PRODUCTION OF BACTERIAL CELLULOSE BY *ACETOBACTER XYLINUM*: EFFECTS OF CARBON/NITROGEN-RATIO ON CELL GROWTH AND METABOLITE PRODUCTION

HENG ZHANG, CHUNTAO CHEN, CHUNLIN ZHU and DONGPING SUN

Chemicobiology and Functional Materials Institute, Nanjing University of Science and Technology, Nanjing, P. R. China
✉ *Corresponding author: Dongping Sun, sundpe301@163.com*

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The effects of carbon/nitrogen ratios on production of bacterial cellulose (BC), by submerged cultivation of *Acetobacter xylinum* NUST4.2, were investigated using central composite design and response surface analysis. The accumulation of metabolite was linked to cell growth. Amongst organic sources, peptone was favorable for BC production, while glucose was observed as the best carbon source. The C/N-ratio of 5.39 encouraged the proliferation of bacteria. Data demonstrated that a low C/N-ratio resulted low production of the target product, and 30.01% of the carbon source was used to produce the by-product of gluconic acid, and only 12.36% was used in the BC production. When the initial C/N-ratio was adjusted to 6.31, the metabolic flux analysis showed that 20.96% of glucose fluxed into bacterial cellulose. The C/N-ratio was optimized and improved BC production was accomplished by shifting the metabolic distribution. Under the optimized conditions, the maximum production (1.57 g/l) of bacterial cellulose was obtained in a 15-liter fermentator, which was by 45% higher than that obtained before.

Keywords: cell growth, bacterial cellulose, carbon/nitrogen ratio, fermentation, Design-expert

INTRODUCTION

Cellulose is the most abundant biopolymer on earth, the major component of plants and a representative of microbial extracellular polymers.[1] Due to the excellent three-dimensional structure, bacterial cellulose (BC) displays so many unique properties, such as high purity, ultrafine structure, high crystallinity, high water holding capacity, large surface area, broad elasticity, mechanical strength and biocompatibility.[2] This biomaterial has been used in different application areas, such as foods, textiles, paper, biomedical materials and loud speaker diaphragms.[3,4] Although bacterial cellulose finds applications in several fields, the low productivity of this biomaterial makes manufacturers prefer plant cellulose. Strategies to enhance the production of BC focused on screening high-producing strains, optimizing cultivation process, developing perfect reactors, improving the technology of downstream processing.[5-10] Usually, the culture medium plays the most important role in fermentation, which is directly associated with cell proliferation and metabolite accumulation.[11-12] As major culture nutrients, carbon and nitrogen sources have a significant influence on cell proliferation and metabolite biosynthesis. Meanwhile, the concentration of these substrates can also regulate secondary metabolism through intracellular metabolism. The carbon to nitrogen (C/N) ratio, the most crucial parameter, is also important in the biological process.[13] A proper C/N-ratio value for *Acetobacter xylinum* (*A. xylinum*) is necessary to optimize bacterial cellulose production. However, there have been no reports on dynamic profiles of BC production by submerged cultivation of *A. xylinum*, and the effect of C/N-ratio has not been revealed as yet. With the advances in science, metabolic flux analysis has been widely used to investigate the intracellular fluxes and provide

feasible methods to improve the desired products.[14-15]

Conventional production optimization route was operated along the one-at-a-time method.[1] This optimized method is time-consuming and cannot provide information on mutual interactions between different factors. Fortunately, the developments of statistics have provided convenient ways to understand how the process factors individually and interactively influence the BC production. Response Surface Methodology (RSM) is been proven to be more satisfactory and effective than other methods. It has been successfully utilized to optimize the composition for the fermentation process.[16-18] This study aimed to investigate the C/N-ratio effect on the BC production and cell growth. This work was undertaken with the following objectives: (1) to screen the optimal concentration of glucose and peptone as the sole carbon and nitrogen sources, (2) to use the Design-expert approach to investigate the proper C/N-ratio of cell growth and cellulose production respectively, (3) to investigate the metabolic flux distribution under different C/N-ratios.

EXPERIMENTAL

Microorganism and inoculation

The microorganism used in this study was *Acetobacter xylinum* NUST4.2 stocked in our laboratory, which was first isolated from natural sources. The strain was stored in an Eppendorf tube containing: 20.0 g/l glucose, 6.0 g/l ammoniumsulfate, 1.0 g/l monopotassium phosphate, 0.4 g/l magnesium sulfate, 3.0 g/l peptone, 2.3 g/l yeast extract and 200 g/l glycerol, at -80 °C in a freezer. To revive the bacteria, 100 µl of cell suspension was added to 50 ml of revival medium and cultivated at 30 °C and 160 rpm for 2 days, and then used for seed culture inoculation. The seed culture was grown in a 250 ml shake flask containing 50 ml of liquid medium and incubated at 30 °C on a rotary shaker (160 rpm) for 3 days.

