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SHORT COMMUNICATION

Properties of bacterial cellulose produced in a pilot-scale spherical type bubble column bioreactor

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Abstract−The saccharogenic liquid obtained by the enzymatic saccharification of food wastes was used as a medium for production of bacterial cellulose (BC). The enzymatic saccharification of food wastes (SFW) was carried out by the cultivation supernatant of *Trichoderma inhamatum* KSJ1. 5.6 g/L of BC was produced in a new modified 50 L bubble column bioreactor by *Acetobacter xylinum* KJ1. The productivity was similar to that of a modified 10 L bubble column bioreactor (5.8 g/L). When pure oxygen was supplied into the scaled-up culture conditions, 6.8 g/L (12% enhancement) of BC was produced, indicating a very useful method for BC mass production. The oxygen uptake rate (OUR) and q_{O2} (specific oxygen uptake rate) were 0.214 mg-DO/L·min and 0.257 mg-DO/g-cell·min, respectively. The physical properties, such as morphology, molecular weight, crystallinity, and tensile strength of BCs produced in static culture (A), 10 L (B) and 50 L (C) modified bubble column cultures were investigated. All BCs showed fibrils with highly networking structure. The number average molecular weight of BCs in A, B and C was 2,314,000, 1,878,000, and 1,765,000, respectively. All of the BCs had a form of cellulose I representing pure cellulose. The relative degree of crystallinity showed the range of 79.6-86.0%. Tensile strengths of BC sheet in A, B and C were 1.75, 1.21, and 1.19 kg/mm^2, respectively. In conclusion, BC production by the modified bubble column culture mode of 50 L brought more favorable results in terms of the physical properties and its ease of scale-up.

Key words: Saccharification of Food Wastes (SFW), Bacterial Cellulose (BC), Pilot Scale Production, Modified Bubble Column Bioreactor, Physical Properties

INTRODUCTION

Bacterial cellulose (BC) produced by *Acetobacter xylinum* is a pure cellulose aggregate which does not include any impurities, such as hemicellulose, pectin, and lignin, differing from other plant-derived cellulose. Additionally, BC has higher degree of crystallinity than wood pulp and a reticular microfibrillar structure with the fiber diameter of about 0.1 μm. Therefore, BC has a very high surface area, a high moldability, and a strong tensile strength. These excellent physical properties of BC make it possible to study its actual use for the speaker diaphragm, tourniquet, or dietary fiber; and because of its low toxicity and chemical stability, it has been used in manufacturing an artificial skin as well as membrane for separation [1,2]. Particularly, considering that BC is an environmentally friendly material, it is expected that BC has unbounded versatility and potential for development.

However, when shear stress is applied to the *Acetobacter xylinum* during culture, a *Cel*$^-$ mutation occurs, resulting in a significant reduction in BC productivity [3]. For this reason, the static culture method has been used to obtain BC in spite of low productivity, long culture time, and great labor power. To overcome these problems and to produce BC on an industrial scale, various studies have been conducted, such as the selection of microbes which are genetically stable, developing the culture medium, and adding lactate, pyruvate, ethanol, etc., to the culture medium [4,5]. Studies on the microbial culture and on the control of operating conditions to reduce BC production cost are still continuing. Nevertheless, the productivity of BC is still too low and its production cost is too high for industrial use.

We have already developed an optimized culture technique for mass production of BC that greatly reduces the cost of BC production, thanks to a low-cost sugar generated from the hydrolysis of fibrous macromolecular substances included in food wastes, and used as a medium for BC production [6]. We have confirmed that the productivity and physical properties of BC produced from the saccharogenic liquid and BC produced from a commercial complex medium, HS medium, are similar.

In this study, according to the technology development for mass production, we investigated the proprieties of BC according to scale-up method, comparing the productivity and physical properties of BC produced through various different methods, such as the static culture and the modified bubble column culture using a 10 L and 50 L spherical airlift-type bioreactor designed to enhance the ability of oxygen transfer and BC productivity.

