation zone were plotted against the log concentrations of BGS, straight lines were obtained for both DHNA and ACNQ as shown in Fig. 2-3 (B). The difference in the diameter of growth-stimulation zone between DHNA and ACNQ was about 15 mm at each concentration tested. The growth-stimulatory activity of DHNA was found to be markedly higher than that of ACNQ. Because a large portion of BGS produced by *P. freudenreichii* was reported to be DHNA (40), DHNA was used as the standard BGS in further studies. Moreover, the concentration of BGS produced by several propionibacterial strains was calculated on the basis of the linear relationship between the diameter of growth-stimulation zone and the log DHNA concentration. Using the diameter of the growth zone measured in the bioassay, BGS was determined.

2-3-2. BGS production by anaerobic culture

For the comparison of the properties of BGS production among propionibacterial strains, *P. freudenreichii*, *P. shermanii*, *P. acidipropionici* (JCM 6427 and JCM 6432), and *P. jensenii* (JCM 6433 and JCM 6438) were anaerobically cultivated in TPY medium. *P. acidipropionici* (previous name: *Propionibacterium arabinosum*) JCM 6427 and *P. jensenii* (previous name: *Propionibacterium zeae*) JCM 6438 grew under anaerobic condition, but produced no BGS (data not shown). Figure 2-4 shows the results of cultivations of the other four propionibacterial strains at pH 6.5 using 30 g of glucose as a sole carbon source. On the basis of turbidity, the cells of three strains other than *P. freudenreichii* were observed to grow logarithmically at the initial stage of the cultivation (24 h), followed by a gradual decrease in growth rate. This decrease in growth rate seems to

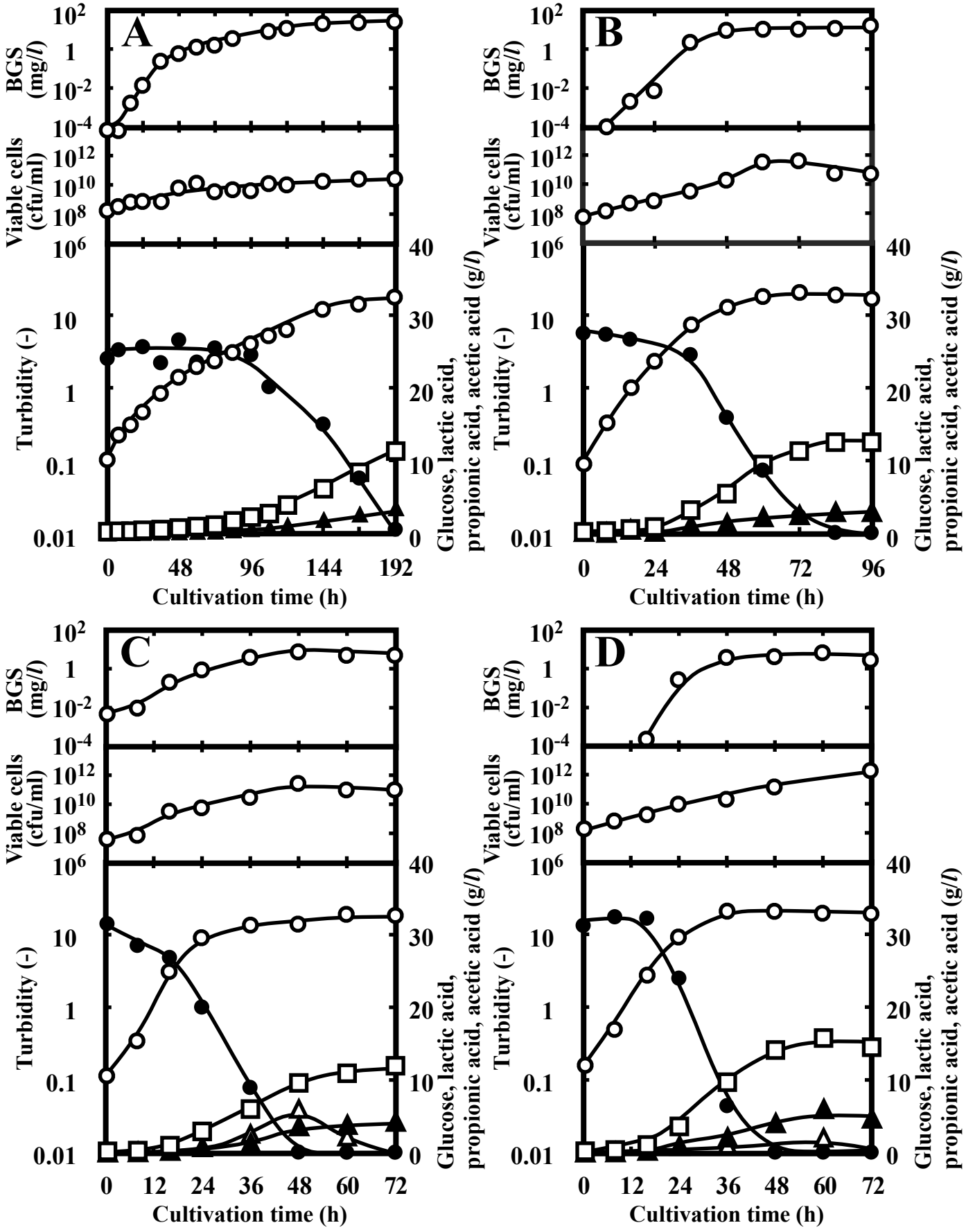


Fig. 2-4. BGS production by anaerobic cultivation of propionibacterial strains. *P. freudenreichii* ET-3 (A), *P. freudenreichii* subsp. *shermanii* PZ-3 (B), *P. acidipropionici* JCM 6432 (C), and *P. jensenii* JCM 6433 (D) were anaerobically cultivated. Symbols: open circles, BGS concentration, viable cells, and turbidity at 660 nm; closed circles, glucose; open triangles, lactic acid; closed triangles, acetic acid; open squares, propionic acid.

be not caused by the depletion of nutrients in the medium, but by the accumulation of organic acids (mainly propionic and acetic acids), similarly to other organic acid-producing bacteria described previously (56, 57). As shown in Fig. 2-4A, although the initial growth rate of *P. freudenreichii* was the lowest among the four strains, BGS concentration increased gradually as the cells grew and then remained almost constant at 23 mg/*l* after 144 h. The content of DHNA in the culture supernatant at 192 h was determined by measuring DHNA concentration by HPLC. The DHNA concentration was 16 mg/*l*, which corresponds to 70% of the total BGS concentration. In anaerobic cultivation, the final cell concentration of *P. shermanii* was as high as that of *P. freudenreichii*, but *P. shermanii* produced 15 mg/*l* BGS. The percentage of DHNA (12 mg/*l*) with respect to the total BGS was about 80%. In the anaerobic cultivation of *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433, glucose was consumed completely after 48 h and the maximum turbidities were obtained within 36 h, suggesting that the growth rates of *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433 were higher than those of *P. freudenreichii* and *P. shermanii.* The BGS concentrations in the supernatants prepared from the cultures after 36 h were approximately 6.8 and 6.1 mg/*l* for *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433, respectively. In the same samples, DHNA concentrations were approximately 5.2 and 5.3 mg/*l* for *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433, respectively. No ACNQ was detected in all of the supernatant samples by HPLC. In anaerobic cultivation, the major moiety (70-87%) of BGS produced by the four propionibacterial strains was found to be DHNA. The results show the possibility that propionibacterial strains produce as yet unknown BGSs other than DHNA and ACNQ.

2-3-3. BGS production by aerobic culture

Presently, it is well established that the response of propionic acid bacteria to oxygen can vary widely among strains. *P. acidipropionici* (referred as *Propionibacterium pentosaceum* in the Ref. 58) can grow on the surface of solid medium in air, whereas

P. freudenreichii and *P. jensenii* cannot (58). In liquid medium, *P. shermanii* and *P. freudenreichii* grow more slowly under aeration than *P. acidipropionici*, and the extent of inhibition of cytochrome synthesis is higher in the two strains more sensitive to oxygen (58). In the production of vitamin B_{12} by *P. freudenreichii*, a cyclic operation in which anaerobic and aerobic conditions are alternatively implemented has been developed by Ye *et al*. to improve productivity (59). They showed that aerobic fermentation is advantageous in decreasing the concentration of growth-inhibitory propionic acid in culture broth and that a long-term aerobic fermentation causes a substantial inhibition of cell growth as well as the vitamin B_{12} synthesis. I examined the effect of oxygen supply on BGS production by propionibacterial strains. *P. acidipropionici* JCM 6427 cultivated under aerobic condition showed a growth rate as high as that under anaerobic condition, but the concentration of BGS produced was negligible (data not shown). No growth of *P. jensenii* JCM 6438 was observed under aerobic condition (data not shown). Figure 2-5 shows the results of aerobic cultivations of the other four propionibacterial strains at pH 6.5 when air was sparged at 0.3 vvm throughout the fermentation. *P. freudenreichii* and *P. shermanii* hardly grew under aerobic condition as shown in Figs. 2-5A and 2-5B, respectively. When *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433 were cultivated, their cell growth rates under aerobic condition were not significantly different from those under anaerobic condition. As shown in Fig. 2-5C, the cell concentration of *P. acidipropionici* JCM 6432 in aerobic cultivation was as high as that in anaerobic cultivation (Fig. 2-4C). The cell concentration of *P. jensenii* JCM 6433 in aerobic cultivation (Fig. 2-5D) was 1.2-fold that in anaerobic cultivation (Fig. 2-4D). The metabolites accumulated in anaerobic cultivation of propionibacterial strains were mainly propionic and acetic acids, whereas acetic acid was almost exclusively formed as an end-product in aerobic cultivation as shown in Figs. 2-5C and 2-5D. In aerobic cultivation, *P. acidipropionici* JCM 6432 produced acetic acid, and little or no other organic acids were detected throughout the cultivation. In the aerobic cultivation of *P. jensenii* JCM

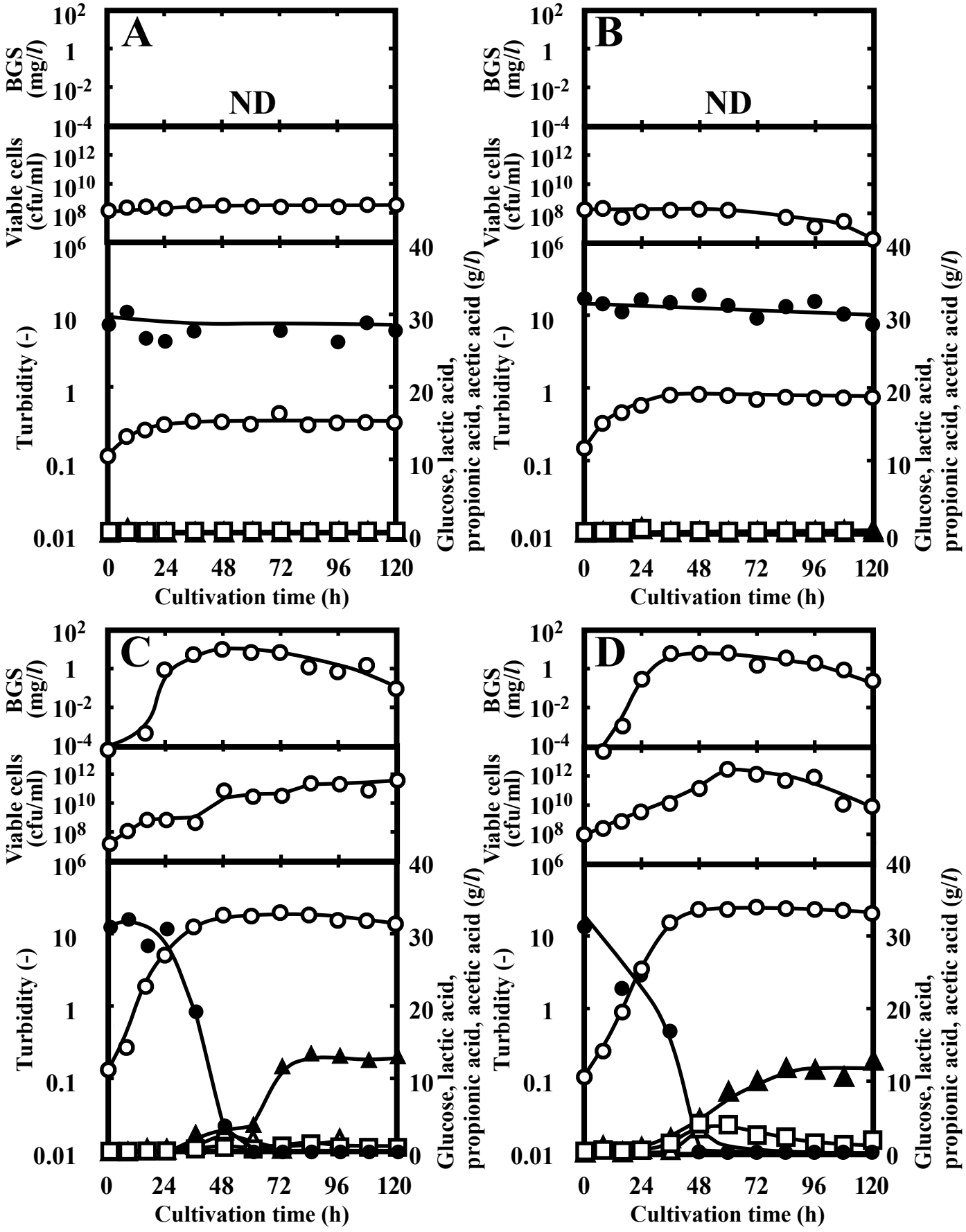


Fig. 2-5. BGS production by aerobic cultivation of propionibacterial strains. *P. freudenreichii* ET-3 (A), *P. freudenreichii* subsp. *shermanii* PZ-3 (B), *P. acidipropionici* JCM 6432 (C), and *P. jensenii* JCM 6433 (D) were aerobically cultivated. Meanings of symbols are the same as those shown in Fig. 2-4. ND, Not detected.

6433, propionic acid was produced as an intermediate in the presence of residual glucose. Then, propionic acid concentration decreased gradually after glucose was completely consumed. On the contrary, acetic acid concentration gradually increased and reached the maximum of 12.8 g/*l* (Fig. 2-5D). Although a difference in cell growth rate was not observed between aerobic and anaerobic cultivations, the concentrations of BGS obtained in the aerobic cultivation of *P. acidipropionici* JCM 6432 (Fig. 2-5C) was higher than that in anaerobic cultivation (Fig 2-4C). When *P. jensenii* JCM 6433 was aerobically cultivated, despite the slight increase in cell concentration as described above, the concentration of BGS obtained was almost as high as that under anaerobic condition. In the aerobic cultivations of *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433, BGS concentration gradually decreased after 60 h. The decrease is probably due to an increase in dissolved oxygen concentration observed after the complete consumption of glucose. The same observation was recently reported by Furuichi *et al.* (35); BGS concentration decreased at the late stage under aerobic condition of *P. freudenreichii* ET-3. In aerobic cultivation, no ACNQ was detected in all of the supernatant samples by HPLC and the major moiety (85-94%) of BGS produced by *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433 was DHNA. The dynamic metabolic changes caused by aeration are an interesting subject of future research in relation to BGS formation as well as production of organic acids as an end-product by propionic acid bacteria. I am now investigating the effect of the level of oxygenation on BGS production by several propionibacterial strains. Further study on the effects of the level of oxygenation in BGS production are shown in Chapter 5.

2-3-4. Comparison of amounts of BGS produced

Table 2-1 shows a comparison of BGS activities of culture supernatant obtained from the cultures of several propionibacterial strains under the anaerobic and aerobic conditions. In the anaerobic and aerobic cultivations of *P. acidipropionici* JCM 6427 and *P. jensenii* JCM 6438, little or no BGS was produced as described above. The metabolites accumulated in

Table 2-1. BGS production by several propionibacterial strains cultivated under anaerobic and aerobic conditions.

Strains used	Aeration	Cultivation time (h)	Turbidity[a] (-)	Propionic acid (g/*l*)	Acetic acid (g/*l*)	BGS[b] (mg/*l*)	DHNA[c] (mg/*l*)	Remarks
P. freudenreichii	N_2/CO_2	192	17.4	11.3	3.5	23.9±2.9	16.0±0.4	Fig. 2-4A
	Air	120	0.4	0.2	0.2	ND[d]	ND	Fig. 2-5A
P. freudenreichii subsp. *shermanii*	N_2/CO_2	96	20.6	12.5	2.9	15.0±2.8	11.9±0.0	Fig. 2-4B
	Air	120	0.8	0.3	0.2	ND	ND	Fig. 2-5B
P. acidipropionici JCM 6432	N_2/CO_2	72	18.7	12.0	4.3	6.8±0.6	5.2±0.0	Fig. 2-4C
	Air	120	19.2	0.7	13.5	9.0±0.7	7.7±0.0	Fig. 2-5C
P. jensenii JCM 6433	N_2/CO_2	72	20.9	14.5	4.7	6.1±0.8	5.3±0.1	Fig. 2-4D
	Air	120	24.5	1.8	12.8	6.4±0.6	6.0±0.1	Fig. 2-5D
P. acidipropionici JCM 6427	N_2/CO_2	24	9.1	0.2	1.4	ND	ND	
	Air	24	9.8	ND	0.4	ND	ND	
P. jensenii JCM 6438	N_2/CO_2	48	10.9	ND	28.9	ND	ND	
	Air	48	0.4	0.3	0.6	ND	ND	

[a] Data indicate the maximum turbidity obtained during the cultivation.

[b] Data indicate the maximum BGS concentration obtained during the cultivation. BGS concentration was determined by the bioassay. Values (± standard deviations) are the averages of triplicate determinations.

[c] Values indicate DHNA concentration in the supernatant samples used in the bioassay. DHNA concentration was determined by HPLC. Data (± standard deviations) are the averages of triplicate determinations.

[d] ND, Not detected.

the anaerobic cultivation of all the propionibacterial strains were mainly propionic and acetic acids, whereas acetic acid was mainly produced as a metabolite in the aerobic cultivation of *P. acidipropionici* JCM 6432 and *P. jensenii* JCM 6433 as shown in Table 2-1. In addition, the different responses of propionic acid bacteria to oxygen supply were observed in terms of cell growth and BGS production. *P. freudenreichii* and *P. shermanii* can grow under anaerobic conditions, but cannot under aerobic conditions. *P. acidipropionici* JCM 6432 produced 6.8 mg/*l* BGS in aerobic cultivation. The BGS concentration was about 1.3 times that in anaerobic cultivation although the maximum cell concentration of this strain in aerobic cultivation was almost similar to that in anaerobic cultivation. On the other hand, aerobic cultivation resulted in an increase in the cell concentration (T_{660} = 24.5) of *P. jensenii* JCM 6433 as compared with that (T_{660} = 20.9) in anaerobic cultivation. However, there was no significant difference in BGS concentration between aerobic and anaerobic cultivations. In both cultivations, no ACNQ was detected in all the supernatant samples by HPLC and the major moiety (70-94%) of BGS produced by the propionibacterial strains was DHNA.

2-4. Conclusion of Chapter 2

In this chapter, I investigated the production of BGS production by propionibacteria under anaerobic and aerobic conditions. The bioassay conditions on the measurement of the concentration of BGS was also optimized. The component concentrations and the pH of the optimum bioassay medium were found to be one-half of the normal values and 8.5, respectively. Using the bioassay method, BGS ranging in concentrations from 0.1 μg/*l* to 1 mg/*l* in samples can be determined using DHNA as the standard BGS compound. I am the first to report the capability of BGS production by propionibacterial strains except for *P. freudenreichii.* In addition, the results obtained in this study suggest that BGS production by several propionibacterial strains, as well as their cell growth and glucose metabolism was

significantly affected by oxygen supply. The different properties occurring in response to aerobic conditions were observed, depending on the propionic acid bacterial strain. Aerobic conditions exerted positive effects on BGS production only by *P. acidipropionici.* To optimize culture conditions for BGS production by each strain, further studies on not only oxygenation level but also the regulation of metabolism to stimulate BGS synthesis in cells, such as the selection of optimal carbon sources, the control of carbon source concentration throughout the cultivation period, and the addition of potential precursors of BGS into the medium, are necessary.

Chapter 3

Production of Bifidogenic Growth Stimulator by Propionibacterial Strains Using Glycerol and Lactic acid, and by Co-cultivations with Lactic Acid Bacteria Using Lactose

Summary

Production of a bifidogenic growth stimulator (BGS) by propionibacterial strains was investigated using glycerol or lactic acid as carbon sources. In the anaerobic cultivation using 30 g/*l* glycerol as a carbon source, *Propionibacterium shermanii* PZ-3, *P. acidipropionici* JCM 6432, and *P. jensenii* JCM 6433 produced BGS at a concentration of 2.4-5.7 mg/*l*. The aerobic conditions resulted in positive effects on BGS production by *P. acidipropionici* and *P. jensenii*. That is, the concentration of BGS obtained in the aerobic cultivation of *P. acidipropionici* and *P. jensenii* were 2.2-fold and 2.0-fold higher than that in anaerobic cultivation, respectively. Using lactic acid as a carbon source, the maximum concentration (1.9 mg/*l*) of BGS was obtained at 84 h in the mono-cultivation of *Propionibacterium shermanii* using 20 g/*l* lactic acid under anaerobic condition. The co-cultivation, in which in the first step lactose was gradually converted only lactic acid by a homo-fermentative lactic acid bacterium and subsequently the lactic acid was used as a carbon source for BGS production by *P. shermanii*, resulted in the successful BGS production. The BGS concentration obtained in the co-cultivations of *P. shermanii* and *Lactobacillus casei* or *P. shermanii* and *Lactobacillus bulgaricus* was 1.8 or 6.4 mg/*l*, respectively.