Experiments on initial glucose and peptone levels and C/N-ratio

The effects of carbon and nitrogen source concentration on the liquid culture of *A. xylinum* NUST4.2 were studied using different concentrations of glucose and peptone. The other culture medium components were as follows: 5.0 g/l monopotassium phosphate, 0.7 g/l magnesium sulfate, 0.2 g/l calcium lactate, 0.6 g/l citric acid and 0.4 g/l sodium carboxymethylcellulose. Experiments were conducted in 250 ml shake flasks filled with 50 ml of the medium. The flasks were held on a rotary shaker and harvested after 4 days. In the experiments on the effects of C/N-ratios, the levels of glucose and peptone in the medium were varied and a statistical approach was applied. The orthogonal tests were developed based on RSM.

Central composite design

Central composite design (CCD) was conducted to screen the optimum C/N-ratio of cell growth and cellulose production. The levels of the variables for these experiments were determined by the preliminary tests of one-factor-at-a-time (OFAT) variations.[19-20] The CCD experimental results were fitted with a second-order model in the form of a quadratic polynomial equation by the multiple regression technique:

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted response (bacterial absorbance, bacterial cellulose yield), β is a constant coefficient, and x_i (i = 1 and 2) are independent variables.

The fitness of the second-order model was expressed by the regression coefficient R^2 and its statistical significance was determined by an F-test. The regression significance was tested by a t-test. The Design-Expert 8.0.7.1 trial versions (Statease Inc., Minneapolis, USA) and Origin 8.0 were used for regression and graphical analyses of the data obtained, respectively.

Harvest of BC and bacteria

The culture broth was centrifuged at 11000 rpm for 15 minutes to remove residual medium. The precipitation was harvested and rinsed in 3‰ (w/v) NaOH solution and 3‰ (v/v) hydrogen peroxide and water bath at 80 °C for 2 hours to eliminate microorganism cells. Then, the floccule was washed with distilled water repeatedly until the pH was neutral. The BC was finally dried on a Petri dish at 70 °C for 12 h until it reached a stable weight. Cell concentration was estimated by measuring the optical density at 490 nm (OD490) after cellulose treatment of the culture broth.

Flux balance model

The metabolic network of central metabolism was constructed according to Tonouchi *et al.* and Zhong *et al.*[21,22]

A stoichiometric model combined with extracellular metabolism was applied to explore intracellular fluxes. The reaction network contained 26 metabolites and 22 reactions with some unknown fluxes. According to the pseudo-steady state hypothesis, mass balances around metabolites can be expressed as:

$$S \cdot v = b \quad (2)$$

where S is the stoichiometric matrix (22*22), v is the vector of 22 unknown reaction rates to be determined, and b is the vector of 22 known reaction rates from substrate consumption, product formation and cell growth rates.

High performance liquid chromatography analysis

The concentration of gluconic acid and acetic acid in the culture broth was analyzed using an Agilent high-performance liquid chromatograph (HPLC) with a UV detector at 210 nm. The HPLC was run on an SMA-H column (300×4.6 mm, SMA, USA), and 5 mM H_2SO_4 was chosen as the mobile phase with a flow rate of 0.6 ml/min. All the samples were treated with 5% (w/v) trichloroacetic acid (TDA) to precipitate protein, and then filtered through a 0.22-μm filter membrane before further analysis.

RESULTS AND DISCUSSION

Screening optimal concentration range of carbon and nitrogen sources

For most organisms, carbohydrates, grease, organic acids, alcohols, amino acids and other organic compounds can be used as carbon sources. However, each microbe prefers to utilize different carbon sources due to their own physiological characteristics. Based on the results of a previous study, glucose was selected as a sole carbon source for this experiment, as it can be transported through the cell membrane easily.[23] Peptone containing abundant nitrogen compounds, as well as many growth factors, was used as a sole nitrogen source. We used single-factor experiments and the ranges of the variables tested were 10-25 g/l peptone, 20-50 g/l glucose.