MATERIALS AND METHODS

1. Production of Bacterial Cellulose

1-1. Enzyme Production and Enzymatic Saccharification of Food Wastes

Inoculating with *Trichoderma inhamatum* KSJ1 on 100 ml YMEB (4 g yeast extract, 10 g malt extract, 4 g glucose, 1.0 L distilled water), the pre-culture for enzyme production was conducted at 30 °C, 120

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rpm, for 3 days. The pre-cultured liquid was inoculated at 2% on the 10 L jar fermentor (BioG, Hamil R&G Co., Korea) in which the fibrous wastes (rice straw and pulp) were contained at 1%, respectively, in substitute for CMC and Avicel used as a carbon source in Mandel's medium [7]. It was then cultured by shaking at 30 °C, 200 rpm, 0.6 vvm, and non-controlled pH for 4 days. The resultant was used as an enzyme liquid for the saccharification of food wastes.

For the enzymatic saccharification reaction, the food wastes were mixed with the enzyme liquid, and the mixture was hydrolyzed by enzyme in 30 L fermentor at 50 °C, 150 rpm for 10 hours. Food wastes were obtained from a student cafeteria at Chonnam National University, and elemental composition by elementar analyzer (Vario EL, Germany) of food wastes used in this experiment was as follows: carbon, 44.5±0.3%, and nitrogen, 2.4±0.2%. Nutrient broth, yeast extract and malt extract, were purchased from Merck, CSL from Sigma, and D-glucose and agar were purchased from Junsei, Japan.

2. BC Production Culture Methods

2-1. Static Culture in 500 ml Flask

Inoculating with *Acetobactor xylinum* KJ1 on 100 ml SFW contained in 500 ml flask, the static culture was conducted at 30 °C for 36 hours, and then, the 4% resultant supernatant liquid was again static-cultured at 30 °C for 36 hours. After inoculating with the pre-cultured broth (4%) agitated by homogenizer (Nissei, A-7, Japan) at 10,000 rpm for 1 min on 100 ml culture medium contained in 500 ml flask, the static culture was also conducted. The culture conditions were shown as follows: temp., 30 °C; pH, 5.25; and time, 5 days.

2-2. Modified Bubble Column Culture of 10 L

The pre-culture was conducted the same as the case of static culture in 500 ml flask. After inoculating with the pre-cultured broth (200 ml) treated by homogenizer (10,000 rpm, 1 min) in 5 L SFW medium of 10 L bioreactor, the modified bubble column culture was conducted under the conditions as follows: temp., 30 °C; initial pH, 5.25; 3 days, and aeration rate of 1.2 vvm (6 L/min).

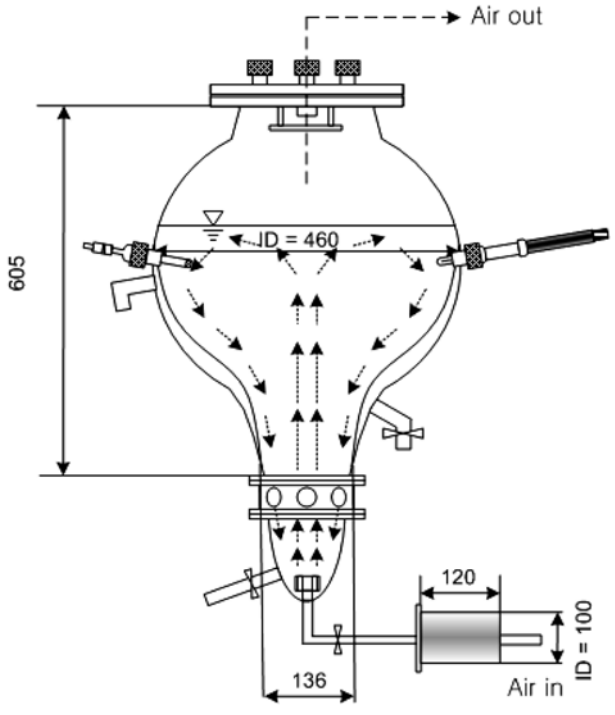

Fig. 1. Schematic diagram of modified 50 L bubble column bioreactor (ID: inner diameter, unit: mm).

2-3. Modified Bubble Column Culture of 50 L

To minimize the shear stress and to maximize the oxygen transfer ability, the culture was conducted by using a modified 50 L spherical bubble column bioreactor with working volume of 30 L (Fig. 1). The pre-culture was conducted the same as the case of static culture in 500 ml flask. After inoculating with the pre-cultured medium (600 ml) treated by homogenizer (10,000 rpm, 1 min) in 30 L SFW medium of 50 L bioreactor, the modified bubble column culture was conducted under the culture conditions as follows: temp., 30 °C; pH, 5.25; 3 days, and aeration rate of 1.0 vvm (30 L/min).