3-1. Introduction

The propionibacteria are best known for their role as dairy starter cultures in which they produce the characteristic flavor and eyes of Swiss-type cheeses (58). Other industrial applications are utilization as probiotics, ensilage inoculum, and production of vitamin B_{12} and propionic acid (46, 58, 60, 61). Kaneko *et al.* (25) found a novel bifidogenic growth stimulator (BGS) produced by *Propionibacterium freudenreichii*. The BGS is not a non-digestible sugar but probably a mixture of 1,4-dihydroxy-2-naphthoicacid (DHNA),

2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) and unknown compounds. Recently, I reported that some propionibacterial strains except for *P. freudenreichii* could produce BGS (62). The selective growth-promotion of bifidobacteria with BGS produced by the propionic acid bacteria and thereby health-promoting effects were recognized to be based on quite different mechanism from that with well-known oligosaccharides such as lactulose and fructo-oligosaccharide. Although tablets containing BGS produced by *P. freudenreichii* are recently commercially available (35), further improvement of BGS production by propionibacteria is necessary.

In the production of useful substances by fermentation using propionibacteria, selection of carbon source is considered to be one of the most important problems. Especially, lactose and glycerol were produced as unavoidable by-products of dairy products and oil and fat industry, respectively. It is necessary to use the waste materials more efficiently from the viewpoints of economics and environment. Propionibacteria can ferment not only a variety of carbohydrates such as lactose, glucose, mannose and fructose but also lactic acid, pyruvate and glycerol. Unlike many other bacteria, propionic acid bacteria can utilize lactic acid more preferentially than lactose and glucose (63, 64). Taniguchi *et al.* reported previously that *Propionibacterium shermanii* slightly grew and hardly produced BGS in a medium containing 30 g/*l* lactose. Taniguchi *et al.* also reported that the high concentration (more than 30 g/*l*) of lactic acid was inhibitory to cell growth and BGS production by propionibacterial strains probably due to what is called substrate inhibition (57). The assimilation properties of carbon source seem to be attributed to a selective habitat for propionibacteria, since they are so abundant in hard, ripened cheeses that contain lactic acid formed as the end-product of lactose fermentation by lactic acid bacteria. Taniguchi *et al.* found that the propionibacterial strains preferentially assimilate lactic acid prior to glucose, independent of the pH of the medium when the medium containing a mixture of lactic acid and glucose was used (57, 65).

In this study, the performance of production of BGS using glycerol or lactic acid as a carbon source were compared among several propionibacterial strains. On the basis of the results obtained, I selected *Propionibacterium shermanii* as a potential organism for BGS production using lactic acid as a carbon source. To utilize efficiently lactose, one of the wastes in the dairy industry, as a carbon source for BGS production, I conducted a co-cultivations of a homofermentative lactic acid bacterium and *P. shermanii*, where lactic acid produced once from lactose by the former was used as a carbon source by the latter. The co-cultivation is considered to allow not only the efficient utilization of lactose for BGS production by propionibacteria but also the alleviation of substrate inhibition by maintaining lactic acid concentration low in culture broth.

3-2. Materials and Methods

3-2-1. Microorganisms and media

P. freudenreichii ET-3, *Bifidobacterium longum* OLB 6001, *Lactobacillus casei* OLL 2218 and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) OLL 1067 obtained from the Food Functionality Institute, Meiji Dairies Corporation Co. (Odawara) were used in this study. *Propionibacterium freudenreichii* subsp. *shermanii* (*Propionibacterium shermanii*) PZ-3 was kindly provided by Prof. N. Nishio of Hiroshima University. *Propionibacterium acidipropionici* JCM 6432 and *Propionibacterium jensenii* JCM 6433, were purchased from Japan Collection of Microorganisms. *B. longum* OLB 6001 was used as a test microorganism for BGS activity assay as reported previously (62). Propionic acid bacteria were cultivated using TPY medium (pH 6.5) as described previously (57, 62). The cultures were incubated at 37ºC for *P. freudenreichii* and *P. shermanii*, and 30ºC for *P. acidipropionici* and *P. jensenii*. In the mono-cultivations of the four propionibacterial strains, 20-30 g/*l* L-lactic acid (lactic acid) or 30 g/*l* glycerol was used as a

carbon source. In addition, 30 g/*l* lactose or 30 g/*l* galactose was used as a carbon source in mono-cultivation or co-cultivation of *P. shermanii*.

3-2-2. BGS production by anaerobic or aerobic cultivations

The four propionibacterial strains were precultivated statically in test tubes containing TPY medium for 36-48 h. The precultivated cells of propionic acid bacteria were inoculated into TPY medium at an initial turbidity in the 0.1-0.2 range at 660nm. Initial substrate (glycerol or lactic acid) concentration was adjusted at 30 g/*l* or 20 g/*l*, respectively. The cultures were incubated at 37ºC for *P. freudenreichii* and *P. shermanii*, and 30ºC for *P. acidipropionici* and *P. jensenii*. Cultivations for BGS production were done in a fermentor (TBR-1, Chiyoda Seisakusho Co., Nagano) with a working volume of 700 ml. The pH was maintained at 6.5 by adding 4 N NaOH. A mixed gas of N_2 and CO_2 at a volume ratio of 9:1 was sparged at 0.3 vvm under anaerobic conditions. In an aerobic cultivation, air was sparged at 0.3 vvm throughout the aerobic fermentation as described previously (62).

3-2-3. Selection of optimal *Lactobacillus* strains used for co-cultivation

Fourteen lactic acid bacteria were precultivated statically in test tubes containing TPY medium for 24-48 h. All strains were inoculated into TPY medium with 15 g/*l* lactose at 37ºC. Turbidity at 24 h, maximum specific growth rate (μ_{max}), residual sugar concentration, and optical purity of lactic acid were compared.

3-2-4. Mono-cultivation and co-cultivation

In mono-cultivations, *P. shermanii* was cultured under anaerobic condition using 30 g/*l* lactose or 30 g/*l* galactose as a carbon source. In a co-cultivation, *P. shermanii* and *L. casei* or *P. shermanii* and *L. bulgaricus* were inoculated simultaneously into TPY medium with 30 g/*l* lactose. The initial turbidity (about 6×10^7 cells/ml: turbidity of about 0.1 at 660 nm) of *P. shermanii* was usually adjusted to be about 30-fold as high as those (about 2×10^6 cells/ml: turbidity of about 0.02 at 660 nm) of *Lactobacillus* strains because of a slow growth rate of the former. Other culture conditions were the same as described in Chapter

3-2-2.

3-2-5. Analytical methods

The cell concentration was measured by the turbidity at 660 nm. The number of viable cells for *P. shermanii* and *Lactobacillus* strains was measured by the plate culture method using the following media. TPY agar medium (pH 6.5) and sodium lactate agar medium (pH 7.0) (66) were used for propionibacterial strains in mono-cultivation and for *P. shermanii* in co-cultivation, respectively. MRS agar medium (pH 6.8) and RCA agar medium (pH 5.3) were used for *L. casei* and *L. bulgaricus* in co-cultivation, respectively (66). The supernatant obtained by centrifugation (25,700×*g*, 10 min) was analyzed for measurement of BGS, lactose, galactose, lactic acid, propionic acid, and acetic acid concentrations as described previously (57, 62). BGS concentration was measured by a modification of agar diffusion plate method as reported previously (62). Data are averaged from triplicate determinations.

3-3. Results and Discussion

3-3-1. BGS production using glycerol as a carbon source

Figure 3-1 shows the results of cultivations of the four propionibacterial strains with pH controlled at 6.5 when 30 g/*l* glycerol was used as a carbon source under anaerobic conditions. Only *P. freudenreichii* could not grow in this condition (Fig. 3-1A). *P. shermanii* was not able to consume glycerol completely within 240 h, but the BGS concentration reached 5.7 g/*l* at 240 h (Fig. 3-1B). The final cell turbidity of *P. acidipropionici* was the highest among those of the four propionibacterial strains. *P. acidipropionici* and *P. jensenii* were able to consume glycerol perfectly during 96 h and 240 h, respectively (Figs. 3-1C and 3-1D). The almost similar BGS concentrations were obtained in the cultivations of *P. acidipropionici* and *P. jensenii* (2.5 mg/*l* and 2.4 mg/*l*).

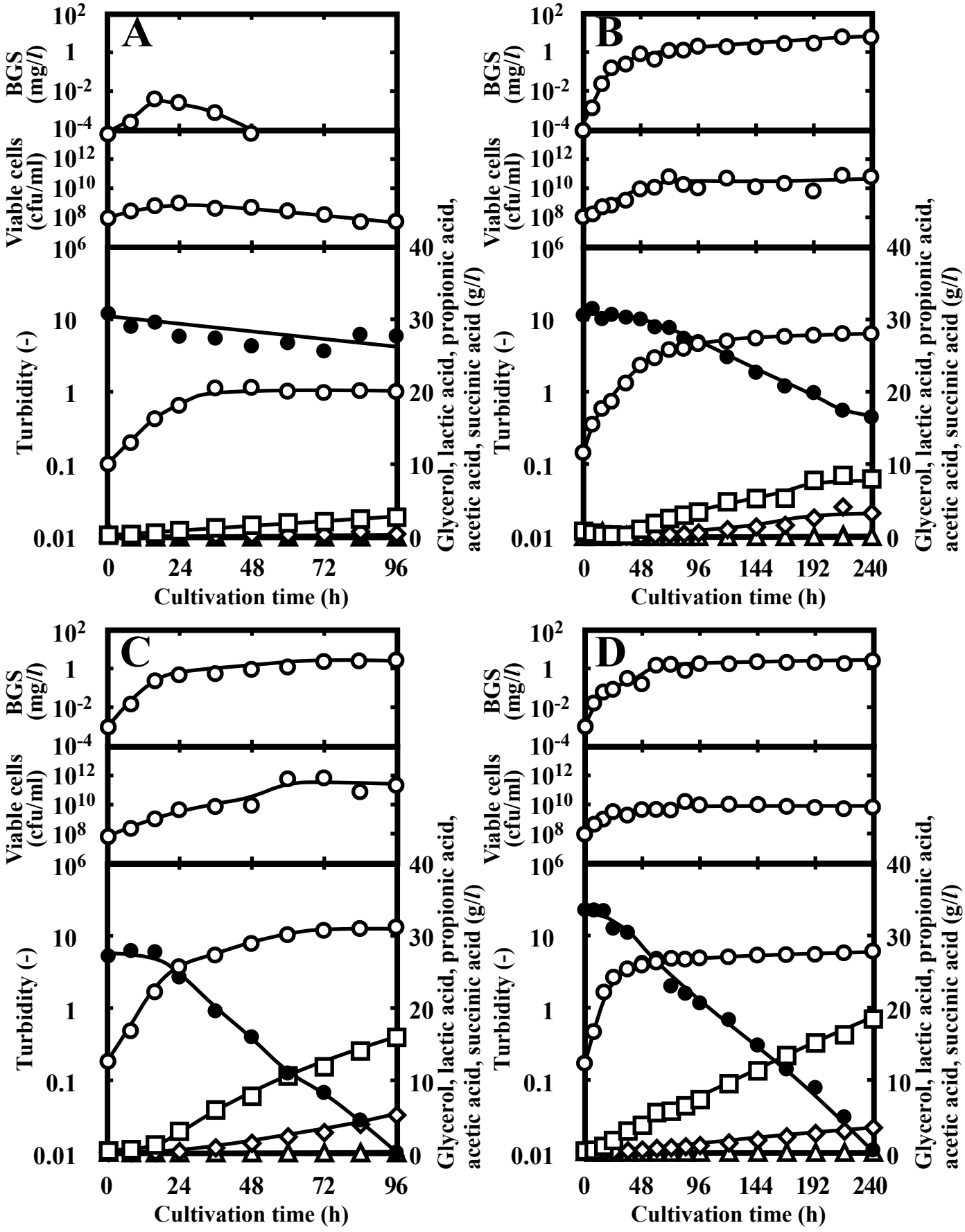


Fig. 3-1. BGS production by anaerobic cultivation of propionibacterial strains using 30 g/*l* glycerol as a carbon source . *P. freudenreichii* (A), *P. shermanii* (B), *P. acidipropionici* (C), and *P. jensenii* (D) were anaerobically cultivated. Symbols: open circles, turbidity at 660 nm; closed circles, glycerol; open triangles, lactic acid; closed triangles, acetic acid; open squares, propionic acid, opened diamonds, succinic acid.

Figure 3-2 shows the results of cultivations of the four propionibacterial strains using glycerol as a carbon source under the aerobic condition. *P. freudenreichii* and *P. shermanii* could not grow in the aerobic cultivations (Figs. 3-2A and 3-2B). The final cell turbidities of *P. acidipropionici* and *P. jensenii* under the aerobic condition (Figs. 3-2C and 3-2D) were higher than those under the corresponding anaerobic condition (Figs. 3-1C and 3-1D). The BGS concentration in these cultivations were approximately 5.5 mg/*l* for *P. acidipropionici* and 4.9 mg/*l* for *P. jensenii*, and these values were more than twice as high as that in the corresponding anaerobic cultivations, respectively.

Table 3-1 shows a comparison of BGS activities of culture supernatant obtained in the anaerobic and aerobic cultivations using glycerol as a carbon source. Under the anaerobic condition, *P. shermanii* was one of the most favorable BGS producer (5.7 mg/*l*), but the rates of cell growth and glycerol consumption were very slowly. On the basis of BGS productivity per cultivation time, *P. acidipropionici* was selected as one of the most excellent BGS producer (2.6×10^{-2} mg$\cdot l^{-1}\cdot h^{-1}$). Under the aerobic condition, *P. freudenreichii* and *P. shermanii* were not able to produce BGS due to little or no cell growth. *P. acidipropionici* produced 5.5 mg/*l* BGS in the aerobic cultivation. The BGS concentration was about 2.2 times that in the anaerobic cultivation. On the other hand, the aerobic condition resulted in an increase in the cell concentration (T_{660} = 24.8) of *P. jensenii* JCM 6433 as compared with that (T_{660} = 6.0) in the anaerobic cultivation. In the aerobic condition, the BGS concentration was about 2.0-fold higher than that in the anaerobic condition.

3-3-2. BGS production using lactic acid as a carbon source

Taniguchi *et al*. (57) reported previously that propionic acid bacteria can utilize lactic acid more preferentially than glucose, and that the growth of *P. freudenreichii* was inhibited by lactic acid at the initial concentration of more than 30 g/*l* probably due to what is called substrate inhibition. For comparison of properties of BGS production among propionibacterial strains, *P. freudenreichii*, *P. shermanii*, *P. acidipropionici*, and *P. jensenii*,

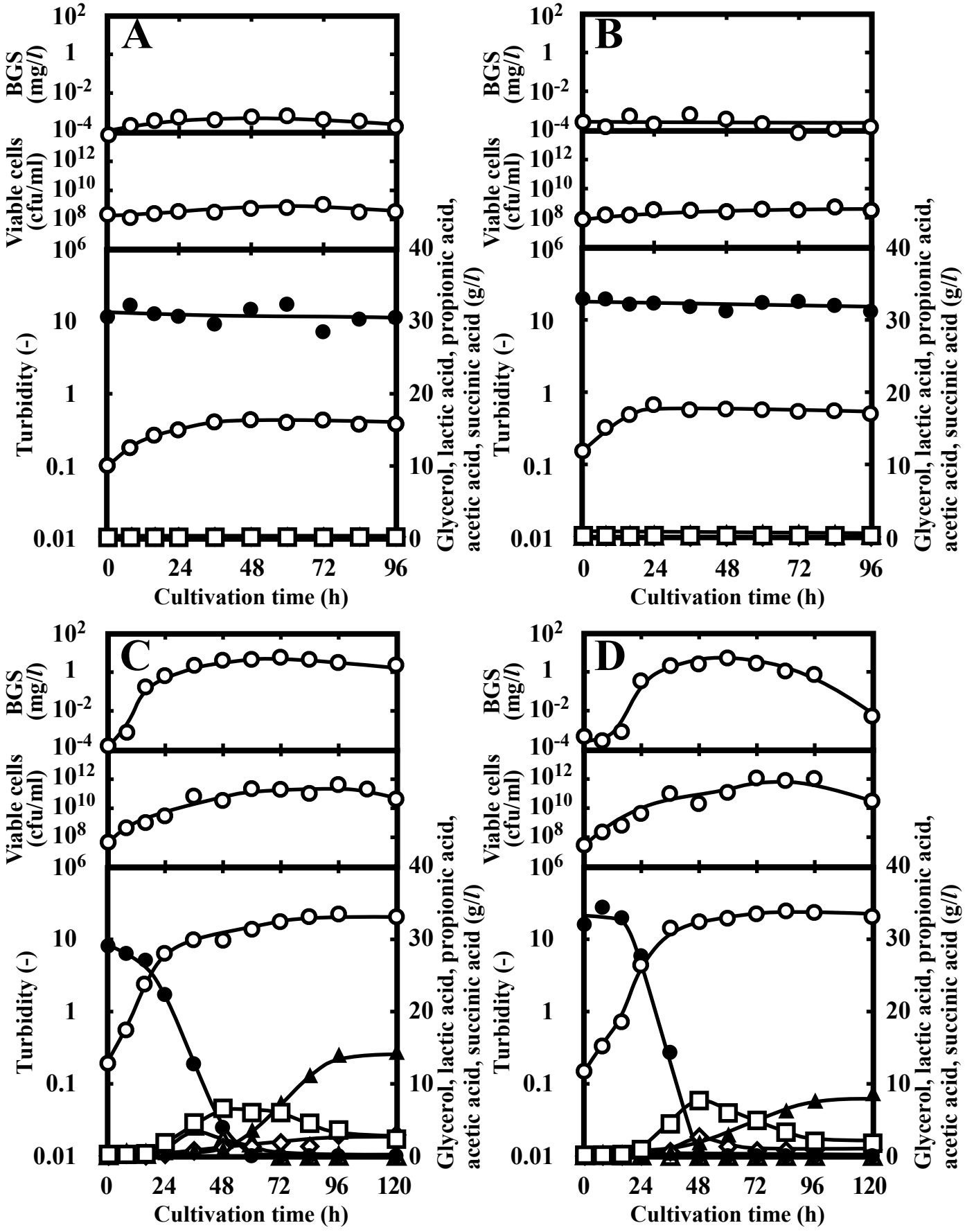


Fig. 3-2. BGS production by aerobic cultivation of propionibacterial strains using 30 g/*l* glycerol as a carbon source. *P. freudenreichii* (A), *P. shermanii* (B), *P. acidipropionici* (C), and *P. jensenii* (D) were aerobically cultivated. Meanings of symbols are the same as those shown in Fig. 3-1.

Table 3-1. BGS production by several propionibacterial strains cultivated under anaerobic and aerobic conditions using 30 g/*l* glycerol as a carbon source.

Strains used	Aeration	Cultivation time (h)	Turbidity[a] (-)	Propionic acid (g/*l*)	Acetic acid (g/*l*)	BGS[b] (mg/*l*)	DHNA[c] (mg/*l*)	Remarks
P. freudenreichii	N_2/CO_2	96	1.1	2.7	0.2	0.0±0.0	ND[d]	Fig. 3-1A
	Air	96	0.4	0.2	0.1	0.0±0.0	ND	Fig. 3-2A
P. shermanii	N_2/CO_2	240	6.3	8.0	0.4	5.7±0.2	5.5±0.0	Fig. 3-1B
	Air	96	0.6	0.2	0.2	0.0±0.0	ND	Fig. 3-2B
P. acidipropionici JCM 6432	N_2/CO_2	96	13.7	16.0	0.7	2.5±0.3	2.1±0.0	Fig. 3-1C
	Air	96	22.6	3.6	14.1	5.5±0.6	5.5±0.0	Fig. 3-2C
P. jensenii JCM 6433	N_2/CO_2	240	6.0	18.5	0.1	2.4±0.1	2.4±0.0	Fig. 3-1D
	Air	96	24.8	1.8	8.8	4.9±0.2	4.9±0.0	Fig. 3-2D

[a] Data indicate the maximum turbidity obtained during the cultivation.

[b] Data indicate the maximum BGS concentration obtained during the cultivation. BGS concentration was determined by the bioassay. Values (± standard deviations) are the averages of triplicate determinations.

[c] Values indicate DHNA concentration in the supernatant samples used in the bioassay. DHNA concentration was determined by HPLC. Data (± standard deviations) are the averages of triplicate determinations.

[d] ND, Not detected.

were anaerobically cultivated in TPY medium. Figure 3-3 shows the results of cultivations of the four propionibacterial strains with pH controlled at 6.5 when 20 g/*l* lactic acid was used as a carbon source under the anaerobic condition. In the mono-cultivations of the four strains, the almost similar profiles of turbidities and numbers of viable cells were obtained. That is, the cells of the four strains grew logarithmically at the initial stage of the cultivation, followed by gradual growth. The decrease in the growth rate seems to be not caused by depletion of nutrients in the medium, but by the accumulation of organic acids (mainly propionic and acetic acids). The final concentrations of propionic and acetic acids reached 9.6-11.7 g/*l* and 3.8-4.6 g/*l*, respectively and the concentration ratio of acetic acid to propionic acid was around 0.4, independent of propionibacterial strains used. As shown in Fig. 3-3, BGS concentrations increased gradually as the cells grew and then they were maintained at an almost constant level. The final cell turbidities of *P. acidipropionici* and *P. jensenii* were higher than those of *P. freudenreichii* and *P. shermanii*. However, *P. freudenreichii* and *P. shermanii* produced 1.6 and 1.9 mg/*l* BGS whereas concentrations of BGS obtained at 72 h in the cultivations were approximately 0.5 and 0.8 mg/*l* for *P. acidipropionici* and *P. jensenii*, respectively.