Figure 1 (a) and (b) depicts the effect of initial glucose and peptone concentrations on cell growth (by OD490) and BC production. Cell concentration decreased with an increase of initial glucose content. BC yield increased in parallel with an increase of initial glucose concentration. The BC yield on sugar decreased when the initial glucose concentration was more than 40 g/l. The results show that a high initial glucose concentration (45 g/l) was unfavourable to BC biosynthesis. Such a phenomenon was observed in submerged cultivation of medicinal mushroom *Cordyceps militaris*.[19] The osmotic pressure resulted from a high glucose concentration may be adverse to the secondary metabolite biosynthesis, meanwhile the cell growth is also injured. Some experts believe that the carbon could cause catabolite repression (caused by glucose), as reported for *Saccharomyces cerevisiae*.[24]

Nitrogen sources are mainly used for microbial cell material and some nitrogen compounds. Previous experiments showed that *Acetobacter xylinum* NUST4.2 does not grow in the absence of organic nitrogen (data not shown). In order to find out the optimum concentration of the nitrogen source, we chose the peptone as the sole nitrogen source, which is the most beneficial to BC production. The results are shown in Figure 1 (b). Cell growth and BC production presented a similar response to different peptone concentration. 15 g/l of peptone produced the maximal yield of BC. *Acetobacter xylinum* produced less biomass when the peptone concentration was more than 20 g/l. So, in the later experiments, we chose the optimal glucose concentration of 25 to 45 g/l and peptone concentration of 12.5 to 22.5 g/l.

Optimization of C/N-ratio by response surface methodology

To increase BC production further, it is necessary to consider not only the concentrations of both carbon and nitrogen sources, but also their balance in the medium. Thus, experiments were conducted to study the combined effect of the carbon source (glucose) and nitrogen source (peptone), using the statistical methodology of central composite design (CCD).

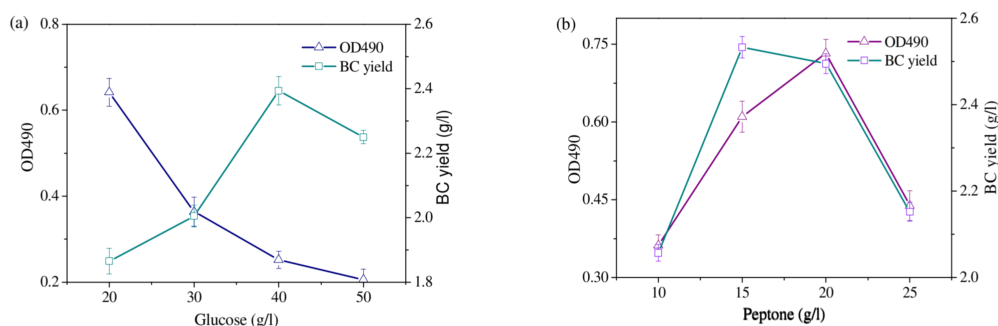

Figure 1: Effects of glucose (a) and peptone (b) concentrations on bacterial absorbance (OD490) and bacterial cellulose yield

Table 1
Experimental design and responses of the central composite design (CCD)

Runs	x_1 Glucose (g/l)	x_2 Peptone (g/l)	C/N-ratio	y_1 OD490	y_2 BC (g/L)
1	45.00 (+1)	22.50 (+1)	5.011	0.708	2.921
2	45.00 (+1)	12.50 (-1)	9.020	0.506	2.342
3	25.00 (-1)	22.50 (+1)	2.784	0.496	1.631
4	25.00 (-1)	12.50 (-1)	5.011	0.438	1.781
5	49.14 (+1.414)	17.50 (0)	7.035	0.517	2.680
6	20.86 (-1.414)	17.50 (0)	2.987	0.382	1.511
7	35.00 (0)	24.57 (+1.414)	3.569	0.673	2.130
8	35.00 (0)	10.43 (-1.414)	8.408	0.412	1.600
9#	35.00 (0)	17.50 (0)	5.011	0.792	2.699
10	35.00 (0)	17.50 (0)	5.011	0.789	2.753
11	35.00 (0)	17.50 (0)	5.011	0.785	2.700
12	35.00 (0)	17.50 (0)	5.011	0.791	2.730
13	35.00 (0)	17.50 (0)	5.011	0.790	2.714

#Runs 9, 10, 11, 12 and 13 were replicates at the center point

Table 2
ANOVA results for bacterial concentration and BC production obtained from CCD