3. OUR Measurement

OUR (oxygen uptake rate) was measured by dynamic method [8]. It was calculated from the decreasing rate of dissolved oxygen without supply of any other oxygen after the saturated state of dissolved oxygen when the culture reached exponential growth. k_La was measured from the change rate of dissolved oxygen by supplying air again at the critical concentration of dissolved oxygen.

4. Analyses of BC Properties

4-1. Morphological Observation

Morphology of BC depending on the culture condition was observed by using SEM (scanning electron microscope) (JSM-5400, Japan). A small section of BC was placed on the SEM sample holder and coated with gold by vacuum deposition. An accelerating voltage of 20 KV was employed to get the SEM images.

4-2. Molecular Weight

Weight average molecular weight (Mw) of BC was evaluated by the size-exclusion chromatography method with a high-performance gel permeation chromatography (GPC) system (Viscotek, GPCmax). It is hard to dissolve BC in common GPC solvent due to the hydrogen bonding of OH groups. Therefore, the derivative of BC (cellulose nitrate) was made by nitration according to the methods described by Alexander and Mitchell [9]. At first, 10.1 g phosphorous pentoxide was slowly dissolved in 25 ml nitric acid (90% conc.). 0.1 g BC was put into this solution (4 ml), and reacted at 20 °C for 20 min. The resultant product was carefully washed with distilled water several times, and neutralized with 5% sodium carbonate solution. And the crude product was washed 3 times with distilled water, boiled in distilled water for 20 min, and immersed in ethanol for 10 min. After drying in vacuum oven, the final product was obtained. Molecular weight was checked with 0.5% cellulose nitrate/tetrahydrofuran (THF) solution using an RI detector. Eluent was also THF, and the molecular weight standard material was polystyrene.

4-3. Relative Crystallinity Index

X-ray diffraction patterns of BC samples were measured with X-ray diffractometer (Rigaku D-max 1200, Japan). The radiation was Ni-filtered Cu-Kα of 1.54 nm wavelength. The X-ray unit operated at 20 kV. Angular scanning was varied 2-60°, and data were collected by using a 2-step scan mode with angular intervals of 0.05°. The relative crystallinity index (Cr I) was estimated by Segal's method [10], using the following equation:

$$\text{Cr I} = 1 - h_{am}/h_{cr} = 1 - h_{am}/(h_{tot} - h_{am}),$$

where hcr is the crystalline peak intensity corresponding to the (002)

plane at 2θ=22.5° for cellulose I, ham is the peak intensity of amorphous fraction at 2θ=18° for cellulose I, and htot is total height.

4-4. Tensile Strength

Tensile strength of BC was measured according to ASTM Standard D 638 [11], with a tensile tester (United, STM-5). A suspension containing disintegrated BC was filtrated, and washed with distilled water several times. The remaining BC above the filter paper was detached and dried at higher temperature under pressure. The thickness of resultant sheet was about 0.25 mm. The sheet was cut into ribbon (8 mm×80 mm) form for measurement. The elongation rate was 1.0 mm/min.

RESULTS AND DISCUSSION

1. Productivity of BC Depending Upon the Culture Modes

The productivity of BC depending upon the various culture conditions was examined. In the case of static culture in 500 ml flask using saccharogenic liquid medium (SFW), the productivity was 5.7 g/L. While, the modified bubble column culture was applied, it was changed. In 10 L flask with SFW added 0.4% agar, it was 5.8 g/L, and in 50 L bioreactor, 5.6 g/L. It has been well known that the productivity of BC is much dependent on the shear stress during culture. Decreasing the shear stress in BC productivity is very important. By adding the agar in SFW, the viscosity of medium would be increased and the shear stress would also be decreased. Therefore, it is thought that, in the modified bubble column culture with addition of agar, the *Cel*$^-$ mutant is generated to a low degree on account of low shear stress, resulting in the increase of BC productivity [3].