Figure 3-4 shows the results of cultivations of the four propionibacterial strains using 20 g/*l* lactic acid was used as a carbon source under the aerobic condition. *P. freudenreichii* and *P. shermanii* couldn't grow under the aerobic cultivations (Figs. 3-4A and 3-4B). The cell concentration of *P. acidipropionici* and *P. jensenii* in aerobic cultivation (Figs. 3-4C and 3-4D) were 2.9-fold and 3.2-fold that in the corresponding anaerobic cultivations (Figs. 3-3C and 3-3D), respectively. The BGS concentration reached 3.1 mg/*l* at 60 h for *P. acidipropionici* and 1.2 mg/*l* at 48 h for *P. jensenii*, respectively. The BGS concentration gradually decreased after 60 h in both cultivations probably due to an increase in dissolved oxygen concentration observed after the complete consumption of glucose. The same observation was recently reported (35); the BGS concentration decreased at the late stage of

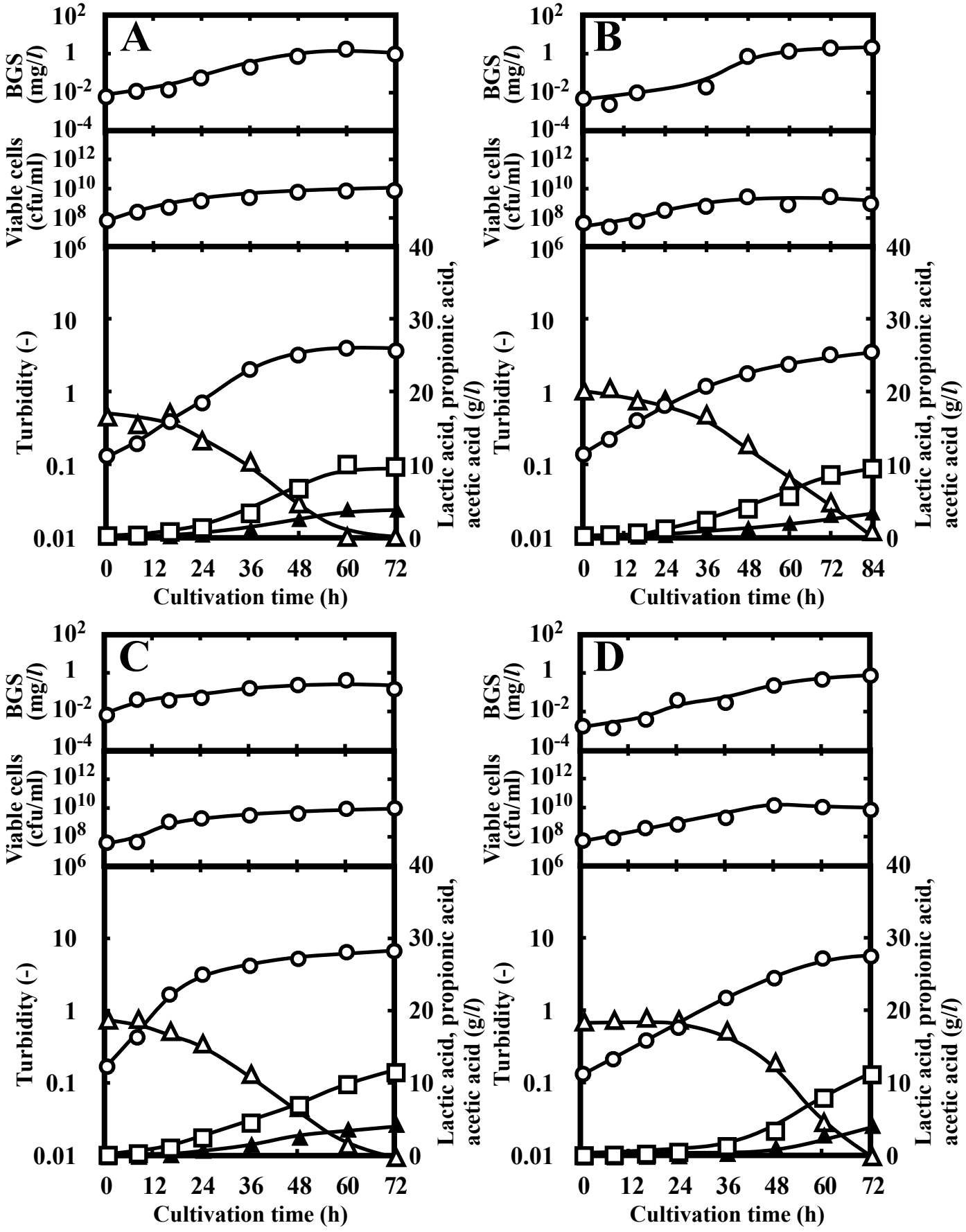


Fig. 3-3. BGS production by anaerobic cultivation of propionibacterial strains using 20 g/*l* lactic acid as a carbon source. *P. freudenreichii* (A), *P. shermanii* (B), *P. acidipropionici* (C), and *P. jensenii* (D) were anaerobically cultivated. Symbols: open circles, BGS, viable cells and turbidity at 660 nm; open triangles, lactic acid; closed triangles, acetic acid; open squares, propionic acid.

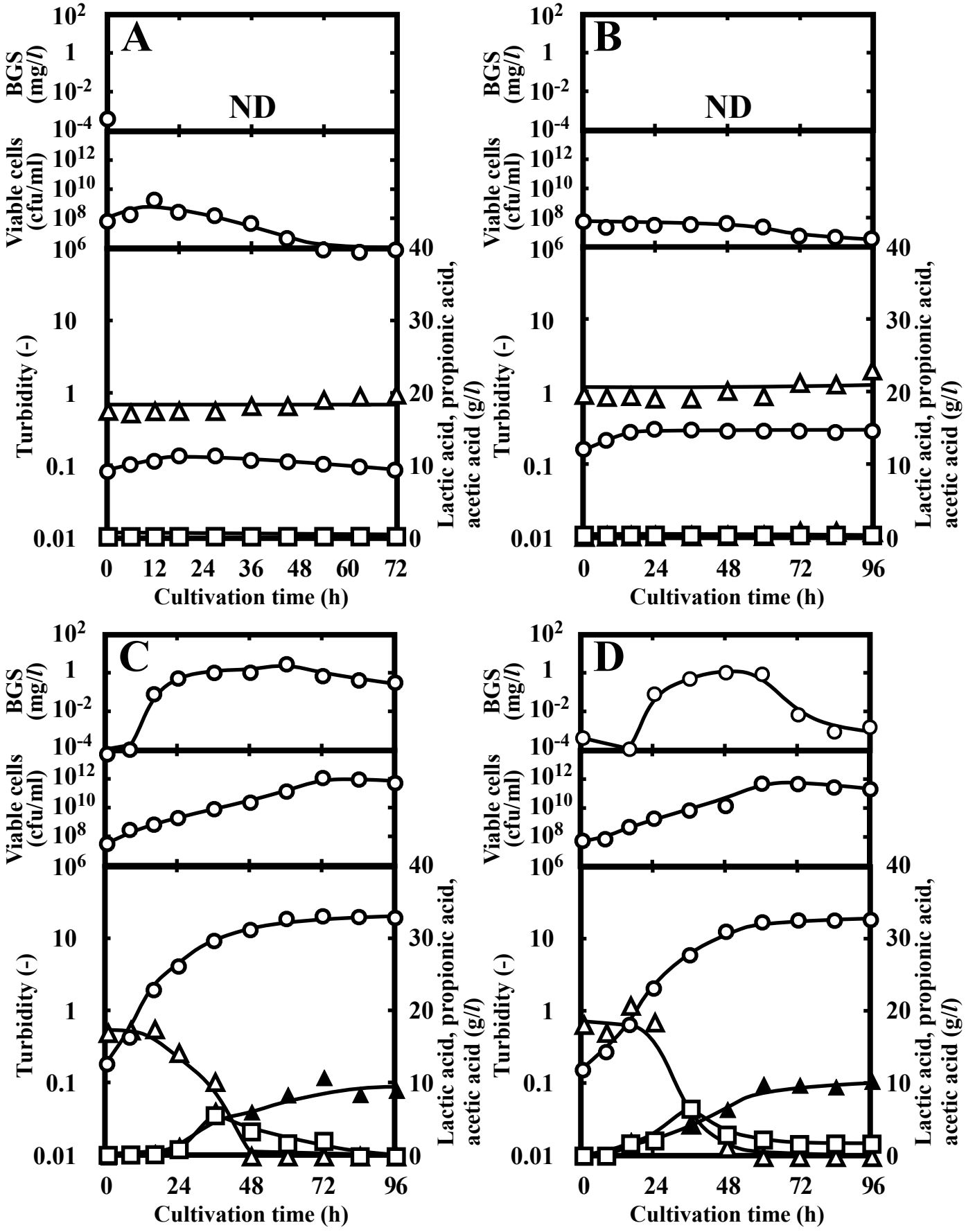


Fig. 3-4. BGS production by aerobic cultivation of propionibacterial strains using 20 g/*l* lactic acid as a carbon source. *P. freudenreichii* (A), *P. shermanii* (B), *P. acidipropionici* (C), and *P. jensenii* (D) were aerobically cultivated. Meanings of symbols are the same as those shown in Fig. 3-3. ND, Not detected.

the cultivation under the aerobic condition of *P. freudenreichii* ET-3.

Table 3-2 shows a comparison of BGS activities of culture supernatant obtained in the anaerobic and aerobic cultivations using lactic acid as a carbon source. Under the anaerobic condition, *P. freudenreichii* and *P. shermanii* produced 1.6 mg/*l* and 1.9 mg/*l* BGS, respectively. *P. acidipropionici* and *P. jensenii* produced BGS at a low concentration of 0.5-0.8 mg/*l*. Under the aerobic condition, *P. freudenreichii* and *P. shermanii* were not able to produce BGS, similarly to the cultivations using glucose and glycerol as a carbon source as described above. The cell concentrations of *P. acidipropionici* (T_{660} = 20.9) and *P. jensenii* (T_{660} = 18.8) were increased remarkably as compared with those in the anaerobic cultivations (T_{660} = 7.1 and 5.8), respectively. Furthermore, *P. acidipropionici* produced 3.1 mg/*l* BGS at 60 h in the aerobic cultivation. The BGS concentration was about 6.2 times that in the corresponding anaerobic cultivation.

These results suggest that some propionibacterial strains have potential for use of glycerol and lactic acid as a carbon source and for production of BGS under anaerobic and aerobic conditions

3-3-3. BGS production by co-cultivation using lactose as a carbon source

To utilize positively the performance of propionibacteria capable of using lactic acid more preferentially than lactose and glucose, it is necessary to convert lactose to lactic acid by lactic acid bacteria. However, it was reported that lactic acid bacteria produced acetoin and acetic acid, growth-inhibitory metabolites, from glucose under the aerobic condition (67). Therefore, I performed BGS production in anaerobic condition. On the basis of the results described above, I selected *P. shermanii* as one of the most favorable strains for BGS production using lactic acid as a carbon source.

I investigated a co-cultivation where in the first step lactose was gradually converted only lactic acid by a homo-fermentative lactic acid bacterium and subsequently the lactic acid was used as a carbon source for BGS production by *P. shermanii*. Firstly, I screened a

Table 3-2. BGS production by several propionibacterial strains cultivated under anaerobic and aerobic conditions using 20 g/*l* L-lactic acid as a carbon source.

Strains used	Aeration	Cultivation time (h)	Turbidity[a] (-)	Propionic acid (g/*l*)	Acetic acid (g/*l*)	BGS[b] (mg/*l*)	DHNA[c] (mg/*l*)	Remarks
P. freudenreichii	N_2/CO_2	72	3.9	9.6	3.8	1.6±0.1	1.5±0.0	Fig. 3-3A
	Air	96	0.1	0.1	0.2	ND[d]	ND	Fig. 3-4A
P. shermanii	N_2/CO_2	84	3.4	10.6	4.0	1.9±0.3	1.6±0.1	Fig. 3-3B
	Air	96	0.3	0.2	0.7	ND	ND	Fig. 3-4B
P. acidipropionici JCM 6432	N_2/CO_2	72	7.1	11.7	4.6	0.5±0.0	0.5±0.0	Fig. 3-3C
	Air	96	20.9	ND	9.3	3.1±0.1	3.1±0.1	Fig. 3-4C
P. jensenii JCM 6433	N_2/CO_2	72	5.8	11.3	4.4	0.8±0.0	0.7±0.0	Fig. 3-3D
	Air	96	18.8	1.8	10.6	1.2±0.2	1.1±0.2	Fig. 3-4D

[a] Data indicate the maximum turbidity obtained during the cultivation.

[b] Data indicate the maximum BGS concentration obtained during the cultivation. BGS concentration was determined by the bioassay. Values (± standard deviations) are the averages from triplicate determinations.

[c] Values indicate DHNA concentration in the supernatant samples used in the bioassay. DHNA concentration was determined by HPLC. Data (± standard deviations) are the averages of triplicate determinations.

[d] ND, Not detected.

homo-fermentative lactic acid bacterium suitable for converting lactose to lactic acid in TPY medium. Table 3-3 shows the comparison of lactic acid production from lactose by homo-lactic acid bacteria. Lactic acid yield per lactose consumed ($Y_{LA/S}$) and maximum specific growth rate (μ_{max}) in TPY medium with lactose were employed as criteria for selecting lactic acid bacteria favorable for the BGS production using lactose as a carbon source by co-cultivation with *P. shermanii*. The fourteen *Lactobacillus* strains, which were purchased from National Bio Resource Center (NBRC) and stored in our laboratory, were tested on the basis of the criteria described above. The $Y_{LA/S}$ and μ_{max} for *L. casei* OLL 2218 were 0.73 g/g and 0.13 h^{-1}, respectively and the μ_{max} for *L. bulgaricus* OLL 1067 was 0.23 h^{-1} in TPY medium. *L. bulgaricus* converted 15 g/*l* lactose to 5 g/*l* lactic acid and 8 g/*l* galactose. The two strains with the growth properties were selected for co-cultivation with *P. shermanii*.

The co-cultivation was done by simultaneous inoculation of *P. shermanii* and *L. casei* or *P. shermanii* and *L. bulgaricus* into TPY medium containing 30 g/*l* lactose and subsequent incubation as described in Materials and Methods. Figure 3-5 shows the production of BGS by the co-cultivations of a combination of *P. shermanii* and *L. casei* as well as *P. shermanii* and *L. bulgaricus*. As shown in Fig. 3-5A, lactose was completely consumed up to 48 h by *L. casei* while only lactic acid was once produced as an intermediate in the initial period and reached a maximum concentration of 12 g/*l* at 36 h, followed by gradual decrease in the lactic acid concentration. On the contrary the concentrations of propionic and acetic acids gradually increased and reached maximum values of 13 g/*l* and 6.4 g/*l* at 96 h, respectively. The μ_{max} obtained in the initial cultivation time was of 0.16 h^{-1}, which is 2-fold that (0.08 h^{-1}) in mono-cultivation using lactic acid as a carbon source (Fig. 3-3B). The high growth rate seems to be attributed to avoidance of growth inhibition by lactic acid at a high concentration. The number of viable cells of *P. shermanii* was kept at a high level of more than 10^{10} cells/ml throughout the cultivation except for the initial phase. The BGS concentration was

Table 3-3. Comparison of lactic acid production by several lactic acid bacteria using 15 g/*l* lactose as a carbon source.

Strains used	Lactic acid (g/*l*)	Residual lactose (g/*l*)	Residual galactose (g/*l*)	Turbidity (-)	μ max (h^{-1})	Optical Isomer D：L
L. casei subsp. *casei* NBRC 15883	6.6	4.3	ND	2.5	0.141	10： 90
L. casei OLL 2206	2.9	10.9	ND	1.6	0.164	7： 93
L. casei OLL 2218	7.6	4.6	ND	3.6	0.128	0：100
L. delbrueckii subsp. *bulgaricus* OLL 1067	5.0	ND	7.8	3.2	0.225	100： 0
L. lactis subsp. *lactis* NBRC 12007	0.7	14.3	ND	0.5	0.141	0：100
L. lactis OLL 2603	2.4	4.1	5.9	0.5	0.155	100： 0
L. lactis OLL 2612	6.7	3.3	ND	2.9	0.254	100： 0
L. paracasei subsp. *paracasei* JCM 8132	0.9	14.1	ND	0.4	0.125	1： 99
L. paracasei NBRC 15889	1.0	14.5	ND	0.4	0.128	1： 99
L. plantarum subsp. *plantarum* JCM 1149	1.0	14.2	ND	0.4	0.118	47： 53
L. plantarum NBRC 15891	1.0	14.7	ND	0.6	0.164	48： 52
L. rhamnosus KY-3	1.5	13.8	ND	0.5	0.102	0：100
L. rhamnosus NBRC 3425	1.0	13.7	ND	0.7	0.405	0：100
L. sakei NBRC 15893	1.0	13.4	ND	0.5	0.174	1： 99

All strains were cultured anaerobically for 24h at 37 ºC. Turbidity was determined by optical density at 660 nm.
ND, Not detected.

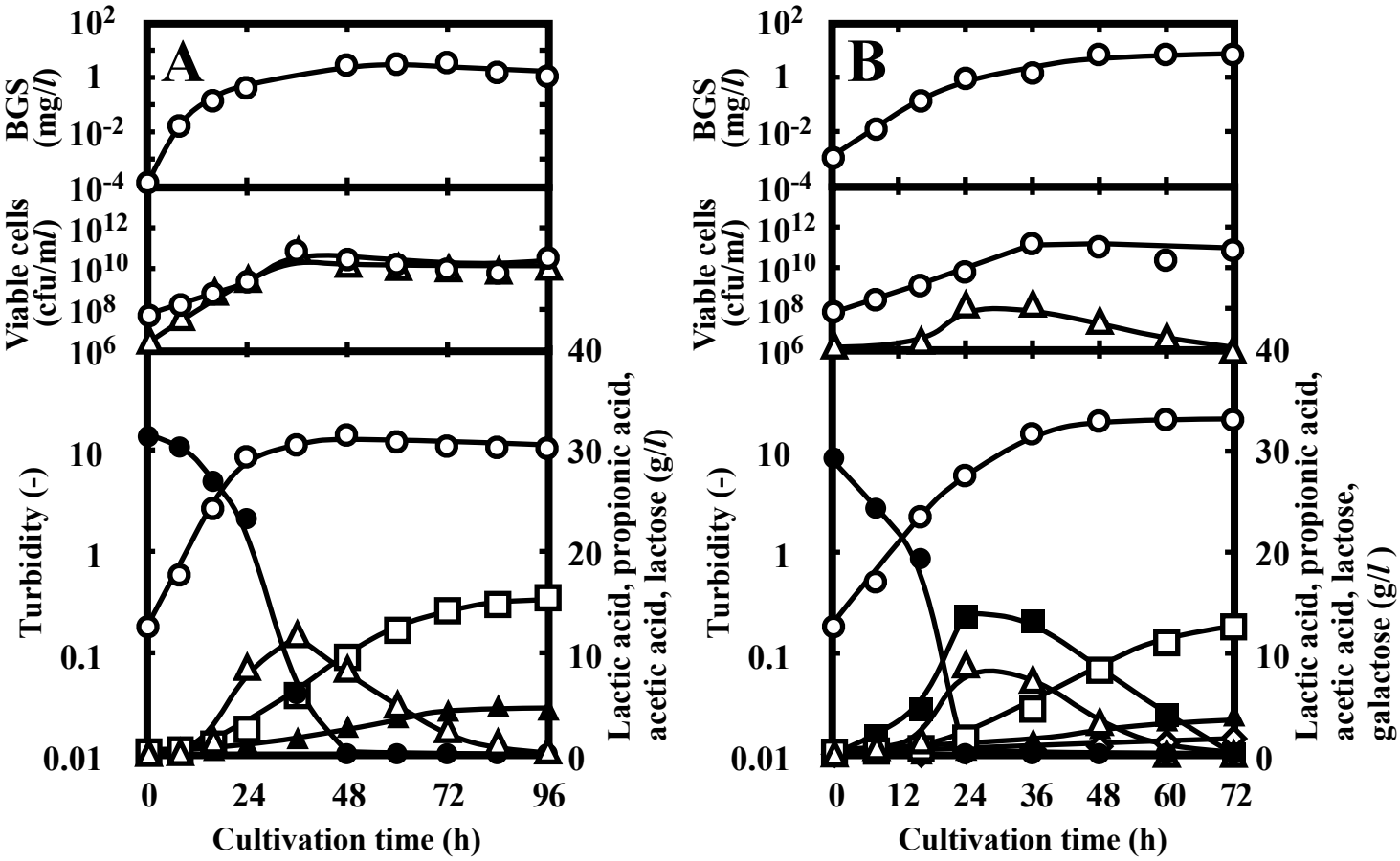


Fig. 3-5. BGS production by co-cultivation of *P. shermanii* and a homolactic acid bacterium using lactose as a carbon source. Co-cultivation was carried out by combining *P. shermanii* with *L. casei* (A) or *L. bulgaricus* (B). The cell concentration of *P. shermanii* was usually adjusted to be about 30-fold as high as those of Lactobacillus strains. No or little galactose was detected in the co-cultivation of *P. shermanii* with *L. casei* (A). Symbols: open circles, BGS, viable cells of *P. shermanii* and turbidity at 660 nm; closed circles, lactose; open triangles, lactic acid and viable cells of *L. casei* (A) or *L. bulgaricus* (B); closed triangles, acetic acid; open squares, propionic acid; closed squares, galactose.

approximately 1.8 mg/*l* during 48-96 h. The co-cultivation of *P. shermanii* and *L. casei* resulted in successful BGS production using TPY medium containing lactose as a carbon source. When *P. shermanii* and *L. bulgaricus* were simultaneously inoculated in TPY medium with lactose, lactose was rapidly converted up to 24 h by *L. bulgaricus* as shown in Fig. 3-5B. *L. bulgaricus* produced not only lactic acid but also galactose because galactose is not uptaken by *L. bulgaricus*. Lactic acid once accumulated was gradually converted to propionic and acetic acids, and galactose remained in the culture broth was gradually consumed by *P. shermanii* after lactic acid was almost depleted. In the co-cultivation, the number of viable cells of *L. bulgaricus* increased to 10^8 cells/ml in the middle phase of 24-36 h, and then it gradually decreased. The μ_{max} obtained in the initial cultivation time was 0.19 h^{-1} for *P. shermanii* and the number of viable cells of *P. shermanii* was kept at a high level of more than 10^{11} for the late phase of 36-72 h. BGS concentration increased gradually even while galactose as well as lactic acid was consumed and reached 6.4 mg/*l* at 72 h. The gradual supply of both lactic acid and galactose by *L. bulgaricus* resulted in positive effect on the cell growth and BGS production by *P. shermanii* in the co-cultivation.

