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Production of bacterial cellulose in static conditions by a simple fed-batch cultivation strategy

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Abstract−The current study investigated fed-batch cultivation for the increased productivity of bacterial cellulose (BC) sheets by *Gluconacetobacter hansenii* PJK in static conditions using chemically defined medium and waste from beer fermentation broth. Fermentations were carried out in a 3 L jar fermenter without any impeller for 30 days. In the proposed fed-batch cultivation, 500 mL of the medium was initially inoculated with pre-culture in a jar fermenter while a fresh medium was fed periodically. BC production was also done by using batch cultivation which was used as a control for comparison. The results obtained revealed an overall of 2-3 times increase in BC production in fed-batch cultivation compared to batch cultivation after 30 days of cultivation. During these experiments, it was found that waste from beer fermentation broth is a superior medium for the BC production using fed-batch cultivation. The production of water soluble oligosaccharides as useful by-products was also monitored during these investigations. Fed-batch cultivation and waste from beer fermentation was also found superior for the production of these by-products. According to literature search and to the best of our knowledge, it is the first report of using fed-batch cultivation for BC production in static conditions.

Key words: Bacterial Cellulose, *Gluconacetobacter hansenii* PJK, Fed-batch Cultivation, Static Culture Conditions, Water Soluble Oligosaccharides

INTRODUCTION

Although cellulose is the main constituent of plant cell wall, some bacteria of the genera *Acetobacter, Rhizobium*, *Agrobacterium*, *and Sarcina* can also produce cellulose called biocellulose or bacterial cellulose (BC) [1]. BC is superior to plant cellulose in purity and supermolecular structure and has unique physical and chemical properties that are lacking in plant cellulose. These include high tensile strength, high water holding capacity, high crystallinity, ultra-fine and finely pure fiber network structure, good transparency, fiber-binding ability, compatibility with the living body, biodegradability and moldability during formation [2-4]. These properties make BC suitable for use in biomedical and industrial applications [5,6].

Gluconacetobacter hansenii PJK, a cellulose-producing bacterium, has been extensively studied for production of BC under various experimental conditions using chemically-defined medium [7-11] as well waste from beer fermentation broth [12,13]. However, the previous studies were mostly focused on the fundamental biotechnology and enhancement of productivity in agitated conditions. Most of the applications, especially in the biomedical field, like artificial skin etc., require BC to be in the form of a continuous sheet which can be obtained in static culture. However, the process by which BC is conventionally produced using static culture is not applicable to large-scale industrial production due to the low productivity. Therefore, further investigation is required in order to improve the BC production in static cultures to a level suitable for commercial applications. In the current study, an attempt was made to investigate the fed-batch cultivation for the increased productivity of BC sheets by *G. hansenii* PJK in static conditions using chemically defined medium. As a matter of fact, BC production from a chemically defined medium is an expensive way; the waste from beer fermentation broth (WBFB) was investigated as an alternative cheap source for the production of BC sheets using the newly developed strategy. This study will be helpful in determining if the BC obtained in new conditions is appropriate for commercial applications. It is well-established that *G. hansenii* PJK is capable of synthesizing water-soluble oligosaccharides (WSOS) during the BC production [8]. Therefore, the production of these useful by-products was also monitored during these investigations. To achieve these goals, fermentations were carried out in a jar fermenter without any impeller in batch and periodically fed-batch cultivation modes using chemically-defined medium and WBFB.

EXPERIMENTAL

1. Microorganisms and Cell Culture

The strain *G. hansenii* PJK (KCTC 10505BP) was grown on a medium containing 10 g of glucose, 10 g of yeast extract, 7 g of peptone, 1.5 mL of acetic acid, and 0.2 g of succinate dissolved in 1 L of distilled water where its pH was adjusted to 5. The agar plates used for keeping the strains were prepared by dissolving 15 g of agar in 1 L of the basal medium. The medium containing ethanol was prepared by adding 1% (v/v) ethanol to the autoclaved medium. Colonies of *G. hansenii* PJK were inoculated into a 50 mL medium in a 250 mL flask which was shaken at 200 rpm and cultured at 30 °C

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for 24 h [14].

2. Fermentations

For batch cultivation, 100 mL pre-culture (5% of working volume) was inoculated into 2 L of the chemically defined medium in a 3 L jar fermenter (Kobiotech Co., Korea) without any impeller. In fed batch cultivation 25 mL pre-culture (5% of initial working volume) was inoculated into 500 mL of the medium (chemically defined or WBFB supplemented with 1% glucose) in a 3 L jar fermenter (Kobiotech Co., Korea) without any impeller. A fresh medium (250 mL) was fed periodically. In either case, the fermentations were carried out at 30 °C with an aeration rate of 1 vvm over the medium for one month. Periodic samples were taken from the culture medium and were analyzed for WSOS contents and culture data.

