Journal of Industrial and Engineering Chemistry 37 (2016) 243–250

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

# The statistical optimization of bacterial cellulose production via semi-continuous operation mode

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ARTICLE INFO

*Article history:*
Received 15 January 2016
Received in revised form 10 March 2016
Accepted 18 March 2016
Available online 26 March 2016

*Keywords:*
Bacterial cellulose
Optimization
Fermentation
Semi-continuous culture
Response surface methodology (RSM)

ABSTRACT

Bacterial cellulose (BC) is highly pure and has a higher crystallinity and molecular weight than plant cellulose. Therefore, BC can be used in many different areas such as biotechnology, pharmaceutical, cosmetics. Because of the price of BC, the productivity of BC is an important parameter for industrial scale applications. In this study, BC was produced in static culture using a semi-continuous operation mode; the conditions were optimized using response surface methodology (RSM). The collected BC was characterized by Fourier-transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), and tensile strength. Optimization parameters were selected as glucose concentration, surface area/volume ratio, surface area and incubation day intervals. The optimum values for incubation day intervals, volume changing ratios, glucose concentrations and surface area/volume ratios were 7 days, 66%, 50 g/L and 1.22 $cm^{-1}$, respectively. BC productivity reached 0.284 g/L/day under optimal conditions, while the model equation proposed 0.289 g/L/day. RSM is essential for determining the optimum values of parameters for BC production compared with the one-variable-at-a-time method. The semi-continuous operation mode is alternative and a good candidate for the industrial scale production of BC.



## Introduction

Cellulose is the most abundant biopolymer in nature. Due to its structure and the ease with which cellulose can be obtained, it is used in many industrial areas. Bacterial cellulose (BC) is produced by microorganisms and has enhanced physiochemical properties compared to native cellulose, such as high crystallinity, a high molecular weight and high purity [1]. Therefore, there is a demand for the use of BC in specific areas, such as the food, biomedical, biotechnology and cosmetics fields [2–7]. To meet this demand, researchers have focused on increasing the production rates of BC in different types of cultures and processes [8–12].

The selection of static or agitated cultures is strongly dependent on the microbial strain. Impeller type and speed are the most important parameters in agitated cultures, and researchers commonly focus on the optimization of these parameters [13,14]. The surface area/volume ratio and the surface area are essential parameters for static cultures because they highly affect the transport of oxygen in the medium [15]. In addition to that different applications are presented based on manipulation microorganism using knock-out gene in *Gluconoacetobacter xylinus* to prevent the production of gluconic acid from glucose, addition of Vitamin C to inhibit the production of organic acids and using buffer medium to stabilize the pH that was decreased by gluconic acid production [16–18]. Four main types of operation modes (i.e., batch, fed-batch, semi-continuous and continuous systems) are used in biochemical industries [19]. In particular, the fed-batch mode is highly attractive due to its higher productivity values compared with the batch mode. The productivity of BC can be elevated in the fed-batch mode with the addition of a carbon source (i.e., corn steep liquor, glucose, sucrose, beer waste or other fermentation wastes) and the maintenance of the exact concentration ranges of the substrate without substrate inhibition [20–22]. Semi-continuous operation mode is based on medium replenishing at exact ratio and certain time. Fundamentally, this changing ratio set up between 33 and 66% (v/v) of medium. Changing time may be decided by concentration of certain compound or growth phase of microorganism. The advantages of semi-continuous operation mode are giving higher yield and

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http://dx.doi.org/10.1016/j.jiec.2016.03.030
1226-086X/

productivity than batch mode and easy to operate separation and purification processes because of low amount of medium [19]. BC productions in semi-continuous operation mode were recently reported [23,24]. Lin et al. [23] used a rotating disc bioreactor; BC was removed every 5 days. However, instead of changing the medium with different volume changing ratios, the authors completely replaced the medium with fresh medium. Çakar et al. [24] observed the effect of volume changes and incubation days on static culture and found that a 0.5 volume changing ratio with fresh medium every 7 days per round was optimal for obtaining high productivity with *Gluconoacetobacter xylinus* cultured in molasses medium. The development in both studies was based on the production of BC. Therefore, unclear parameters remain, such as the exact number of required incubation days, the optimal initial concentration of the carbon source and the exact volume changing ratio.

Response surface methodology (RSM) is common method used in statistical optimization techniques. The most important advantage of RSM is the ability to design fewer experiment sets, thereby making the process less time consuming and reducing material costs. However, the selection of the range of parameters is essential to obtain an acceptable optimized model. To define and optimize the process, different type of statistical optimization methods have been proposed, such as central composite design (CCD), Box–Behnken design, two-level factorial design, Placket–Burmann design and the Taguchi method. Placket–Burmann and the Taguchi method are mainly used for the selection of important parameters. Two-level factorial design provides optimized results, but the accuracy is not as high as that obtained with the CCD and Box–Behnken methods [25–27].