3-3-4. Comparison of BGS production between mono-cultivation and co-cultivation

Figure 3-6 shows the BGS production by *P. shermanii* using lactose and galactose as a carbon source. Using lactose (Fig. 3-6A) or galactose (Fig. 3-6B) as a carbon source, the cultivation time required for complete consumption of the carbon sources was extended as compared with that in the cultivation using lactic acid or glucose (62). The BGS concentration in the supernatant prepared from the culture was approximately 2.1 mg/*l* for the cultivation using lactose and 3.6 mg/*l* for the cultivation using galactose. Table 3-4 shows a comparison of amounts of BGS produced by the mono and co-cultivations. Of the propionibacterial strains tested in mono-cultivations using 20 g/*l* lactic acid as a carbon source, *P. shermanii* was the most suitable strain for BGS production. In the mono-cultivation of *P. shermanii*, the BGS concentration was 1.9 mg/*l*, which is higher than those in the

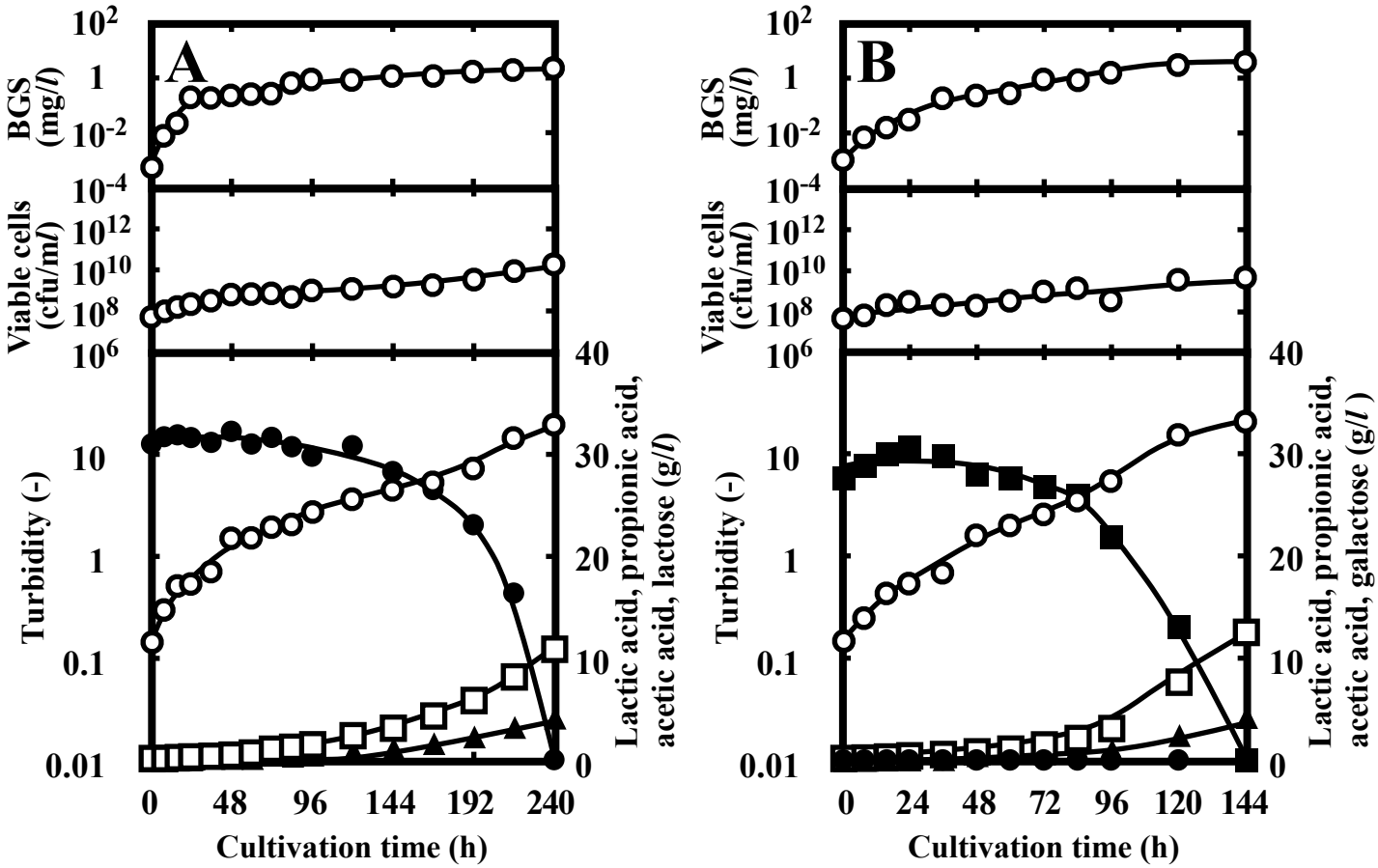


Fig. 3-6. BGS production by mono-cultivation of *P. shermanii* using lactose (A) and galactose (B) as a carbon source. No lactic acid was detected. Meanings of symbols are the same as those shown in Fig. 3-5.

Table 3-4. Comparison of the amounts of BGS produced by *P. shermanii* among different culture methods.

Culture methods	Strains used	Carbon source		Cultivation time (h)	Cell concentration[a] (cells/ml)	Propionic acid (g/*l*)	Acetic acid (g/*l*)	BGS[b] (mg/*l*)	Remarks
		Substrate	Concentration (g/*l*)						
Mono-culture	*P. shermanii*	Lactic acid	20	84	2.6×10^{9}	10.6	4.0	1.9±0.3	Fig. 3-3B
		Lactic acid	30	84	2.2×10^{8}	1.7	0.8	0.2±0.0	
		Lactose	30	240	1.7×10^{10}	10.9	4.2	2.1±0.0	Fig. 3-6A
		Galactose	30	144	4.3×10^{9}	12.5	4.3	3.6±0.2	Fig. 3-6B
Co-culture	*P. shermanii* + *L. casei*	Lactose	30	96	6.6×10^{10} [c]	15.4	4.5	1.8±0.1	Fig. 3-5A
	P. shermanii + *L. bulgaricus*	Lactose	30	72	1.4×10^{11} [c]	12.6	3.9	6.4±0.2	Fig. 3-5B

[a] Data indicate the maximum cell concentration obtained during the cultivation.

[b] Data indicate the maximum BGS concentration obtained during the cultivation. BGS concentration was determined by the bioassay. Values (± standard deviations) are averages from triplicate determinations.

[c] Data indicate the maximum cell concentrations of *P. shermanii* in the co-cultivation.

mono-cultivations of the other strains as shown in Table 3-2. *P. shermanii* slightly grew and hardly produced BGS in TPY medium containing 30 g/*l* lactic acid (data not shown). In the co-cultivation of *P. shermanii* and *L. casei* using 30 g/*l* lactose as a carbon source, the BGS concentration was 1.8 mg/*l*. The value is almost identical to that in the mono-cultivation of *P. shermanii* using 20 g/*l* lactic acid as a carbon source. When *P. shermanii* and *L. bulgaricus* were inoculated in the co-cultivation, the BGS concentration was 6.4 mg/*l* and the highest BGS concentration was obtained among different cultivations. BGS obtained in the co-cultivation of *P. shermanii* and *L. bulgaricus* was 3.0-3.4-fold those in the mono-cultivations using lactic acid and lactose and 1.8-fold that in the mono-cultivation using galactose. The highest yield of BGS obtained in the co-cultivation of *P. shermanii* and *L. bulgaricus* appears to be due to the assimilation of not only lactic acid but also galactose by *P. shermanii*. The co-cultivation, in which lactic acid produced once from lactose by *Lactobacillus* strains was used as a carbon source by propionibacterial strain, is expected to be utilized as a potential cultivation method for production of useful substances by propionic acid bacteria.

3-4. Conclusion of Chapter 3

In this chapter, I investigated BGS production using glycerol and lactic acid as a carbon source by cultivations of propionibacterial strains. The results obtained in this study show that lactic acid is available as a carbon source for production of BGS by *P. shermanii.* The co-cultivation of *P. shermanii* and *Lactobacillus* strains was useful for BGS production using lactose as a carbon source.

Chapter 4

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

Summary

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} $mg\cdot l^{-1}\cdot h^{-1}$. The BGS productivity per cultivation time in continuous cultivation with filtration was 1.9-fold that (9.4×10^{-2} $mg\cdot l^{-1}\cdot h^{-1}$) 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} $mg\cdot l^{-1}\cdot h^{-1}$) 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, I produced BGS continuously at a high level of 47 mg/*l*. The BGS productivities per cultivation time (3.5 $mg\cdot l^{-1}\cdot h^{-1}$) and the total volume of medium used (1.7×10^{-1} $mg\cdot l^{-1}\cdot h^{-1}$) 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*.

4-1. Introduction

Propionibacterial species are characterized as Gram-positive, non-spore- forming, nonmotile, facultatively anaerobic or aerotolerant, and rod-shaped bacteria (30, 31). 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 (30, 31, 68). They also may contribute to natural fermentations of silage and olives (60) and can produce a variety of industrially important products such as propionic acid, vitamin B_{12}, and bacteriocins (69-71). Recently, propionic acid bacteria have received much attention as both probiotics beneficial for human health (46, 72) and producers of prebiotics stimulating selectively the growth of beneficial intestinal bacteria such as bifidobacterial species (35, 42).

In 1994, a novel bifidogenic growth stimulator (BGS) produced by *Propionibacterium freudenreichii* was found by Kaneko *et al.* (25), who showed that BGS is not a nondigestible sugar but a mixture of 1,4-dihydroxy-2-naphthoic acid (DHNA), 2-amino-3-carboxy-1,4-naphthoquinone, and unknown compounds. I have recently reported that BGS, mainly DHNA, is produced by propionibacterial strains such as *Propionibacterium freudenreichii* subsp. *shermanii* (*Propionibacterium shermanii*), *P. acidipropionici*, and *P. jensenii* except for *P. freudenreichii* (62). 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 (48). 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 maintenance 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. Taniguchi *et al.* 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 (56, 61, 73-76). Although continuous bioreactor systems with membrane separation have disadvantages inherent in practical applications such as scale-up and membrane maintenance (77), Taniguchi *et al.* successfully applied a bioreactor system to the production of useful materials, such as starter cultures (56, 73), vitamin B_{12} (61), intracellular enzyme (74, 75), and bacteriocin (76), 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 (63). Taniguchi *et al.* 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 (57). Recently, Taniguchi *et al.* 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 (65).

In the present work, I 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, I used an on-line lactic acid controller for maintaining the concentration of lactic acid suitably low in fed-batch cultivation. Furthermore, I investigated the efficient production of BGS by the propionibacterial strain using the continuous bioreactor system coupled to an on-line lactic acid controller.

4-2. Materials and Methods

4-2-1. 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 37ºC 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).

4-2-2. 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 maximum specific growth rate (μ_{max}), turbidity at 660 nm, lactic acid consumption, and BGS production for 48 h.

4-2-3. 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.

4-2-4. Continuous cultivation with a filtration device

The bioreactor system described previously (56, 61, 74-76) was used for continuous cultivation with a filtration unit in this study (Fig. 4-1). 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 (61). 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}.

4-2-5. 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 (Fig. 4-2). 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

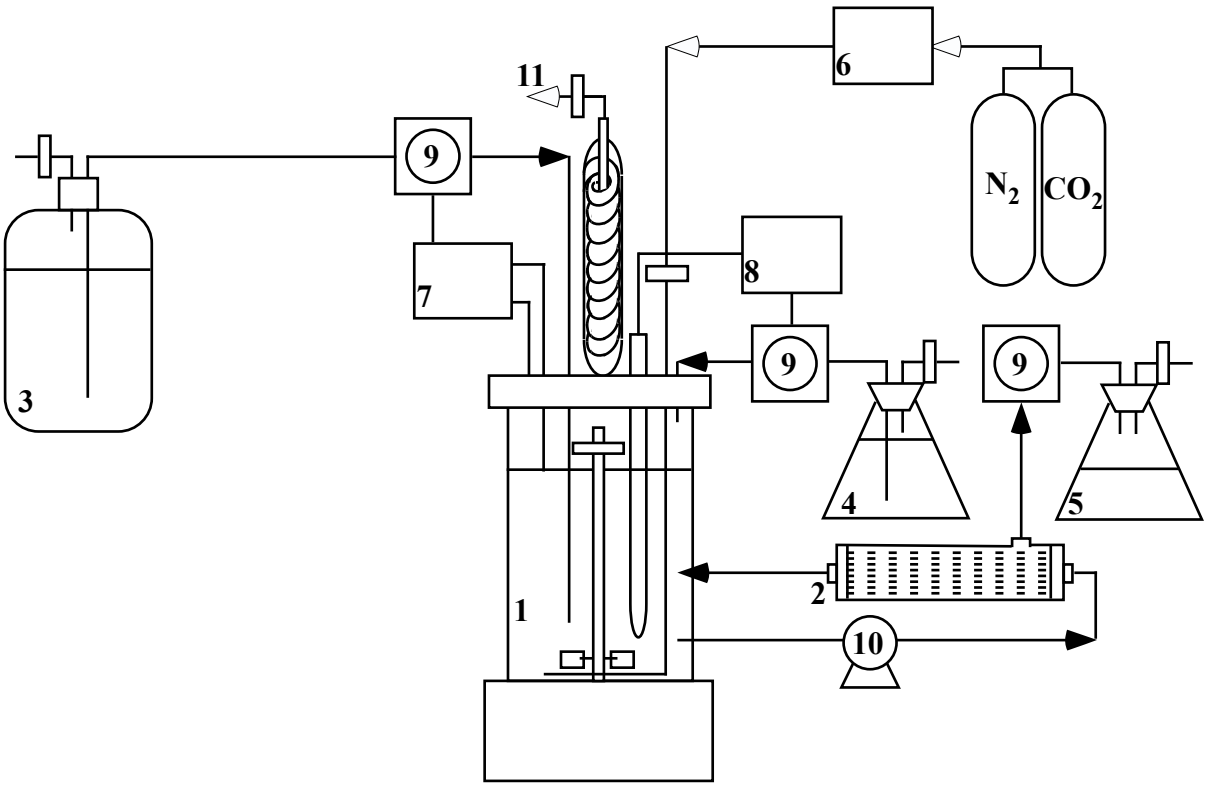


Fig. 4-1. Schematic diagram of the membrane bioreactor with a microfiltration module for BGS production.

1. anaerobic reactor
2. cross-flow membrane
3. reserver of fresh medium
4. 4 N NaOH solution
5. product tank
6. gas mixer
7. level controller
8. pH controller
9. peristaltic pump
10. high power roller pump
11. waste gas

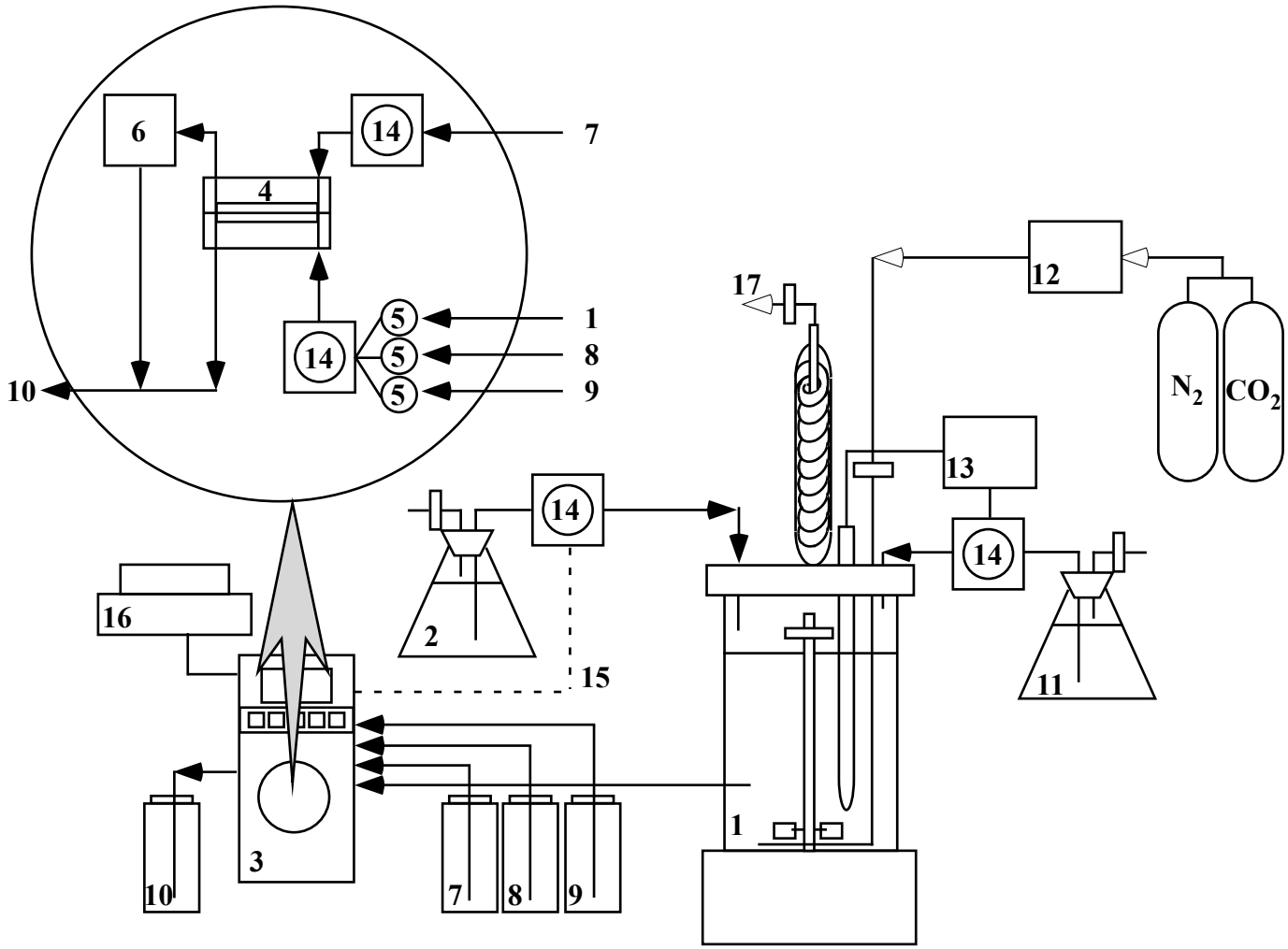


Fig. 4-2. Schematic diagram of the bioreactor with an on-line lactic acid controller for BGS production.

1. anaerobic reactor
2. medium with lactic acid for feeding
3. on-line lactic acid level controller
4. dialysis membrane
5. valves
6. lactic acid electrode
7. buffer solution
8. standard lactic acid solution
9. distilled water
10. waste water
11. 4 N NaOH solution
12. gas mixer
13. pH controller
14. peristaltic pump
15. line for controlling a pump
16. printer
17. waste gas

above.

4-2-6. 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 (Fig. 4-3). 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*.

4-2-7. 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 (62). 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 (55).