3. Isolation and Quantification of WSOS

WSOS was isolated by centrifuging the culture broth for 20 min at 3,000 rpm; the supernatant obtained was treated with 5 volumes of ethanol at 4 °C for 1 h. The precipitates were separated by centrifugation, dissolved in distilled water and re-precipitated with ethanol. After incubation at 4 °C for 1 h and followed by centrifugation, the obtained precipitates were dried at 60 °C until a constant weight was achieved [14]. All the experiments were run in duplicate and averaged.

4. Detection of Cellulose Producing Cells (*Cel*$^+$) and Non-cellulose Producing Mutants (*Cel*$^-$)

Cel$^+$ and *Cel*$^-$ were identified by cultivating them on nutrient agar plates. For this purpose, the culture broth was diluted with a saline solution and spread onto agar plates. These plates were incubated at 30 °C until the appearance of the colonies. Then, the colony-forming units (CFU) were counted. The smooth-type colonies were identified as *Cel*$^-$, while the mucous and rough-type colonies were considered as *Cel*$^+$ as reported previously [10].

5. Harvest and Processing of BC Sheets

The BC sheets were harvested after 30 days cultivation in each case and washed with distilled water repeatedly to remove the residual medium and other impurities. These BC sheets were then treated by boiling in 0.3 N NaOH solution for 15-20 min. The process was repeated until the sheets were completely cleaned. Finally, these sheets were washed thoroughly with distilled water to remove the traces of residual NaOH, and were weighed. Finally, the sheets were stored in containers filled with distilled water.

RESULTS AND DISCUSSION

1. BC Production from Chemically Defined Medium in Static Conditions using Batch and Fed-batch Cultivation Strategy

In static cultivation, BC is produced and forms a thick, leather-like white pellicle at the air-liquid interface of the culture [4]. BC productivity depends on the cultivation method and other culture conditions [15]. Fed-batch cultivation has been widely used for the enhanced production of BC in shaking and agitated conditions [15]. However, according to a literature search and to the best of our knowledge, it has never been tested for BC production in static conditions. It could be easily hypothesized that this cultivation mode may also be useful in enhancing the BC productivity in static conditions. For this purpose, the BC production by *G. hansenii* PJK was studied by using a chemically defined medium in fed-batch cultivation mode

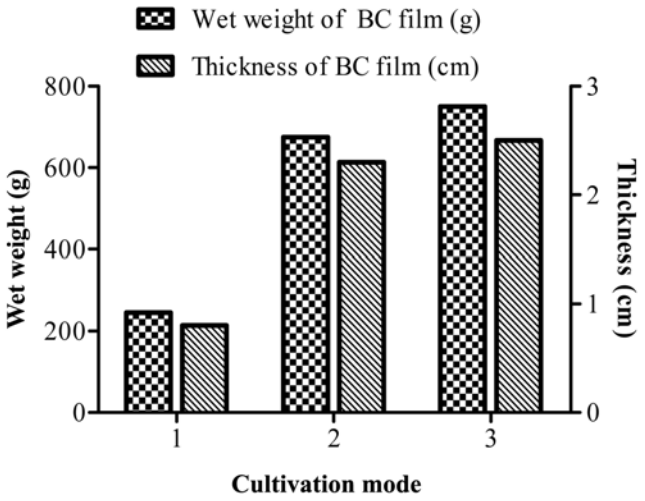

Fig. 1. Wet weight and thickness of the BC films produced by *G. hansenii* PJK. Cells were cultivated for 30 days using 2 L medium in a 3 L jar fermenter at 30 °C and an aeration rate of 1.0 vvm in static conditions. Cultivation modes: 1. Batch cultivation using chemically-defined medium; 2. Fed-batch cultivation using chemically-defined medium; 3. Fed-batch cultivation using waste from beer fermentation broth.

in a fermenter using batch cultivation as a control. In the proposed fed-batch cultivation, 500 mL of the medium was initially inoculated with pre-culture in a 3 L jar fermenter without any impeller. A fresh medium was fed periodically. The results obtained are shown in Fig. 1. Using batch cultivation, a total of 245.41 g (wet weight) with a 0.8 cm thickness of BC sheet was obtained after 30 days of cultivation. In contrast, a drastic increase in weight and thickness of the BC sheets was observed when the fed-batch cultivation strategy was adopted while keeping the other culture conditions constant (Fig. 1). In this mode of production, the wet weight of the BC sheet increased to 675.20 g with a final thickness of 2.3 cm after 30 days of cultivation time (Fig. 1). Overall, a 2-3 times increase in BC production was observed in fed-batch cultivation compared to batch cultivation.