Furthermore, in a 50 L bioreactor, the productivity of BC was much increased to 6.8 g/L by the injection of oxygen together with air. This means that BC productivity enhanced up to 12%, compared with the air supplemented culture. From these results, we concluded that the sufficient supply of oxygen as well as air also played an important role in BC productivity. The value of OUR measured at 13 hours-culture in which the dry cell weight was 0.834 g-dry cell/L was 0.214 mg DO/L·min and q_{O2} was calculated to be 0.257 mg-DO/g-cell·min.

At Shoda laboratory at the Tokyo Institute of Technology, it was reported that BC productivity was 5.1 g/L in a 50 L Internal-Loop Airlift Reactor using Corn steep liquor-fructose (CSL-Fru) added with agar for 67 hours culture [12]. And Chao etc. [13] also reported that it was 8.0 g/L in an airlift reactor using air and oxygen for 67 hours culture. These productivities are almost at a similar level to ours. However, we concluded that it was not an appropriate method for large scale production, because a highly concentrated solution state culture was not possible, due to the accumulation of bulk BC solid in circulation part.

Therefore, in modified 50 L bubble column culture, it might be helpful in BC production by adding the viscous polysaccharide or

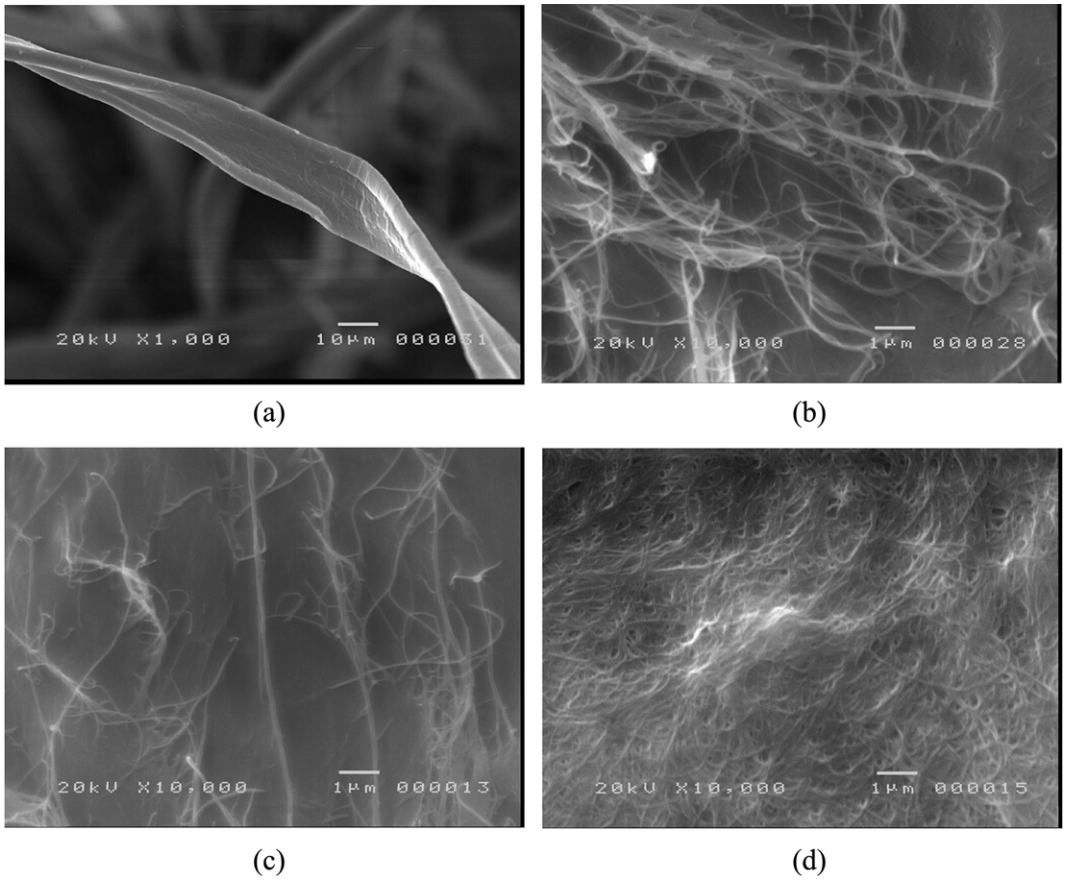

Fig. 2. SEM micrograph of cotton cellulose (×1,000) and bacteria cellulose (×10,000) produced at various culture conditions using SFW. (a): cotton cellulose, (b): modified 50 L bubble column, (c): modified 10 L bubble column, (d): static culture.

supplying oxygen in air, and supposed to be most effective in BC productivity with low cost-large production.