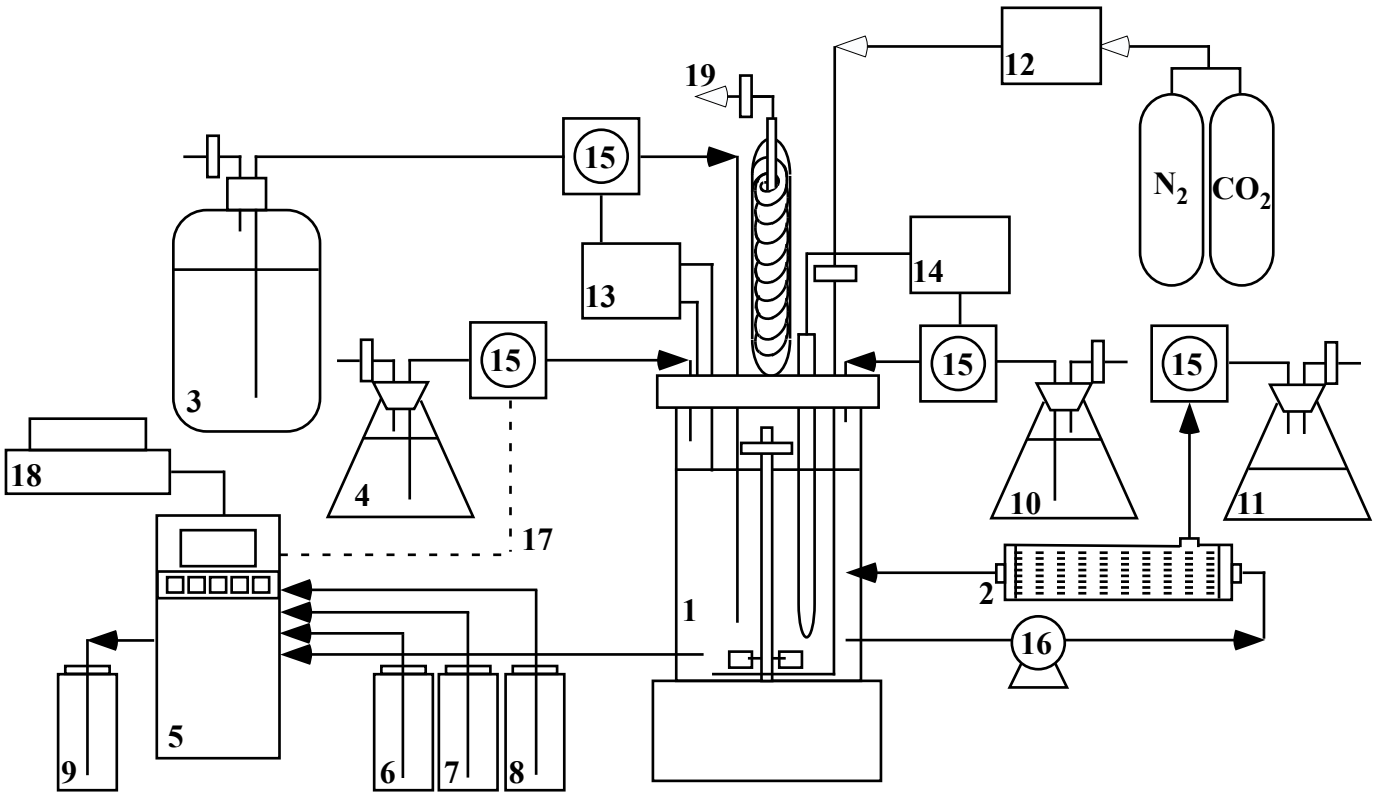


Fig. 4-3. Schematic diagram of the membrane bioreactor with a microfiltration module and an on-line lactic acid controller for BGS production.

1. anaerobic reactor
2. cross-flow membrane
3. reserver of fresh medium
4. medium with lactic acid for feeding
5. on-line lactic acid level controller
6. buffer solution
7. standard lactic acid solution
8. distilled water
9. waste water
10. 4 N NaOH solution
11. product tank
12. gas mixer
13. level controller
14. pH controller
15. peristaltic pump
16. high power roller pump
17. line for controlling a pump
18. printer
19. waste gas

4-3. Results and Discussion

4-3-1. Inhibitory effect of lactic acid on cell growth

Propionic acid bacteria can utilize lactic acid more readily than lactose and glucose (53, 63, 77). 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 4-4 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.

4-3-2. Batch cultivation

Figure 4-5 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

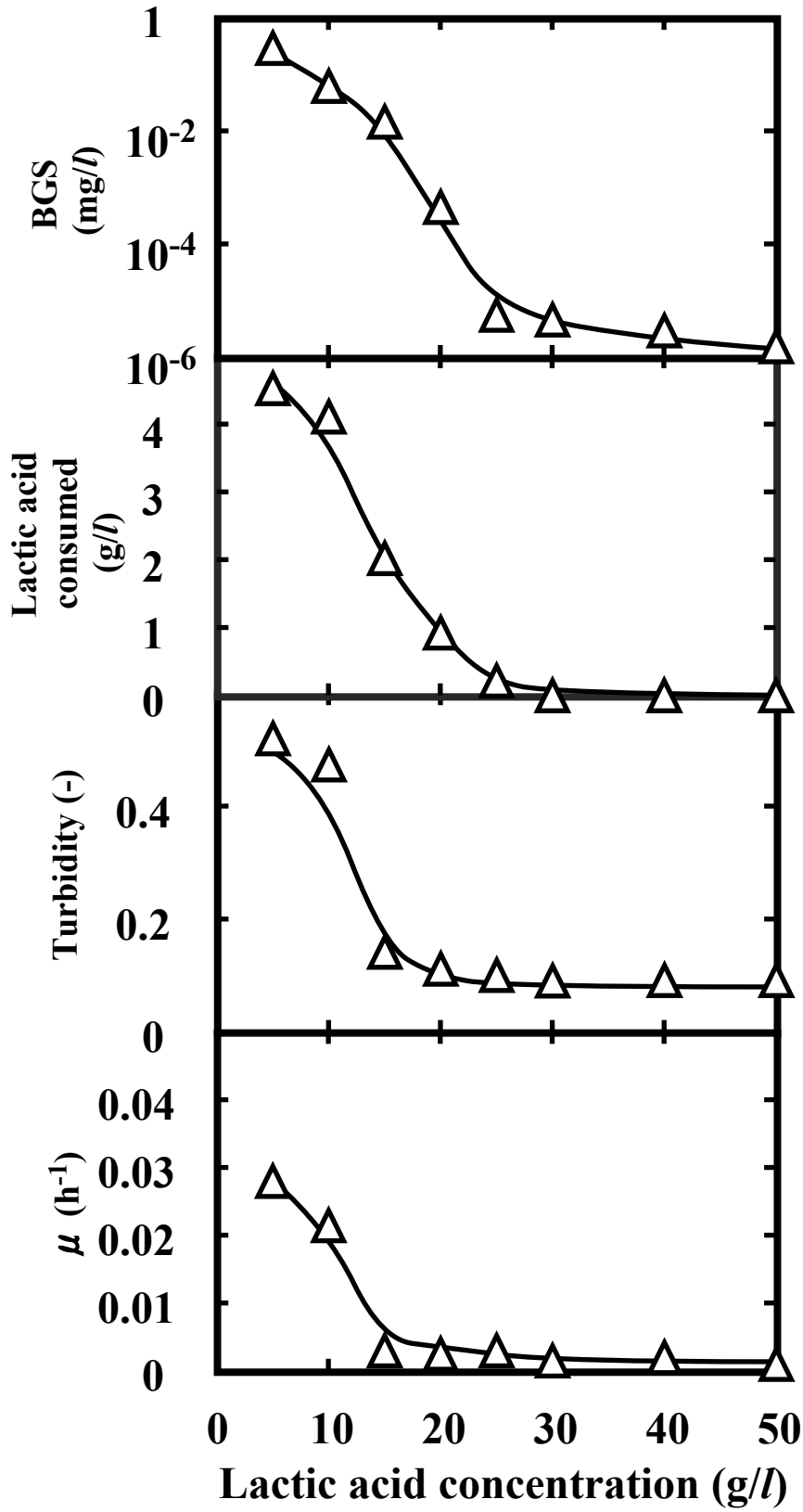


Fig. 4-4. Effect of initial lactic acid concentration on cell growth, lactic acid consumption, and BGS production by *P. shermanii*. The cultivation was carried out in test tubes for 48 h. The specific growth rates were examined on the basis of the initial increase in turbidity at 590 nm.

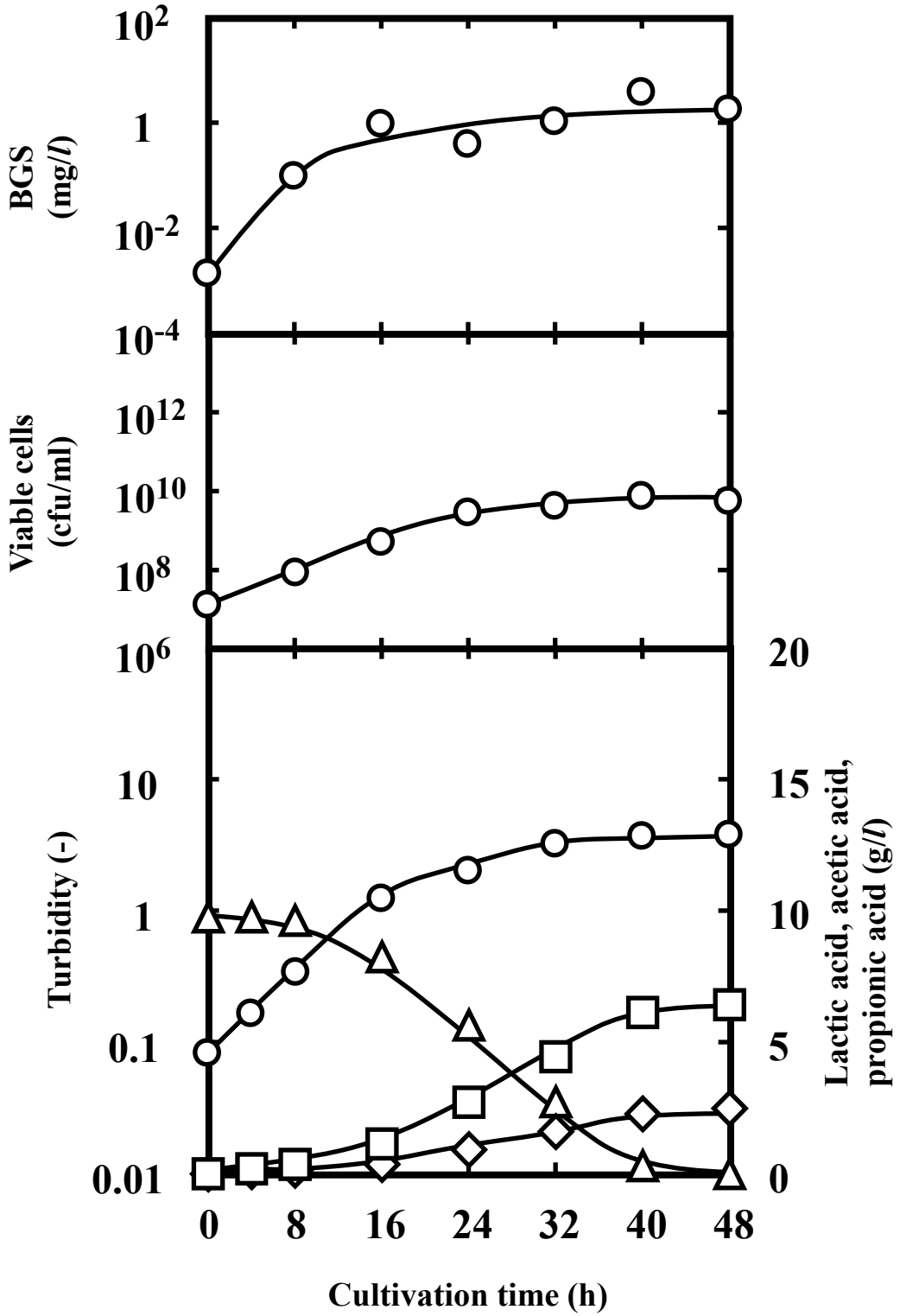


Fig. 4-5. Batch cultivation of *P. shermanii*. Symbols: open circles, BGS, viable cells and turbidity at 660 nm; open triangles, lactic acid; open diamonds, acetic acid; open squares, propionic acid.

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 (56, 61, 73-76). 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. BGS concentration increased gradually as the cells grew and then remained nearly constant at 3.8 mg/*l* after 40 h.

Table 4-1 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 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.

4-3-3. Continuous cultivation with a filtration device

I 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 4-6 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

Table 4-1. Inhibitory effects of the initial concentrations of metabolites on the cell growth of *P. shermanii*.

Initial conditions		μ_{max}	Turbidity[a]	Lactic acid	BGS [a]
Propionic acid (g/*l*)	Acetic acid (g/*l*)	(10^{-2} h^{-1})	(660 nm) (-)	consumed[a] (g//*l*)	(10^{-2} mg/*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	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] Data were obtained at 48 h of cultivation time.

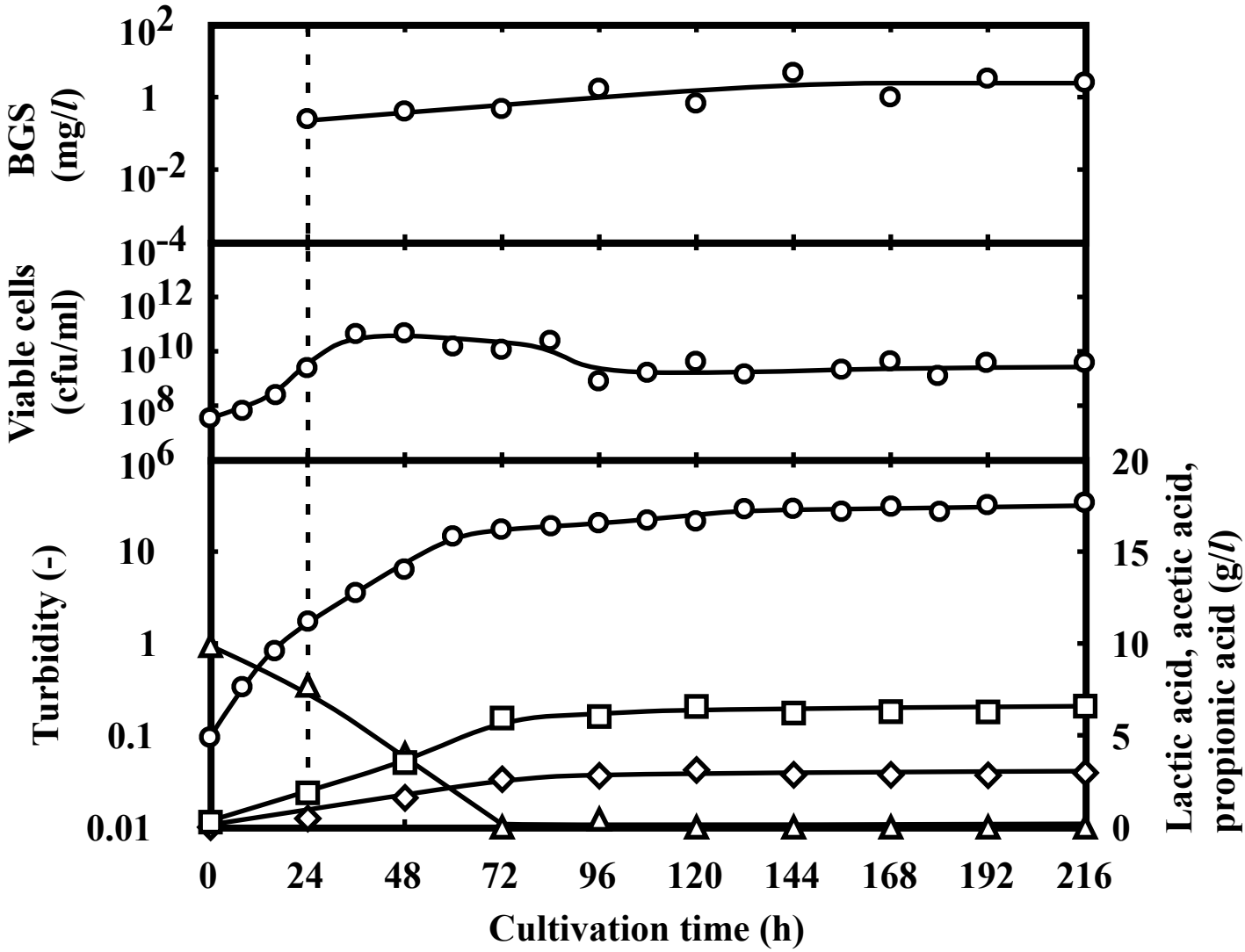


Fig. 4-6. Continuous production of BGS using a bioreactor with a microfiltration module. Filtration was started at 24 h as indicated by the dotted line. *D* was maintained at 0.075 h^{-1}. The same volume of fresh medium with 10 g/*l* lactic acid as that of the filtrate was supplied. Symbols are the same as those shown in Fig. 4-5.

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. 4-5); 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 $1\text{-}5\times10^{9}$ cfu/ml after the depletion of lactic acid, as shown in Fig. 4-6. 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*.

4-3-4. 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 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, I 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 (78), 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 4-7 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

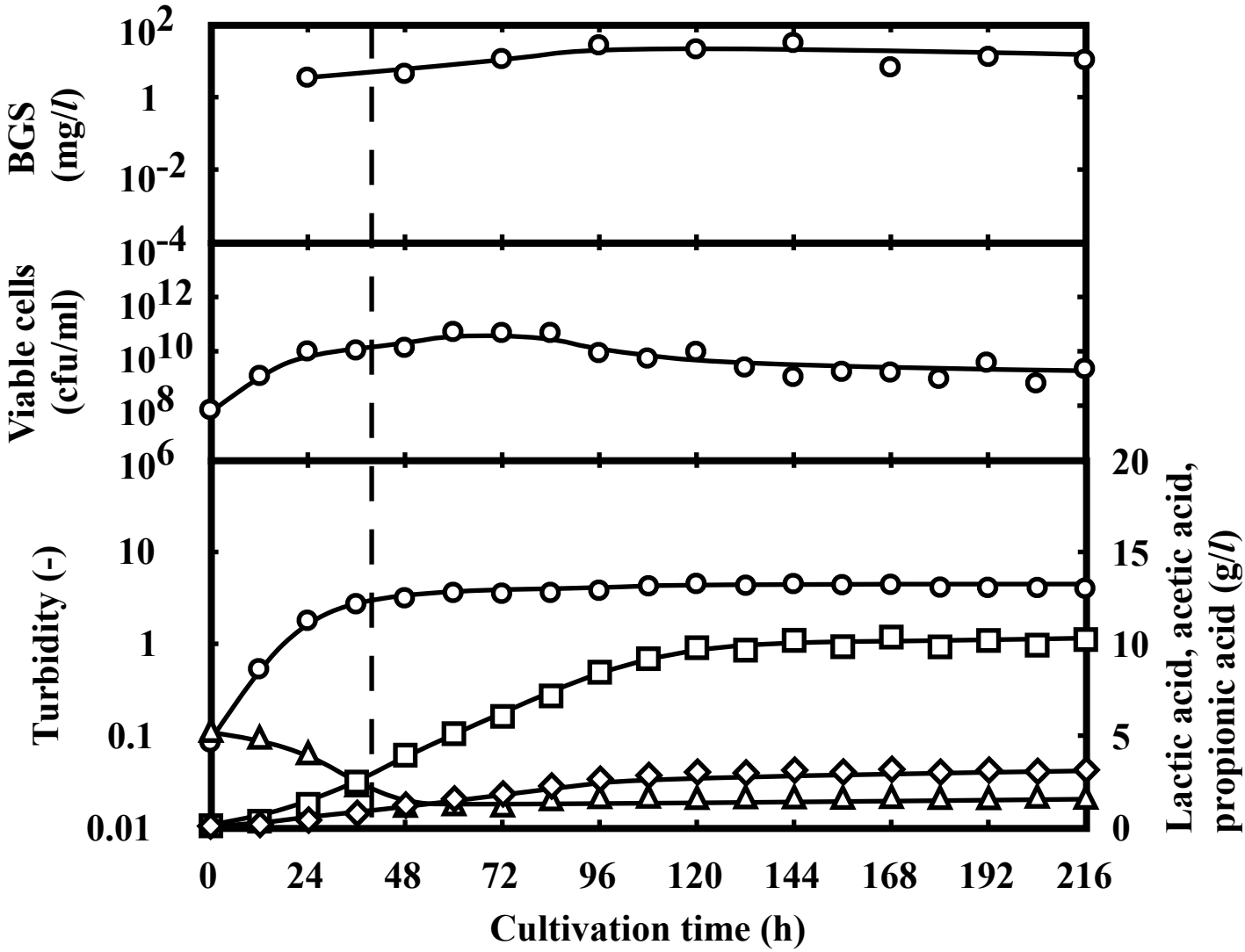


Fig. 4-7. Fed-batch production of BGS using a bioreactor with an on-line lactic acid controller. Control of lactic acid concentration in the culture broth was started at 42 h as indicated by the broken line. The concentration of lactic acid was maintained at about 3 g/*l* by feeding fresh medium with 50 g/*l* lactic acid. Symbols are the same as those shown in Fig. 4-5.

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. 4-7. 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 $1\text{-}4\times10^{9}$ 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.