It is well known that BC productivity is higher in shaking and agitated conditions than the static conditions [8]. However, many practical applications of BC like artificial skin and as food packaging material require it to be in a continuous film or sheet form. Such type of continuous films could be obtained under static conditions. In this scenario, a 2-3 times increase in BC production in static culture obtained in the current study by adopting the fed-batch cultivation rather than the batch cultivation is of significant importance.

2. BC Production from WBFB in Static Conditions using Fed-batch Cultivation Mode

BC productivity was successfully enhanced in fed-batch cultivation using a chemically defined medium (Fig. 1). However, BC production from a chemically defined medium represents an expensive way that may hinder its industrial application. Therefore, the waste from beer fermentation broth (WBFB) was tested as an alternative and cheap source as medium for bacterial growth for the BC production using fed-batch cultivation. Our previous studies have shown that *G. hansenii* PJK is capable of producing BC from WBFB in flask culture [13]. WBFB contains 68.11 g/L carbon, 17.19

g/L nitrogen and 116.14 g/L hydrogen [16]. It also contains 29.44 g/L total carbohydrate, 0.67 g/L total protein and 45.8 g/L ethanol [16]. The fed-batch cultivation was also tested for the production of BC by using WBFB as a medium for the bacterial growth. The results obtained were compared with those obtained with a chemically defined medium in batch and fed-batch cultivation mode. It was found that WBFB is even a better medium than the chemically defined medium for the production of BC sheets in fed-batch. A 750 g of BC sheet was obtained with 2.5 cm thickness after 30 days of cultivation using WBFB (Fig. 1). These values clearly show an improvement in BC production than that obtained with a chemically defined medium. It is also noteworthy that WBFB is not only a cheap source for the production of BC, but it is also an effective way for the production of valuable products and for environmental cleaning.

3. Production of WSOS in Batch and Fed-batch Cultivation

G. hansenii strains are capable of co-synthesizing WSOS during the BC production from a chemically defined medium [8-11,14] and from WBFB [13,15]. WSOS produced using WBFB were oligomers having α-glucuronic acid as a building block. These WSOS had only a few structural differences from glucuronic acid oligomers produced by using a chemically defined medium. The major dissimilarities included the presence of *O*-acetyl and *O*-methyl groups and a lack of unsaturation in the terminal unit of the non-reducing end of WSOS [17]. WSOS showed a thermal stability better or comparable to the other microbial polysaccharides of industrial potential and may be useful as auxiliary emulsifying agent [18]. In the present experiments, the production of this valuable by-product was also monitored. In most of the cases WSOS production increased with culture time (Fig. 2). In batch cultivation with a chemically defined medium, the WSOS production increased monotonically to 3.30 g/L on 30th day. In fed batch cultivation, the WSOS production increased to 6.65 g/L on the 18th day of cultivation and decreased thereafter reaching 3.54 g/L on the 30th day of cultivation. The production of WSOS on 18th day in this case is almost double as compared to batch cultivation. The WSOS production in fed-batch cultivation using WBFB first increased to 20.85 g/L on 18th day of cultivation and then decreased to 13.20 g/L on the 30th day of cultivation. Here, the WSOS production increased and decreased in the same manner as fed batch cultivation using a chemically defined medium (production of WSOS is maximum on 18th day in both the cases). However, while using WBFB, the WSOS production was three times more as compared to fed-batch cultivation with a chemically defined medium. These results coincide with our previous findings where WBFB was evaluated as a superior medium for the production of WSOS under agitated conditions [15]. The current study also revealed the usefulness of the fed-batch cultivation for the WSOS production over the batch production (Fig. 2).