2. Morphological Observation of BC

The precise morphology of BC according to the culture condition was observed by SEM (scanning electron microscope) (JSM-5400, Japan). Fig. 2 shows the SEM micrographs of cotton cellulose (CC) and BC; (a) is from CC, and (b), (c) are BCs from the modified bubble column cultures, and (d) is from the static culture. In the case of BC produced from modified bubble column culture, we were able to find the well developed fibrils with highly networked structure. The diameter of BC was approximately 0.1-0.2 μm. The diameter of CC was the level of 16-22 μm (average: about 20 μm). Therefore, it was found that the diameter of BC was ultrafine, compared with that of general fiber. We were also able to find the fibril structure in a static cultured system. However, it was much aggregated with a dense mesh structure. We considered that fibrils were aggregated during culture without shear force.

In general, due to the structural characteristic of BC as an ultrafine and highly-pure fiber network, it has unique properties, including high mechanical strength, high water absorption capacity, and high crystallinity.

3. Degree of Polymerization of BC

The molecular weight (Mw) of polymeric materials is one of the most important properties. The Mw of BC was measured by the GPC method. The retention time of BC from the static culture, the modified bubble column culture in 10 L reactor, in 50 L reactor using the SFW medium, and cotton cellulose (CC) was 14.77, 14.82, 14.88, and 15.32 min, respectively. The average Mw and polydispersity of various BC and CC determined by the calibration curve of polystyrene as standard material is shown in Table 1. As expected, the Mw of BC is much larger than that of CC, and the static cultured BC has the largest Mw among various BCs.

Table 1. Molecular weight and its distribution of cotton cellulose and bacteria cellulose produced at various culture conditions using SFW

Sample	Mn	Mw	Mz	Polydispersity
BC (static)	2,314,000	3,265,000	4,235,000	1.410
BC (10 L)	1,878,000	2,822,000	3,955,000	1.503
BC (50 L)	1,765,000	2,722,000	3,714,000	1.542
Cotton cellulose	1,233,000	2,529,000	3,959,000	2.051

It is well known that, when the shear force is applied, *A. xylinum* produces BC together with water-soluble polysaccharide such as acetan and/or xylan. This water-soluble polysaccharide interferes with the hydrogen bonding between microfibrils to reduce the length of the microfibrils, and thereby reduces the Mw of BC [14]. According to Moon et al. [6], BC produced by shaking culture method has lower level of Mw. This water-soluble polysaccharide, in particular, is produced at increased levels by shear stress during the shaking culture. In this experiment, however, BC produced by modified bubble column culture in 50 L as well as 10 L reactor has relatively higher level of Mw as in the case of the static culture system. And the polydispersity, an index of polymeric uniformity, of BC is a level of 1.4-1.5, while that of CC is 2.0. This means BC consists of comparatively uniform polymer chains. From these re-