4-3-5. 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, I used the continuous cultivation system with a filtration device and a lactic acid controller for BGS production by *P. shermanii*. Figure 4-8 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. 4-8, both the

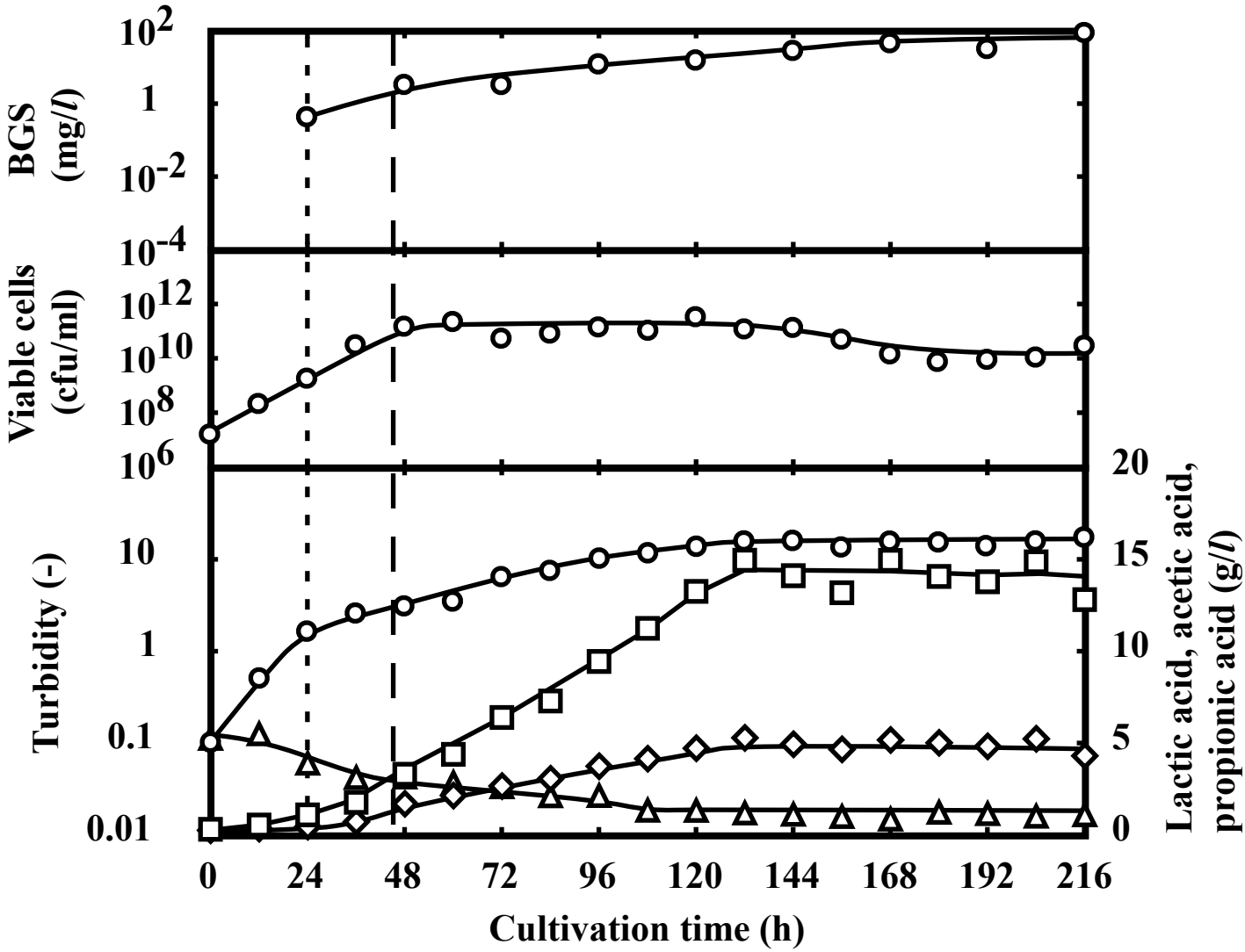


Fig. 4-8. Continuous production of BGS using a bioreactor with a microfiltration module and an on-line lactic acid controller. Filtration was started at 24 h, as indicated by the dotted line. *D* was maintained at 0.075 h^{-1}. The same volume of fresh medium with 10 g/*l* lactic acid as that of the 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. 4-5.

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 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-line lactic acid controller (Fig. 4-7). 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. 4-8) was 12-20 fold those in both the batch cultivation (Fig. 4-5) and continuous cultivation with filtration (Fig. 4-6), and 1.5-fold that in the fed-batch cultivation with the lactic acid controller (Fig. 4-7), respectively.

4-3-6. Comparison of BGS productivity among different cultivations

Table 4-2 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 4-2), 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 4-2). 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. 4-6. 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 4-2), 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 4-2). 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

Table 4-2. Comparison of BGS productivity among different cultivation methods.

Run no.	Cultivation method	Cultivation time T (h)	Total amount of lactic acid S_t (g)	Total amount of Culture medium 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})	$B_t/S_t/T$ (10^{-3} mg·g^{-1}·h^{-1})	$B_t/V_t/T$ (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. 4-5
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. 4-6
3	Fed-batch cultivation with a lactic acid controller	216	26	1.1	4.4	1.1	20.5	6.3	30.7[a]	33.2	21.4[a]	5.9	14.0	Fig. 4-7
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]	391.4	351[b]	-	16.8	Fig. 4-8

[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.

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 (54, 74, 76). 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.

Chapter 5

Analysis of Changes in Metabolisms of Propionibacteria by Oxygen Supply

Summary

Dairy propionibacterial strains produce a bifidogenic growth stimulator (BGS) which is not a nondigestible sugar but a mixture of 1,4-dihydroxy-2-naphthoic acid, 2-amino-3-carboxy-1,4-naphthoquinone and unknown compounds. In this study, I compared the amount of BGS produced by *Propionibacterium jensenii* that was cultivated under dissolved oxygen (DO)-controlled condition as well as under anaerobic and aerobic conditions. *P. jensenii* was able to grow more efficiently under aerobic and DO-controlled conditions than under anaerobic condition. However, BGS concentration decreased in DO-controlled cultivation because the oversupply of oxygen caused DHNA oxidation. To clarify the positive effect of oxygen supply on the BGS biosynthesis, I investigated the changes in proteins expressed in the propionibacterial cells under aerobic conditions by the proteomic and enzyme activity analyses in the cell-free extracts. Almost enzymes related to glycolysis were down-regulated under aerobic conditions, but a few enzymes for glucose metabolism of *P. jensenii* were up-regulated in the aerobic cultivations. Isochorismate synthase activity was higher under aerobic conditions than under anaerobic conditions.

5-1. Introduction

In 1994, Kaneko *et al.* (25) found a novel bifidogenic growth stimulator (BGS) produced by *Propionibacterium freudenreichii*. The BGS is not a nondigestible sugar but a mixture of 1,4-dihydroxy-2-naphthoic acid (DHNA), 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ), and unknown compounds. DHNA is a precursor of menaquinone (vitamin K) (40, 43). Yamasaki *et al.* (41) proposed that ACNQ functions as a mediator of electron transfer from NAD(P)H to O_2 and H_2O_2 in bifidobacterial cells. They also speculated that the exogenous oxidation of NADH is an efficient way for bifidobacteria to store pyruvate and to

generate ATP. Satomi *et al.* (52) reported that a significant increase in the number as well as in the frequency of occurrence of bifidobacterial cells in fecal samples of healthy human adults was observed during the intake period of the culture powder of *P. freudenreichii* containing the BGS. A recent preliminary report has shown that 4 weeks of ingesting BGS tablets resulted in clinical and endoscopic improvements in patients with active ulcerative colitis with no side effect (54).

Microorganisms have outstanding capability for adaptation to changes in environment. Especially, existence of oxygen is one of the important factors for growth of bacteria similarly to pH and temperature (79, 80). Various species of bacteria show positive or negative reaction against oxygen supply in culture broth. Several propionibacteria could grow under aerobic conditions as described previously (62). It is well established that propionibacterial responses to oxygen supply can vary widely among the strains. Of the four species tested (*Propionibacterium acidipropionici*, *Propionibacterium thoenii*, *P. jensenii*, and *P. freudenreichii*), only the former two could grow on the surface of solid medium in the air by Canzi *et al.* (81). Also in our previous study, *P. acidipropionici* and *P. jensenii* could grow under aerobic condition in liquid medium, and the concentrations of BGS obtained in the aerobic cultivations were 1.1-1.3-fold than that in anaerobic cultivations (62). The similar results obtained by Furuichi *et al.* (35) that *P. freudenreichii* ET-3 produced BGS more efficiently under aerobic condition because the metabolic flux was changed.

In this chapter, I compared the amount of BGS produced in dissolved oxygen (DO)-controlled cultivation with those produced under anaerobic and aerobic conditions. Moreover, to clarify the positive effect of oxygen supply on BGS biosynthesis, I investigated the changes in proteins expressed in the propionibacterial cells under aerobic conditions by proteomic and enzyme activity analyses in the cell-free extracts.

5-2. Materials and Methods

5-2-1. Microorganisms

P. jensenii 6433 was purchased from Japan Collection of Microorganisms and used as a producing strain of BGS in this study. *Bifidobacterium longum* OLB 6001 obtained from Meiji Dairies Corporation (Odawara) was used as a test microorganism for BGS activity assay. The microorganism was anaerobically precultured statically in test tubes at 30ºC 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) for 36-48 h (62).

5-2-2. Batch cultivations

The precultured cell was inoculated into TPY medium at an initial turbidity of about 0.1 at 660 nm. Cultivation was carried out in a fermentor with a working volume of 700 ml (TBR-1, Chiyoda Seisakusho, Nagano) coupled to a unit of pH and DO controllers (PW-1, Chiyoda Seisakusho, Nagano). The pH was maintained at 6.5 by adding 4 N NaOH using a peristaltic pump coupled to a pH controller. Anaerobic and aerobic cultivations were carried out as described in Chapter 2. DO-controlled cultivation was performed as follows: air or a mixed gas of N_2 and O_2 at a variable volume ratio was sparged at 0.3 vvm throughout the fermentation. Furthermore, agitation speed was regulated by a DO controller. The DO in culture broth was maintained at 3 ppm.

5-2-3. Preparation of cell free extract

Figure 5-1 indicates the procedure for preparation of cell free extract of *P. jensenii*. Cells were harvested by centrifugation (16,000×*g*, 20 min) and resuspended with phosphate buffer saline (PBS, pH 6.8) to wash the cells. The suspension was harvested by centrifugation (16,000×*g*, 20 min). Washing operation was performed twice and the washed cells were freezed at -80ºC and thawed. Cells were resuspended with PBS (pH 6.8) and glass beads (BZ-01, As One, Osaka), and homogenized by sonication (200 W, 10 min). The

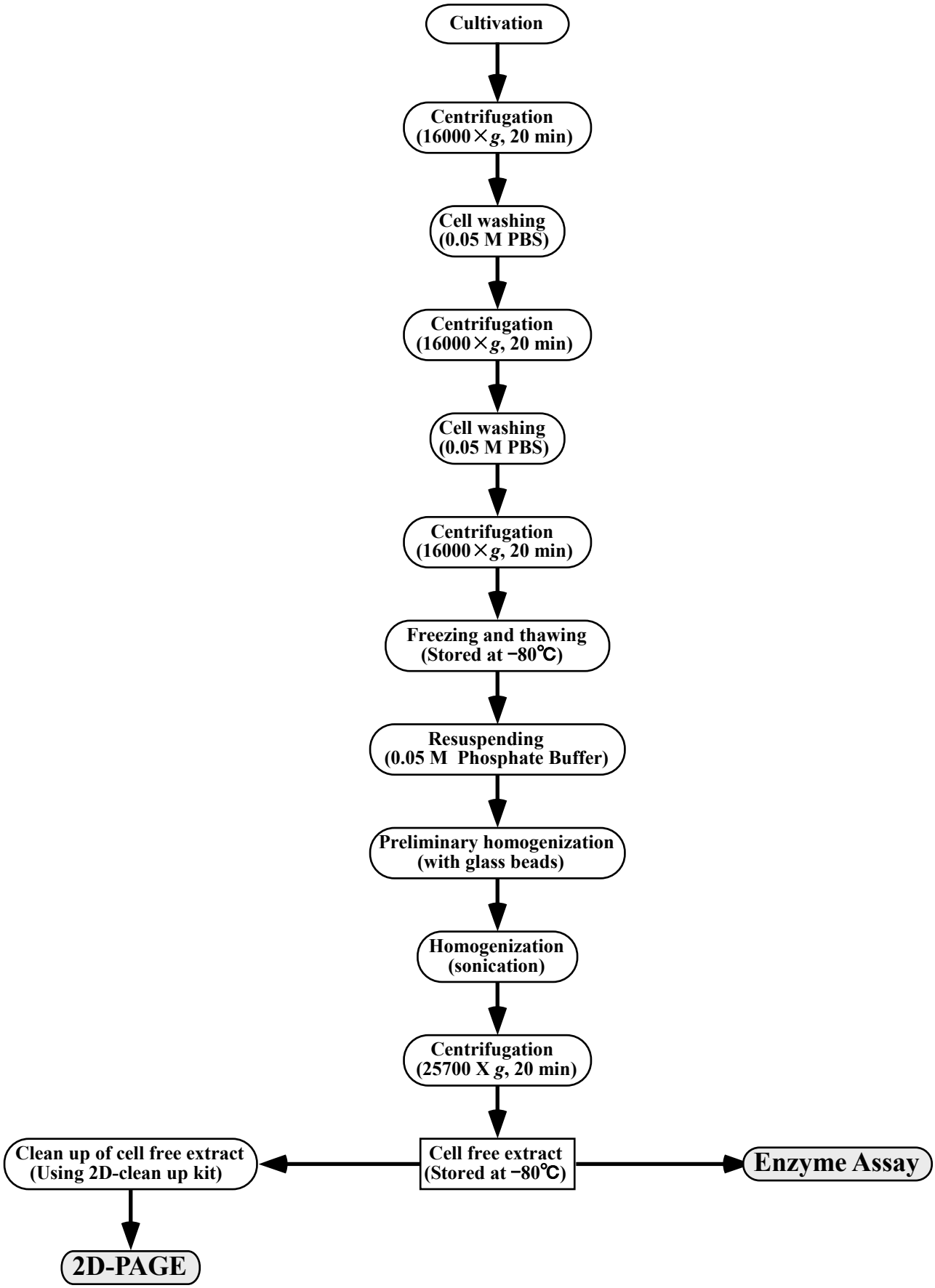


Fig. 5-1. Preparation of cell free extract.

suspension was then centrifuged at 25,700×*g* for 20 min to remove cell debris. The supernatant obtained was stored at -80ºC until use in enzyme assay and two-dimensional electrophoresis (2D-PAGE) described below.

5-2-4. Enzyme assays

L-Lactate dehydrogenase (EC 1.1.1.27) activity in the supernatants was assayed in a reaction mixture of 3.36 ml containing 100 mM glycylglycine buffer (pH 10), 44 mM L-glutamic acid, 4.7 mM β-nicotinamide adenine dinucleotide (β-NAD), and 47 units of glutamate-pyruvate-transaminase (GPT) (EC 2.6.1.2). Fumarate hydratase (EC 4.2.1.2) activity was determined in a reaction mixture of 3.0 ml containing 50 mM phosphate buffer (pH 7.3) and 75 mM L-malic acid. Succinate dehydrogenase (EC 1.3.99.1) activity was assayed in a reaction mixture of 3.0 ml containing 40 mM succinic acid, 100 mM sodium phosphate buffer (pH 7.6), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (w/vol) BSA, and 5 mM $K_3Fe(CN)_6$. Isochorismate synthase (EC 5.4.4.2) activity was assayed in a reaction mixture of 1.25 ml containing 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2 \cdot 6H_2O$, and 0.8 mM bariumchorismate. In the assay, methanol-*sec*-butanol (1:1) was used as a reaction terminator. Specific activities of enzymes are expressed as micromoles of substrate utilized or product formed per minute per milligram of protein. Spectrophotometric assays except for isochorismate synthase were carried out using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto) by the following conditions: L-lactate dehydrogenase, 25ºC, 340 nm; fumarate hydratase, 25ºC, 250 nm, and succinate dehydrogenase, 25ºC, 455 nm. Isochorismate synthase activity was determined by the method of Poulsen *et al.* (82) using an HPLC system (Shimadzu, Kyoto). The HPLC analysis was performed at 40 ºC and a flow rate of 1 ml/min on a 4.6 mm × 250 mm ZORBAX SB-C8 column with a particle size of 5 μm. The eluent consisted of 50 mM H_3PO_4 (final concentration) in H_2O-MeOH (13:7) and the pH was adjusted to 2.5 with 4 M NaOH in the water phase before addition of MeOH. The eluent was filtered through a 0.45 μm (PTFE) filter and degassed under vacuum.

5-2-5. Two-dimensional electrophoresis

The first dimensional separation was performed on IPG dry strip (pH 4-7, 11 cm, GE Healthcare, UK) using an Ettan IPGPhor II electrophoresis unit (GE Healthcare). Cell free extract samples were partially purified by using 2-D Clean-Up Kit (GE Healthcare) and suspended in an isoelectrofocusing IPG sample buffer containing 8 M urea, 0.5% (vol/vol) IPG buffer, 0.002% (vol/vol) bromophenol blue, 2% (w/vol) 3-[(3-cholamidopropyl) dimethylammonio]- propanesulfonate and 18 mM dithiothreitol (DTT). Protein concentration was determined using the Lowry method (83) with bovine serum albumin (BSA) as a standard in order to load 200 μg protein in 200 μl IPG sample buffer on the strips. The dried IPG strips were actively rehydrated for 12 h at 25ºC in the ceramic strip holder. The following voltage gradient was applied: 500 V for 2 h; from 500 V to 1000 V for 2 h; from 1000 V to 6000 V for 5 h; and 6000 V for 1.67 h. After isoelectrofocusing electrophoresis, strips were equilibrated for 15 min in a equilibrating buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (vol/vol) glycerol, 2% (w/vol) sodium dodecyl sulfate (SDS), 0.002% (vol/vol) bromophenol blue, and 65 mM DTT.

The second-dimensional separation was carried out on an AE-8150 my Power 500 (ATTO, Co., Tokyo) using 10% or 15% acrylamide separating gels without stacking gel at a stepwise voltage of 20 V to 250 V for 3.5 h, and then, a constant voltage of 250 V for about 1 h. Standard proteins for SDS-PAGE (Bio-Rad Laboratories, Inc., California) were used as markers of molecular weight. Gels were fixed and stained with Coomassie Brilliant Blue (CBB).

5-2-6. N-terminal amino acid sequencing

Electrophoretically separated samples were transferred onto polyvinylidene fluoride (PVDF) membranes using a HorizBlot (AE-6677, ATTO). Proteins were visualized by CBB staining. Spots were cut from the membranes and after the destaining step, applied to an automatic protein sequencer (PRSQ-21A, Shimadzu, Kyoto) as described by the manufacturer.

Sequence homologies were searched using the BLAST program (http://blast.genome.jp/).

5-2-7. Analytical methods

Cell concentration was determined by measuring turbidity at 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, DHNA, ACNQ, glucose, lactic acid, acetic acid, and propionic acid concentrations. BGS concentration was determined by the agar diffusion plate assay method using *Bifidobacterium longum* OLB 6001 as reported previously (62). The concentrations of DHNA, ACNQ, glucose, and organic acids were determined by HPLC as reported previously (62).

5-3. Results and Discussion

5-3-1. Cell growth and BGS production under DO-controlled conditions

Figure 5-2 indicates the cultivation of *P. jensenii* at pH 6.5 using 30 g of glucose as a carbon source under DO-controlled (3 ppm) conditions. *P. jensenii* grew exponentially until 36 h. BGS concentration increased gradually as the cells grew and then reached at 3.5 mg/*l* after 48 h. Propionic acid was hardly produced, and only acetic acid was produced during the cultivation time.