In this study, a semi-continuous operation mode was optimized for BC production using CCD with parameters including incubation days intervals, volume changing ratios, initial concentrations of glucose and surface area/volume ratio; these parameters fell within the ranges of 3–10 days, 33–66% (v/v), 10–50 g/L and 0.2–1.5 $cm^{-1}$, respectively. *G. xylinus* was used for BC production, and M1A05P5 was used as the production medium. BC (g/L), cell dry weight (CDW; g/L), glucose consumption were determined. BC was characterized using Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), and tensile strength. This study is the novel one in field of semi-continuous operation in BC production optimization.

## Materials and methods

### *Microorganism culture*

The *G. xylinus* (FC01) strain used in this study was previously isolated and identified by 16S rRNA sequence analysis in our laboratory. *G. xylinus* was grown on M1A05P5 medium: glucose, 10, 30 or 50 g/L; yeast extract, 10 g/L; peptone, 7 g/L; acetic acid, 1.5 ml/L and ethanol, 10 ml/L. The pH was adjusted to 5 with HCl or NaOH [28]. M1A05P5 medium showed higher BC productivity than Hestrin–Schramm medium that was shown in our previous study. It enhanced the growth of BC producing *G. xylinus* via ethanol addition and pH adjustment [28]. The bacterial cultures were incubated at 30 °C under static conditions in bottles with different volumes of medium to reach the desired surface area/volume ratios. After each incubation period, BC was collected from the surface and the volume was changed depending on the semi-continuous process conditions. Incubation day intervals were selected using response surface methodology. The medium was replenished 9, 4 and 3 times for the incubation day interval parameters of 3, 6.5 and 10 days, respectively.

### *Bacterial cellulose, cell dry weight and reduced sugar analysis*

BC was collected as described above. The samples were freeze-dried and treated with NaOH (0.3 M) at 80 °C for 1 h. After BC was washed, the samples were freeze-dried again to calculate the cell dry weight (CDW; g/L) via the difference in weights during the freeze-drying processes. Some cells remained in medium; therefore, we make calculation via correlation to full volume of medium. BC (g/L) was obtained in high purity after the second drying [29]. The spent medium (L) was calculated with the volume changing ratio, total working volume and media replenishing time. The production of BC (g/L/day) was calculated based on the total BC amount and the volume of the production medium and spent medium combined with the total incubation time. Reduced sugar was measured by using 3,5-dinitrosalicylic acid (DNS) method [30].

### *Structural characterization*

Fourier-transform infrared (FT-IR) spectroscopy was performed using the Perkin-Elmer Spectrum 100 Spectrometer. Scans were taken between 4000 and 450 $cm^{-1}$ [31]. The baselines for each sample spectrum were normalized using the software. The Carl Zeiss EVO-40 microscope was used for Scanning electron microscopy SEM analysis under high vacuum at high potential (10 kV). The BC samples were mounted and gold-coated in preparation for SEM imaging. Tensile strength and Young's modulus of selected BC samples were analyzed using an Instron 5900. The samples were weighted and prepared in wet condition [7].

### *Central composite design (CCD)*

Four important parameters (incubation day interval, volume changing ratio, glucose concentration and surface area/volume ratio) were investigated by central composite design in a static culture of bacterial cellulose production. The boundary values of the parameters are presented in Table 1. The optimum incubation day interval was investigated between days 3–10 when BC was newly generated on the media surface and reached steady-state generation. To obtain the optimum volume changing ratio for the semi-continuous process, between 1/3–2/3 of the medium was replenished according to the definition of the semi-continuous process [19]. The glucose concentrations reported in the literature were approximately 10–20 g/L for BC production. To observe the effect of the glucose concentration on production, BC production was performed in glucose concentration conditions between 10 and 50 g/L. Oxygen transport is an important parameter for static culture productions and should be efficiently sustained. Hence, the surface area/volume ratio was maintained in a wide range (0.2 to 1.5 $cm^{-1}$) [15].

Because the parameter ranges were wide, the face centered central composite design was selected in the response surface methodology [26]. The experiment sets of four factors with six center points are listed in Table 2. The CCD of 30 runs with three levels was set using the Design Expert software (7.0, Stat-Ease Inc.,

**Table 1**
Experimental range of the four variables used in FCCCD in terms of actual and coded factors.

| Independent variables | Symbols | Units | Levels | | |
|---|---|---|---|---|---|
| | | | −1 | 0 | +1 |
| Incubation day interval | A | days | 3 | 6.5 | 10 |
| Volume changing ratio | B | %, v/v | 33 | 50 | 66 |
| Glucose | C | g/L | 10 | 30 | 50 |
| Surface area/Volume ratio | D | $cm^{-1}$ | 0.2 | 0.85 | 1.5 |