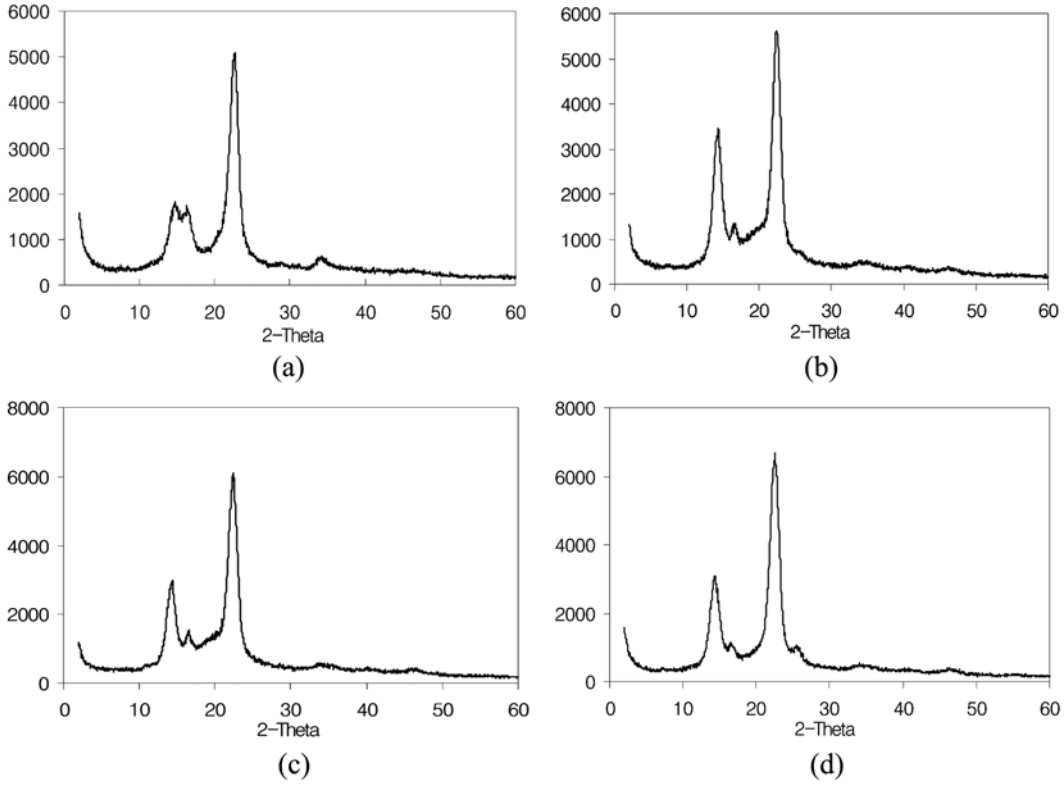

Fig. 3. X-Ray diffractogram of cotton cellulose and bacteria cellulose produced at various culture conditions using SFW. (a): cotton cellulose, (b): modified 50 L bubble column, (c): modified 10 L bubble column, (d): static culture.

sults, we considered that, irrespective of the modified bubble column or static culture, BC produced in state of uniform and higher Mw, differing from the shaking culture condition.

4. Crystallinity of BC

The crystallinity of BC was measured by X-ray diffraction method and the diffractogram is shown in Fig. 3. Irrespective of BC or CC, the sharp peaks appeared around 14.8, 16.9, and 22.5 degrees of 2-theta. This means all cellulose (BC and CC) have cellulose I-type crystal structure of natural cellulose.

We measured the relative degree of crystallinity (RDC) of BC as produced under different culture conditions by Segal's method. The RDC of BC from the static culture, the modified bubble column culture in 10 L reactor, and in 50 L reactor using the SFW medium were 86.0, 80.4, and 79.6%, respectively. According to Moon etc. [6], it was reported that BC produced by shaking culture method had 50% level of RDC. From these results, it was concluded that the crystallinity of BC almost did not change in modified bubble column culture.

5. Tensile Strength of BC

Tensile strength is one of the important mechanical properties of materials. The reformed BC as a sheet (non-woven) was cut into ribbon form and it was measured by using a universal tensile tester. The tensile strength of BC sheet from the static culture, the modified bubble column culture in 10 L reactor, and in 50 L reactor using the SFW medium were 1.75, 1.21, and 1.19 kg/mm^2, respectively. It is well known that tensile strength is much affected by the degree of polymerization of materials. Therefore, it is thought that this result is very reasonable. Nishi et al. [15] suggested that, because of its high mechanical property, a sheet made from BC is expected to be suitable for making acoustic transducer diaphragms.

CONCLUSIONS

We investigated the scale-up method, comparing the productivity and physical properties of BC produced through various different methods, such as the static culture (A) and the modified bubble column culture using a 10 L (B) and 50 L (C). 5.6 and 5.8 g/L of BC were obtained in culture modes of B and C, respectively. The productivity of BC in B and C was almost similar irrespective of size of modified bubble column bioreactor. Furthermore, in 50 L, it much increased to 6.8 g/L by supplement of oxygen.

All BCs produced in cultures A, B, and C have fibrils with highly networked structure. The molecular weight and tensile strength of BC produced from the modified bubble column culture were slightly decreased, compared with those from static culture. The crystallinity was also slightly decreased. However, a significant difference of the properties according to reactor size in the modified bubble column culture was not shown.

Therefore, it was considered that the modified scaled-up (50 L) bubble column culture might be great helpful in the development of low cost-mass production.

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