Figure 5-3 shows the comparisons of cell growth and BGS activities obtained under the different culture conditions. The concentrations of acetic and propionic acid as main metabolites was also indicated in Fig. 5-3. The results of anaerobic and aerobic cultivations were previously shown in Fig. 2-4D and Fig. 2-5D, respectively. The DO concentration in the aerobic conditions was lower than 3 ppm during 8-72 h of cultivation. The turbidity (T_{660} = 27.4) obtained in the DO-controlled cultivation was higher as compared with those in the anaerobic (T_{660} = 20.9) and aerobic cultivations (T_{660} = 24.5). The propionic acid

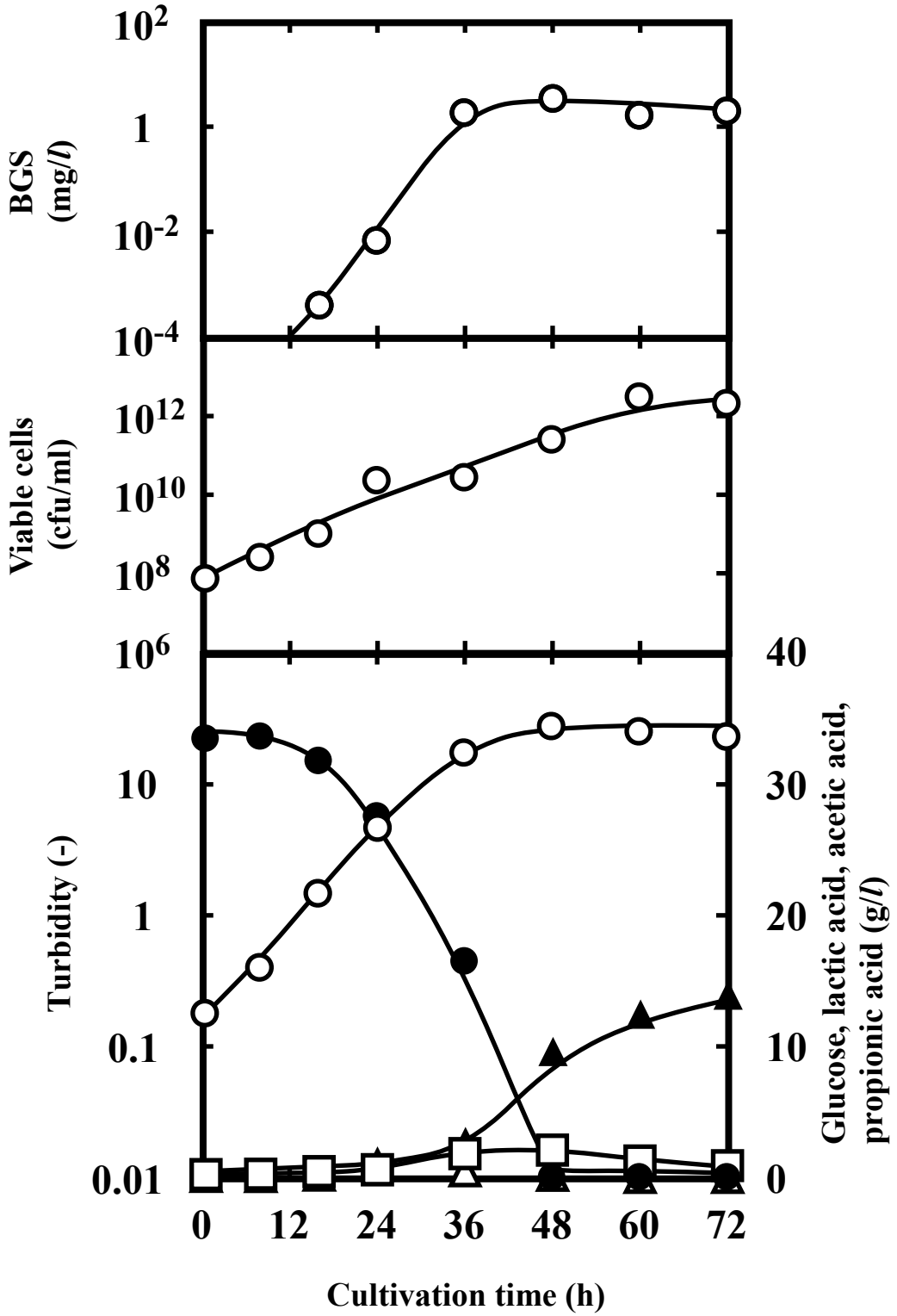


Fig. 5-2. BGS production of *P. jensenii* under DO-controlled (3 ppm) condition. *S*ymbols: open circles, BGS, viable cells and turbidity at 660 nm; closed circles, glucose; open triangles, lactic acid; closed triangles, acetic acid; open squares, propionic acid.

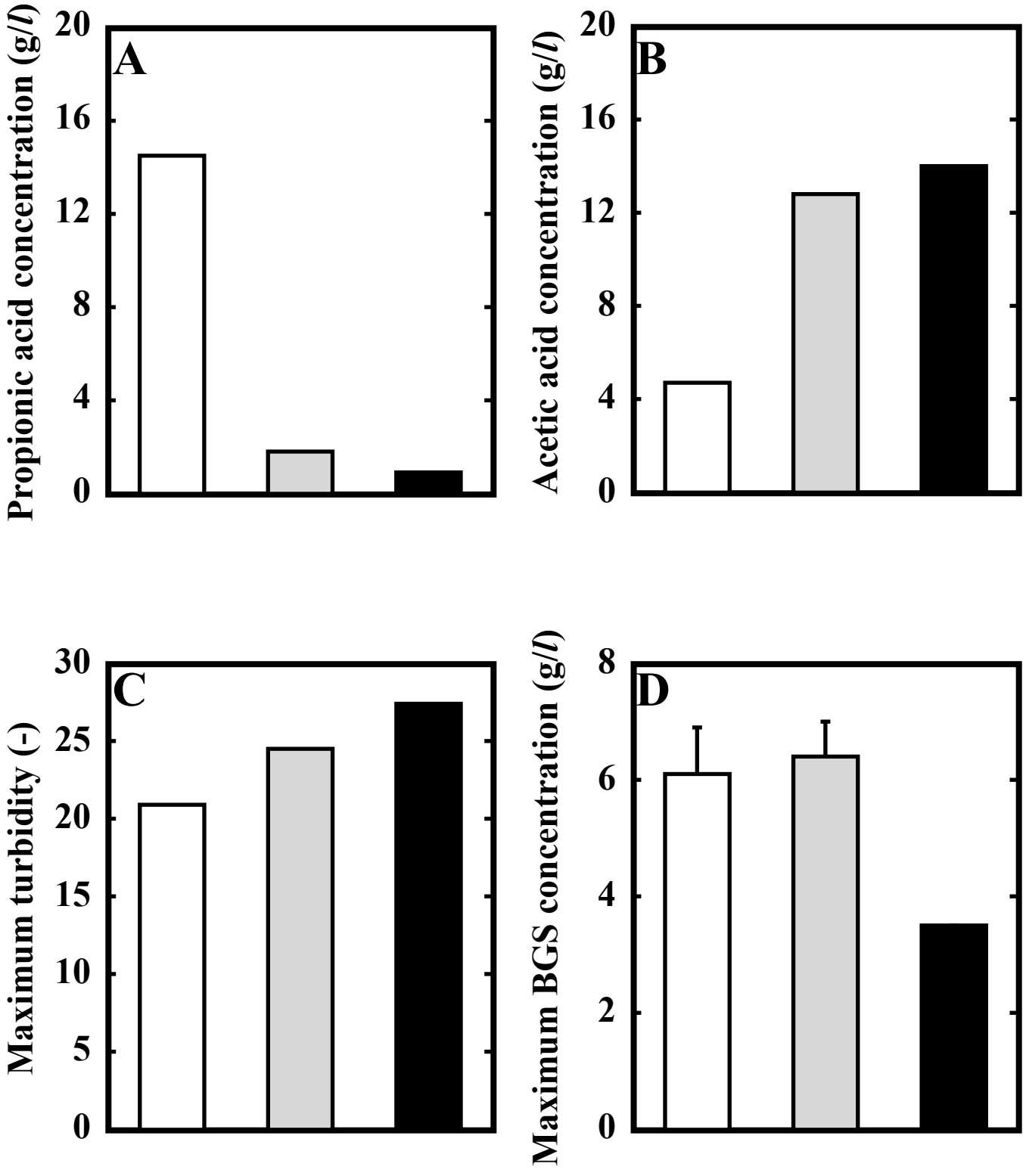


Fig. 5-3. Effect of oxygen supply on the organic acid concentrations, turbidity, and BGS concentraition. Data denotes propionic acid concentration (A), acetic acid concentration (B), maximum turbidity (C), and maximum BGS concentration (D). Symbols: anaerobic condition (open bars), aerobic condition (hatched bars), and dissolved oxygen-controlled condition (solid bars). The error bars indicate the standard deviations.

concentrations were 14.5 g/*l*, 1.8 g/*l*, and 0.9 g/*l* for the anaerobic, aerobic and DO-controlled cultivations, respectively. As the rate of oxygen supply increased, the propionic acid concentrations significantly decreased (Fig. 5-3A) and in contrast, the acetic acid concentrations increased (Fig. 5-3B). The concentrations of acetic acid in the aerobic (12.8 g/*l*) and DO-controlled cultivations (14.0 g/*l*) were 2.7-fold and 3.0-fold that in the anaerobic cultivation (4.7 g/*l*), respectively. The BGS concentration in the aerobic cultivation was slightly higher than that in the anaerobic cultivation, but the increase in rate of oxygen supply resulted in the significant reduction of BGS concentration.

The increase in the cell growth in the aerobic and DO-controlled cultivations seem to be attributable to the gain of energy as well as the alleviation of inhibition of cell growth related to the conversion of propionic acid to acetic acid. Ye *et al.* (84) and Namba *et al.* (85) reported that propionic and acetic acids inhibited the cell growth of *Propionibacaaterium shermanii* and vitamin B_{12} production by *P. shermanii*, and that propionic acid inhibited more strongly than acetic acid. In addition, Furuichi *et al.* (35) suggested that biosyntheses of DHNA and menaquinone were stimulated by the decrease in propionic acid concentration in the cells of *P. freudenreichii*. In this study, propionic acid exerted the inhibitory effect on the cell growth and the BGS production, and the degree of the inhibition was stronger than that of acetate as described in Chapter 4. However, the BGS concentration (3.5 mg/*l*) in the DO-controlled cultivation was significantly lower as compared with those in the anaerobic and aerobic cultivations. The decrease is probably due to DO in the culture broth during cultivation because DHNA is very labile to oxygen. In the aerobic cultivation, since the DO level in the culture broth was maintained at a low concentration (0.0-1.9 ppm) and little or no DHNA seems to be oxidatively degraded, the BGS concentration was slightly higher than that in the anaerobic cultivation. The same observation was recently reported by Furuichi *et al.* (35). From these results, the suitable oxygen supply was found to be useful for the cell

growth of *P. jensenii* and the BGS production by *P. jensenii*. However, the most favorable rate of oxygen supply for BGS production is still under study.

5-3-2. Enzyme activities in cell-free extracts

Table 5-1 shows the activities of isochorismate synthase, fumarate hydratase, succinate dehydrogenase, and L-lactatre dehydrogenase in the cell-free extracts of *P. jensenii* cultivated under the different culture conditions. Isochorismate synthase (reaction **I**) catalyzes the following chemical reaction: chorismate → isochorismate. This reaction exists in the upstream of the DHNA and menaquinone biosynthesis pathway. The specific activities of isochorismate synthase in the aerobic cultivation (4.01×10^{-3} U/mg) and in the DO-controlled cultivation (5.32×10^{-3} U/mg) were 1.2-fold and 1.6-fold that in the anaerobic cultivation (3.43×10^{-3} U/mg), respectively. The increase of this specific enzyme activity seems to result in the enhanced BGS production under the aerobic condition. However, although isochorismate synthase activity in the DO-controlled cultivation was the highest, the BGS concentration was the lowest level due to decomposition of DHNA by DO in the culture broth during the cultivation as described above. Fumarate hydratase (reaction **II**) was found in the cells of *Propionibacterium shermanii* by Krebs and Eggleston (86). The enzyme catalyzes the interconversion between fumarate and malate. Succinate dehydrogenase (reaction **III**) catalyzes the reduction of fumarate to succinate (87). These enzymes relate to the tricarboxylic acid cycle and methylmalonyl CoA pathway. It has shown by Krainova *et al.* (88) that several propionibacteria, grown on glucose, contain all the enzymes of TCA cycle. In addition, propionibacteria have been reported to be capable of utilizing the reverse methylmalonyl CoA pathway (89, 90). The expression of both fumarate hydratase and succinate dehydrogenase was down-regulated when oxygen was supplied. L-Lactate dehydrogenase (reaction **IV**) catalyzes the interconversion of pyruvate and lactic acid with concomitant interconversion of NADH and NAD^+. In the aerobic cultivations using glucose as a carbon source, the up-regulation of L-lactate dehydrogenase expression was observed.

Table 5-1. Enzymatic activity in *P.jensenii* cells under anaerobic condition or aerobic conditions.

Reaction No.	Enzyme commission numbers	Common name	Reaction	Specific activity (U/mg)		
				Anaerobic	Aerobic	DO (3 ppm)
I	5.4.4.2	Isochorismate synthase	Chorismate ⇆ Isochorismate	3.43×10^{-3}	4.01×10^{-3}	5.32×10^{-3}
II	4.2.1.2	Fumarate hydratase	L-Malate ⇆ Fumarate + H_2O	3.98	2.67	1.67
III	1.3.99.1	Succinate dehydrogenase	Succinate + Acceptor ⇆ Fumarate + Reduced acceptor	0.531	0.528	0.366
IV	1.1.1.27	L-Lactate dehydrogenase	L-Lactate + NAD ⇆ Pyruvate + NADH + H^+	1.13	2.50	3.55

These results suggest that *P. jensenii* has the energy regulation-system by converting lactic acid to pyruvate, followed by further metabolism of pyruvate.

5-3-3. Change in protein expression by oxygen supply

To investigate the effect of oxygen supply on the protein expression, I compared the amounts of proteins expressed in the cells obtained in the different cultivations. After the clean-up for removing impurities, I analyzed the cell free extracts of *P. jensenii* by two-dimensional electrophoresis (2D-PAGE), followed by the N-terminal amino acid sequencing of the separated protein spots by a protein sequencer. Figures 5-4, 5-5, and 5-6 indicate the 2D-PAGE of protein expression in the cells obtained in the anaerobic, aerobic, and DO-controlled cultivations, respectively. The six up-regulated proteins under the aerobic conditions were observed, but only one protein was identified with a reliability of 88% (spot 1). Of the three expression-unchanged proteins isolated, two proteins were identified by N-terminal amino acid sequencing with a reliability of 80-90% (spots 2 and 3). The sixteen proteins down-regulated in the aerobic cultivations (Figs. 5-5 and 5-6) were detected and the N-terminal amino acid sequences of the eleven proteins of them were determined with high reliabilities (spots 4-13).

Table 5-2 denotes the results of N-terminal sequence analysis of proteins detected in 2D-PAGE. The fourteen proteins were identified. The activity of aspartate aminotransferase (spot 1, EC 2.6.1.1) catalyzes the reaction of converting oxaloacetate, increased in the cells obtained under the aerobic conditions. DNA binding protein (spot 2) plays an important part in regulation of gene expressions and formation of higher-order chromatin structures. Triose-phosphate isomerase (spot 3, EC 5.3.1.1) plays an important role in glycolysis and catalyzes the reversible reaction of the triose phosphate isomers, dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Glyceraldehyde-3-phosphate dehydrogenase (spots 4 and 5, EC 1.2.1.12) is the enzyme relating to the sixth step of glycolysis and catalyzes the conversion of glyceraldehyde-3-phosphate to D-glycerate-1,3-

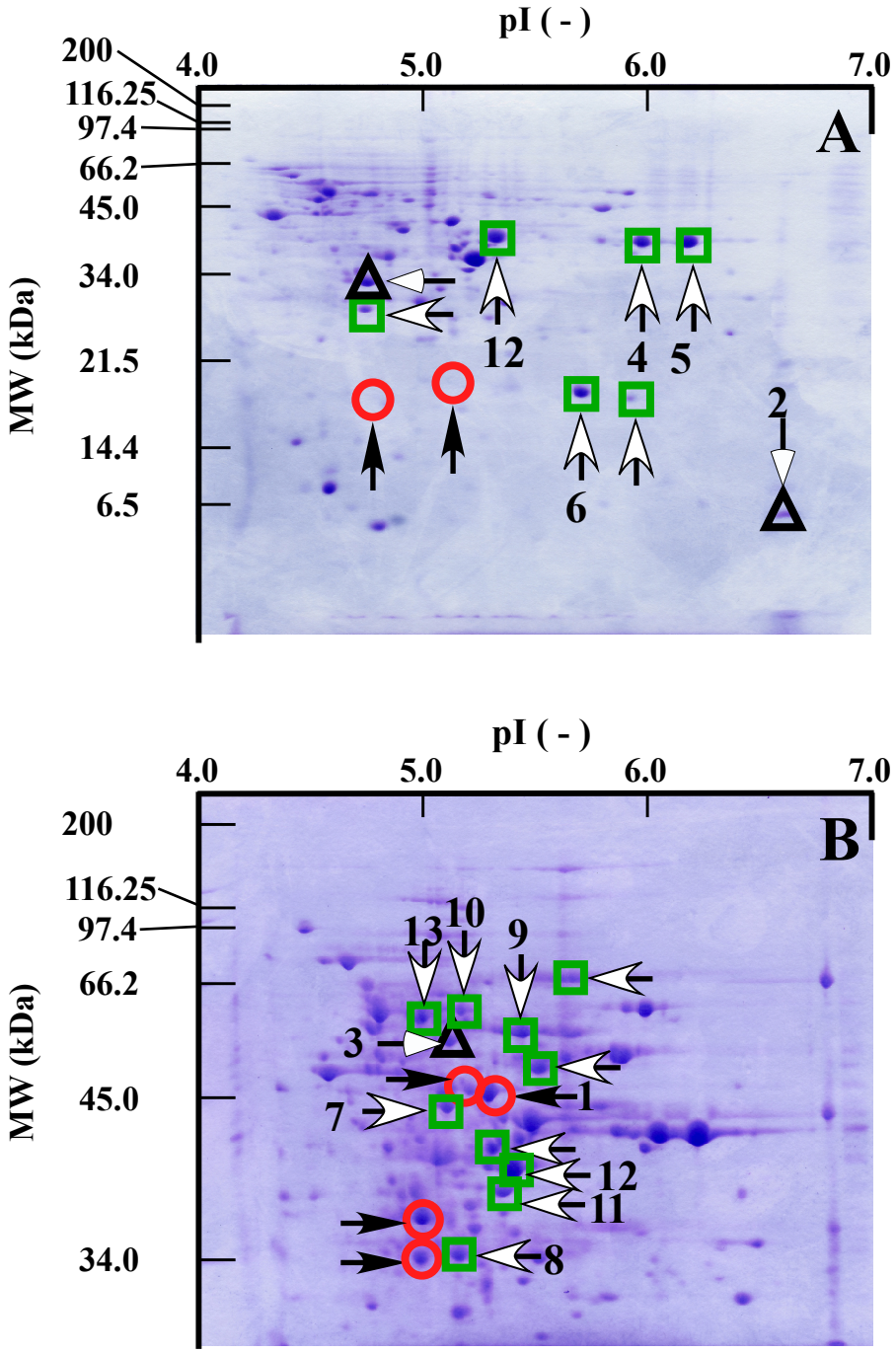


Fig. 5-4. Two-dimensional analysis of protein expression under anaerobic condition. 15 % SDS-PAGE (A) and 10 % SDS-PAGE (B). Symbols denote polypeptides displaying an increased relative rate (open circle), an unchanged relative rate (open triangles), and a reduced relative rate (open squeres) of synthesis during aerobic and DO-controlled (3 ppm) cultivations compared with anaerobic cultivation.

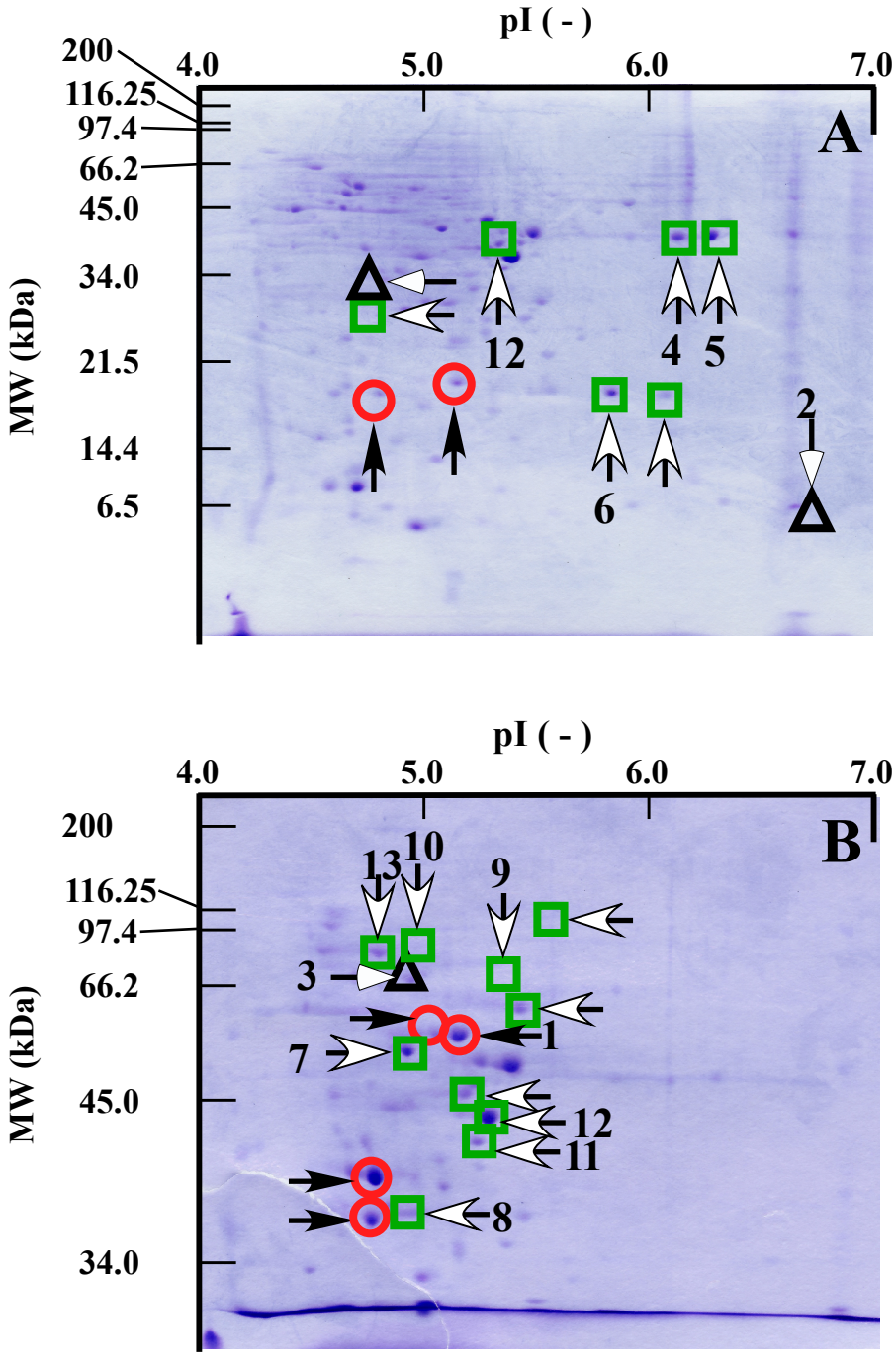


Fig. 5-5. Two-dimensional analysis of protein expression under aerobic condition. 15 % SDS-PAGE (A) and 10 % SDS-PAGE (B). Symbols are the same as those shown in Fig. 5-4.