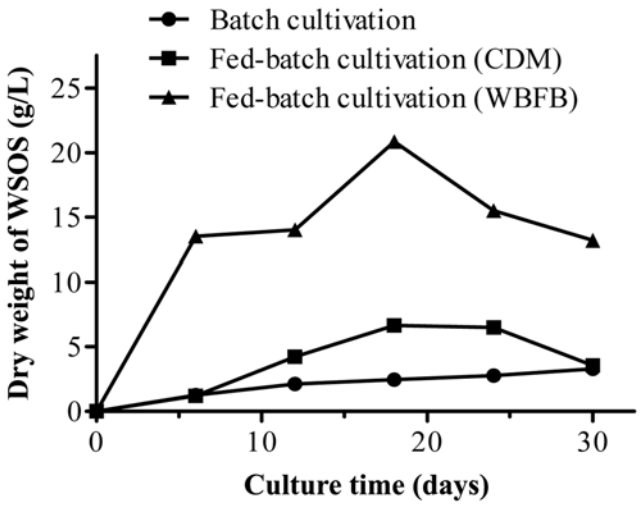

Fig. 2. WSOS production by *G. hansenii* PJK during the course of BC production in a 3 L jar fermenter at 30 °C and an aeration rate of 1.0 vvm in static conditions using different modes and growth media.

Table 1. Live cells density (CFU/mL) of the culture medium during the course of production of BC by *G. hansenii* PJK in a 3 L jar fermenter at 30 °C and an aeration rate of 1.0 vvm in static conditions using different modes and growth media

Culture time (days)	Chemically-defined medium		Waste from beer fermentation broth
	Batch	Fed-batch	Fed-batch
06	2.0×10^4	1.5×10^4	1.2×10^5
12	2.8×10^4	5.2×10^4	4.1×10^5
18	2.9×10^4	6.2×10^4	4.2×10^5
24	6.3×10^4	9.5×10^4	4.1×10^5
30	2.2×10^4	1.4×10^4	2.5×10^5

4. Live Cell Density (CFU/mL), Conversion of Cellulose Producing Cells (*Cel*$^+$) to Non-cellulose Producing Mutants (*Cel*$^-$) and pH of the Culture Broth

The live cell population in all cases increased up to 24 days of cultivation and decreased thereafter as shown in Table 1. On comparison of various cultivation modes and media, it was revealed that the cell population in case of fed batch cultivation with a chemically defined medium was higher compared to batch cultivation. In the case of fed-batch cultivation using WBFB, the cell population was much higher than in a chemically defined medium either in batch or in fed-batch cultivation. Productivity is proportional to the concentration of cells as well as the single cell productivity in a bioreactor [11]. Thus, the higher the cell density, the greater will be the final product. The results obtained in the current study also revealed a similar relationship. The production of BC and WSOS is the highest in case of fed-batch cultivation using WBFB (Fig. 1 and Fig. 2) which also has the highest live cells density (Table 1).

Previously, it has been found that *Cel*$^+$ of *G. hansenii* PJK are converted into *Cel*$^-$ mutants in agitated culture condition [7-11]. *Cel*$^-$ mutants do not have the ability to synthesize BC. In the current experiments, the conversion of *Cel*$^+$ to *Cel*$^-$ was not detected in all cases.

The pH of the culture medium during the course of production of BC by *G. hansenii* PJK in all cases decreased up to some extent (Fig. 3). However, the change was not much significant. The highest decrease in pH of the culture broth was observed in the case of batch cultivation. However, the pH in the case of fed-batch cultivation remained almost constant, which could be due to the periodic addition of the fresh medium. Similar results were also found in

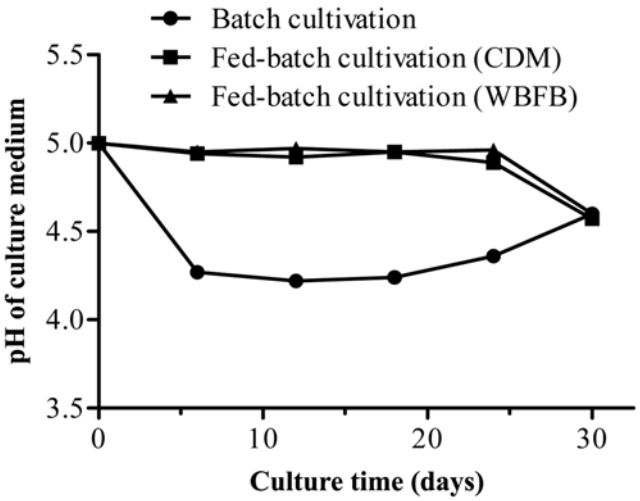

Fig. 3. Variation in pH of the culture medium during the course of production of BC by *G hansenii* PJK in a 3 L jar fermenter at 30 °C and an aeration rate of 1.0 vvm in static conditions using different modes and growth media.

our previous investigations where the pH pattern cycled with the renewal of the culture broth [11].

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