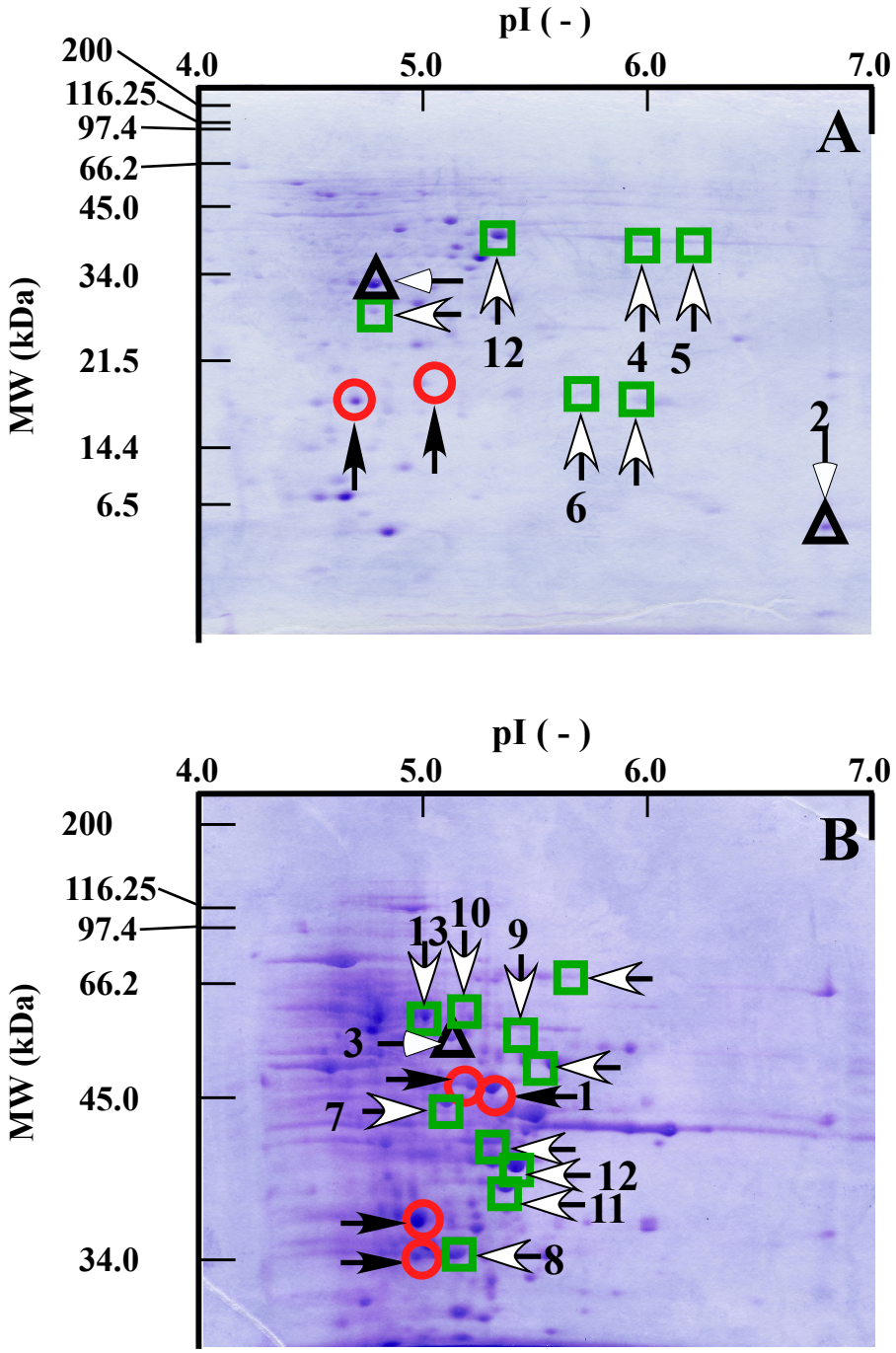


Fig. 5-6. Two-dimensional analysis of protein expression under DO-controlled (3 ppm) condition. 15 % SDS-PAGE (A) and 10 % SDS-PAGE (B). Symbols are the same as those shown in Fig. 5-4.

Table 5-2. N-terminal sequence analysis of proteins expressed in the anaerobic and aerobic cultivations of *P. jensenii* JCM 6433.

Spot number	N-terminal sequence [a]	Database homology	Source	pI [b]	Molecular mass [b] (kDa)	Identity (%)
1	SNEFIPPAKPIIGTVERKAV	Aspartate aminotransferase (EC 2.6.1.1)	*Propionibacterium acnes*	5.3	44	88
2	MTKSELIKAIAKEASLTETQ	DNA-binding protein HU	*Propionibacterium acnes*	6.5	6.5	80
3	SDVVITGFGATTPLGGDAPS	3-Oxoacyl-[acyl-carrier protein]syntase (EC 2.3.1.41)	*Propionibacterium acnes*	5.2	56	90
4	TVKVGINGFGRIGRNFFRAL	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	*Propionibacterium acnes*	6.0	40	100
5	GVKVGINGFGRIGRNFFRAL	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	*Propionibacterium acnes*	6.2	40	100
6	MENFNNDPFAEIDHVGYAVK	Methylmalonyl-CoA epimerase (EC 5.1.99.1)	*Propionibacterium acnes*	5.8	18	95
7	MKSVEDLGDLSGKRVVIDMA	Phosphoglycerate kinase (EC 2.7.2.3)	*Propionibacterium acnes*	5.2	43	94
8	SRKPILAGNYKMNLDHLAGL	Triosephosphate isomerase (EC 5.3.1.1)	*Propionibacterium acnes*	5.4	34	77
9	MKIAVVAMGKIGLDLAVQFA	Putative UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase (EC 1.1.1.-)	*Propionibacterium acnes*	5.6	57	95
10	AELTIRPEEIRDALDHFVQN	ATP synthase subunit α (EC 3.6.3.14)	*Propionibacterium acnes*	5.2	56	95
11	AKIANDVTELIGGTPLVRIN	Cysteine synthase (EC 2.5.1.47)	*Propionibacterium acnes*	5.6	38	90
12	PIATPEVYAEMIDRAKAKAF	Fructose-bisphosphate aldolase (EC 4.1.2.13)	*Frankia* sp. Cc13	5.6	40	80
13	AHPRAGQPAQEQDLIDVDAV	Phosphoglucomutase (EC 5.4.2.2)	*Propionibacterium acnes*	5.1	60	85

[a] Hatched amino acids denote less than 50% of the reliability.

[b] pI and molecular mass values were estimated from the results of 2D-PAGE.

bisphosphate. Recently, Beak *et al.* reported that glyceraldehyde-3-phosphate dehydrogenase regulates generation of H_2O_2 by heat shock and suppresses cell death in yeast and plant cells (91). The enzyme seems to be one of these responsive to some stresses. Methylmalonyl-CoA epimerase (spot 6, EC 5.1.99.1) catalyzes the transformation of methylmalonyl-CoA to the isomer, which can be used in subsequent steps of propionic acid fermentation. Phosphoglycerate kinase (spot 7, EC 2.7.2.3) is a transferase enzyme used in the seventh step of glycolysis. The kinase catalyzes the transformation of a phosphate group from 1,3-bisphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. Cysteine synthase (spot 11, EC 2.5.1.47) has been also known to participate in oxidative (and cold) stress adaptation in *Bacillus subtilis* (92). Fructose-bisphosphate aldorase (spot 12, EC 4.1.2.13) catalyzes the converion of D-fructose 1,6-bisphosphate to glycerol phosphate and D-glyceraldehyde 3-phosphate. Phosphoglucomutase (spot 13, EC 5.4.2.2) is an enzyme that transfers a phosphoryl group on a glucose monomer from the 1' to the 6' position in the forward direction or the 6' to 1' position in the reverse. As described above, the expression of the several enzymes for some bacteria has been reported to be regulated by stresses such as heat, salt, bile and oxygen (93). However, further details of the metabolic response of *P. jensenii* to oxygenation needs to be studied for improvement of BGS production.

Figure 5-7 shows the proposed synthetic pathways of DHNA and menaquinone (58, 89, 94-99). The proteomic analysis and measurement of enzymatic activities in *P. jensenii* showed that most of the enzymes for glucose metabolism were down-regulated while a few enzymes were up-regulated under the aerobic conditions. Especially, three enzymes (glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, and fructose-bisphosphate aldolase) related to glyceraldehyde-3-phosphate were down-regulated under the aerobic conditions. Furuichi *et al.* (98) suggested that glyceraldehyde-3-phosphate is a precursor of isoprenoid unit, and the absence of the isoprenoid precursor stops isoprenoid production and that the termination of isoprenoid production increases DHNA production.

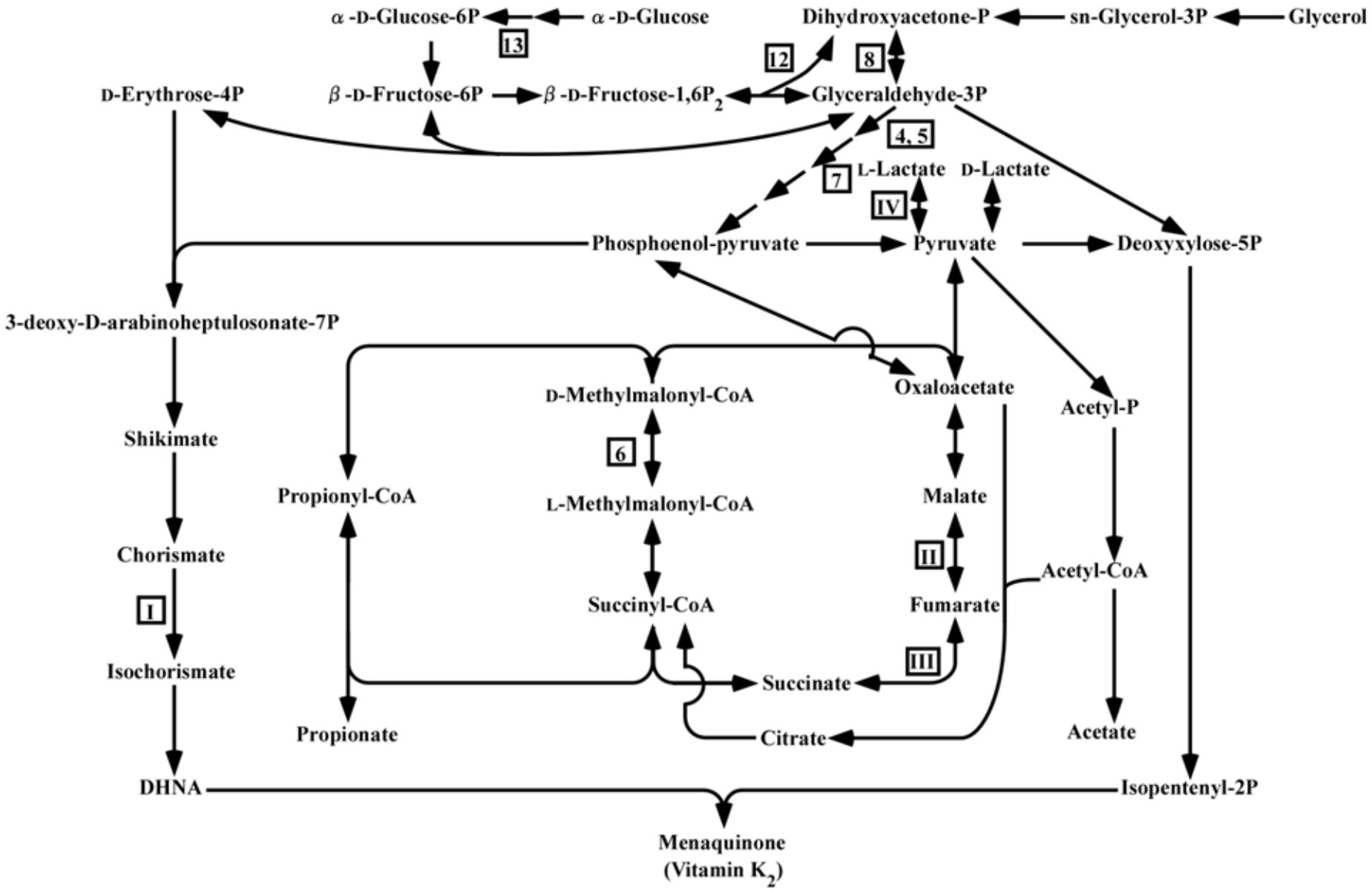


Fig. 5-7. Simplified propionic acid fermentation pathways from glycerol, glucose and lactic acid by propionibacterial strains, and proposed biosynthetic pathways for DHNA and menaquinone production.
Roman figures and Arabic figures denote the enzymes described as Table 5-1 and Table 5-2, respectively.

Therefore, in the aerobic cultivation of *P. jensenii*, the concentration of glyceraldehyde-3-phosphate seems to be lower than that in the anaerobic cultivation. The activity of isochorismate synthase, which is related to the synthesis of the aromatic ring of DHNA, was 1.6-fold higher in *P. jensenii* cells obtained in the aerobic cultivations than that in the anaerobic cultivations. The increase in the activity is considered as one of the reasons for enhanced BGS production in the aerobic cultivation.

5-4. Conclusion of Chapter 5

In this chapter, I found that the rate of cell growth under aerobic conditions was higher than those in the anaerobic cultivations, but the concentration of BGS in the DO-controlled cultivation was less than that in the anaerobic cultivation because DHNA was significantly labile to oxygen. The proteomic and enzyme activity analyses showed the up-regulation for one (isochorismate synthase) of the enzymes related to DHNA synthesis. The findings obtained in this study will be useful in both the screening of the BGS-producing bacteria and the efficient production of BGS.

Chapter 6

Concluding Remarks

This thesis dealt with the efficient production of bifidogenic growth stimulator (BGS) by several propionibacterial strains. The main objectives were (i) optimization of the bioassay method for high sensitive measurement of BGS concentration, (ii) selection of propionibacterial strains suitable for BGS production in the cultivations using different carbon sources, (iii) development of the bioreactor system for BGS production, and (iv) clarification of the positive effect of oxygen supply on the BGS biosynthesis. The principal results of this work can be summarized as follows:

In Chapter 1, the background and the purpose of this work as well as a review of the previous works were given.

In Chapter 2, the bioassay method for the measurement of the concentration of BGS was optimized and dairy propionibacterial strains suitable for BGS production under anaerobic and aerobic conditions were screened.

The optimum component concentrations and pH of the bioassay medium were found to be one-half of the normal values and 8.5, respectively. Using the bioassay method, BGS ranging in concentrations from 0.1 μg/*l* to 1 mg/*l* in samples can be determined using DHNA as a standard BGS compound. BGS production by several propionibacterial strains, as well as their cell growth and glucose metabolism was significantly affected by oxygen supply. The different responses to aerobic conditions were observed among the several propionibacterial strains. Aerobic conditions exerted positive effects on BGS production only by *P. acidipropionici.*

This BGS bioassay method was able to determine BGS concentration more sensitively compared with the analysis method using HPLC. Furthermore, these dairy propionibacterial strains screened in this study have a potential of efficient BGS production.

In Chapter 3, the performance of production of BGS using glycerol or lactic acid as a carbon source was compared among several propionibacterial strains to use the waste materials in food industries.

Four propionibacterial strains selected in Chapter 2 were able to produce BGS using glycerol or lactic acid as a carbon source. Furthermore, for utilization of lactose as a carbon source and improvement of BGS producitivity per cultivation time, I attempted a co-cultivation of a *Lactobacillus* strain, a homofermentative lactic acid bacterium, and *P. shermanii*, in which lactic acid produced once from lactose by the former was used as a carbon source by the latter. The co-cultivation of *P. shermanii* and a *Lactobacillus* strain was useful for BGS production using lactose which is one of the wastes in the dairy industry. Especially, the concentration of BGS obtained in co-cultivation of *P. shermanii* and *Lactobacillus bulgaricus* OLL 1067 was 6.4 mg/*l*. The value was 3.0-3.4-fold those in the mono-cultivations using lactic acid and lactose and 1.8-fold that in the mono-cultivation using galactose.

In Chapter 4, the influences of some organic acids (lactic, acetic, and propionic acids) as a substrate and metabolites on the cell growth and BGS production were investigated. Furthermore, to produce efficiently BGS by *P. shermanii*, the different cultivation methods were investigated. That is, (i) a continuous bioreactor system with a microfiltration module, (ii) a fed-batch cultivation system using an on-line lactic acid controller for maintaining a concentration of lactic acid at a suitably low level in the culture broth, and (iii) a continuous bioreactor system with a microfiltration module coupled to an on-line lactic acid controller were used.

As the initial concentration of lactic acid increased, the consumption of lactic acid gradually decreased similarly to the specific growth rate and the turbidity. The results indicate that the lactic acid concentration in culture broth is necessary to be maintained at less than 10 g/*l* in order to enhance BGS production. 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 cultivation without propionic acid. Thus removal of propionic and acetic acids could be expected to prolong

logarithmic growth phase and to achieve a high BGS production rate. Using the bioreactor system with a filtration device and an on-line lactic acid controller, BGS was produced continuously at a high level of 47 mg/*l*. The BGS productivities per cultivation time (3.5 mg·l^{-1}·h^{-1}) and per total volume of medium used (1.7×10^{-1} mg·l^{-1}·h^{-1}) obtained in this bioreactor system were 37-fold and 2.1-fold those in a conventional batch cultivation, respectively.

The cell growth and BGS production were significantly affected by the concentrations of lactic acid as a carbon source, and propionic and acetic acids as metabolites in the culture broth. The new bioreactor system developed in this study allowed the enhanced BGS productivity not only by controlling the lactic acid concentration at a low level but also by maintaining the concentrations of the inhibitory metabolites at a low level.

In Chapter 5, the effect of oxygen supply on the production of BGS by *P. jensenii* was investigated. The changes in amounts of the proteins expressed in the propionibacterial cells in aerobic and DO-controlled cultivations were also examined by proteomic analysis and measurement of enzyme activities in the cell-free extracts.

The growth rate and cell concentration in the aerobic and DO-controlled cultivations were higher than those in the anaerobic cultivation. However, the maximum BGS concentration in the DO-controlled cultivation (3.5 mg/*l*) was less than those in the anaerobic cultivation (6.1 mg/*l*) and the aerobic cultivation (6.4 mg/*l*) because DHNA was significantly labile to oxygen. The measurement of enzyme activities in the cell-free extracts showed the up-regulation of expression of one (isochorismate synthase) of the enzymes related to DHNA synthesis. Proteomic analysis indicated that the expression of the enzymes related to glyceraldehyde- 3-phosphate, a member of glycolysis, was down-regulated under the aerobic conditions.

The results obtained in this study show that further investigations on (i) use of lactic acid bacteria suitable for an aerobic co-cultivation with propionibacteria, (ii) determination of

an optimum dilution rate in a continuous BGS production using the bioreactor system with a filtration unit and a lactic acid controller, and (iii) determination of optimum oxygen supply rate for BGS production in aerobic and DO-controlled cultivations are necessary to produce BGS further efficiently. The author hopes that the present study contributes to produce efficiently BGS and to screen BGS-producing bacteria from not only propionibacteria but also other ones. DHNA, which is the main component of the BGS in this study, has clinical effects and is useful as an ingredient in functional foods as described in Chapter 1. DHNA has a potential to possess unknown beneficial effects on human health. The author strongly expects that novel BGS except for DHNA are discovered and they are utilized as compounds effective for human health in the near future.

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List of Publications

Papers:

1. Production of Extracellular Bifidogenic Growth Stimulator by Anaerobic and Aerobic Cultivations of Several Propionibacterial Strains.
   Tomoaki Kouya, Katsuhiro Misawa, Masahito Horiuchi, Eri Nakayama, Hiroyoshi Deguchi, Takaaki Tanaka, and Masayuki Taniguchi:
   Journal of Bioscience and Bioengineering, **2007**, 103, 464-471.
2. Production of Extracellular BGS from *Propionibacterium shermanii* Using a Bioreactor System with a Microfiltration Module and an On-line Controller for Lactic Acid Concentration.
   Tomoaki Kouya, Kazuhiro Tobita, Masahito Horiuchi, Eri Nakayama, Hiroyoshi Deguchi, Takaaki Tanaka, and Masayuki Taniguchi:
   Journal of Bioscience and Bioengineering, **2008**, 105, in press.
3. Production of Bifidogenic Growth Stimulator by Co-Cultivation of *Propionibacterium shermanii* and *Lactobacillus* Strain Using Lactose as a Carbon Source.
   Tomoaki Kouya, Masahito Horiuchi, Kazuhiro Tobita, Katsuhiro Misawa, Eri Nakayama, Hiroyoshi Deguchi, Takaaki Tanaka, and Masayuki Taniguchi:
   Journal of Chemical Engineering of Japan, **2008**, 41, in press.

Proceeding:

1. Effect of Oxygen Supply on Production of Extracellular Bifidogenic Growth Stimulator by Several Propionibacterial Strains.
   Tomoaki Kouya, Eri Nakayama, Hiroyoshi Deguchi, Takaaki Tanaka and Masayuki Taniguchi:
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