Parameters	OD490			BC		
	Sum of squares	F value	*p*-value #	Sum of squares	F value	*p*-value
A-Glucose	0.028	33.05	<0.0001	0.30	36.91	0.0005
B-Peptone	0.049	58.98	0.0007	0.22	27.31	0.0012
AB	0.0052	6.18	0.0001	0.046	5.65	0.0492
A^2	0.18	211.53	<0.0001	1.51	184.68	<0.0001
B^2	0.089	106.28	<0.0001	0.44	53.63	0.0002
Model	0.32	76.35	<0.0001	2.33	57.18	<0.0001

#$p<0.05$ are considered significant

Experiments for establishing the effects of C/N-ratio were conducted in 250 ml shake flasks filled with 50 ml of the fermentation medium. The levels of factors for these processes were selected depending on the above results of one-factor-at-a-time variations. Each independent variable was tested at five levels (-α, -1, 0, +1, +α) (α was 1.414, when the variables were 2). The range of the values was matched with industrial practice. The experiment design and the results of 13 runs of the central composite design are shown in Table 1. The two final columns list the results obtained for the absorbance values of bacteria and BC productivity.

Based on Design-expert experiments, two second-order models in the form of quadratic polynomial equations have been proposed, representing the cell growth and BC yield as functions of the more significant variables:

$$y_1=-1.421+0.077x_1+0.164x_2+0.0022x_1x_2-0.0019x_1^2-0.0078x_2^2 \quad (3)$$

$$y_2=-4.832+0.307x_1+0.242x_2+0.0021x_1x_2-0.0047x_1^2-0.010x_2^2 \quad (4)$$

Table 2 lists the results of the ANOVA analysis of experimental responses to carbon and nitrogen sources in terms of cell growth and BC production from *A. xylinum*. The models' *F*-value of 76.35 and 57.18 for these two response variables implied that the models were significant, with a 99.0% level of confidence.

The response surface plots obtained from Eqs. 3 and 4 were used to define the optimal medium components (Fig. 2). The maximum cell optical density (0.889) was obtained for a combination of coded levels of 28.9 g/l (x_1, glucose) and 14.8 g/l (x_2, peptone) (C/N-ratio: 5.39) (Fig. 2 (a)). In contrast, the maximum BC production (2.760 g/l) was obtained under the conditions of 36.6 g/l glucose and 16.01 g/l peptone (C/N-ratio, 6.31), which provided

more appropriate conditions for BC biosynthesis (Fig. 2 (c)). Obviously, the yield of BC was lower under the optimal conditions for cell breed, just 2.404 g/l. According to the contour lines (Fig. 2(b) and (d)), cell growth was favored by low initial concentrations of the substrates. On the other hand, bacterial cellulose was inhibited by high concentrations of glucose and peptone, which may have caused high osmotic pressure. However, there still was a difference. Therefore, a further study is required to understand the metabolic flux under different C/N-ratios.

Microbial metabolism

The metabolisms occurring in the microbial cells are so complex that it is quite difficult to establish a complete network. In this paper, it can be simplified according to the system we investigated. The following major assumptions were introduced in the model: (1) it only considered the main center carbon metabolites; (2) the cellular composition of *Acetobacter xylinum* NUST4.2 was constant and was not subjected to the variations in uptake and product excretion reactions.[25]

Metabolic reactions are catalyzed by a large number of intracellular enzymes, which refers to the hexose monophosphate pathway (HMP), Embden-Meyerhof-Parnas pathway (EMP) and tricarboxylic acid cycle (TCA). Due to the absence or low catalytic activity of 6-phosphofructokinase, glycolysis does not occur completely in *Acetobacter xylinum*.[26] It has been found that glucose cannot be utilized only for cellulose synthesis, because of the amounts of by-products produced through bypass, which decrease the yield of cellulose.[27] For *Acetobacter xylinum* NUST4.2, a part of D-glucose transfers into gluconic acid, while most of the substrate is used for the production of UDP-Glc and glucose 6-phosphate (Glc-6-P). The overflows of excessive gluconic acid lead to reducing the utilization of the target product of carbon source. Meanwhile, the accumulation of acidic compounds also makes the pH decrease, which is not beneficial to fermentation.

The flux distributions under different C/N-ratios are shown in Figure 3. Under the conditions of optimal cell growth (C/N-ratio, 5.39), 12.36% of glucose was incorporated into BC, and most of glucose was fluxed into the by-product gluconic acid (r25, 30.01%). In comparison, about 20.96% of glucose was used for BC synthesis, and only 25.79% of glucose was fluxed into gluconic acid when the C/N-ratio was 6.31. From the metabolic flux analysis, different C/N-ratios can regulate the metabolic distribution in *A. xylinum*.

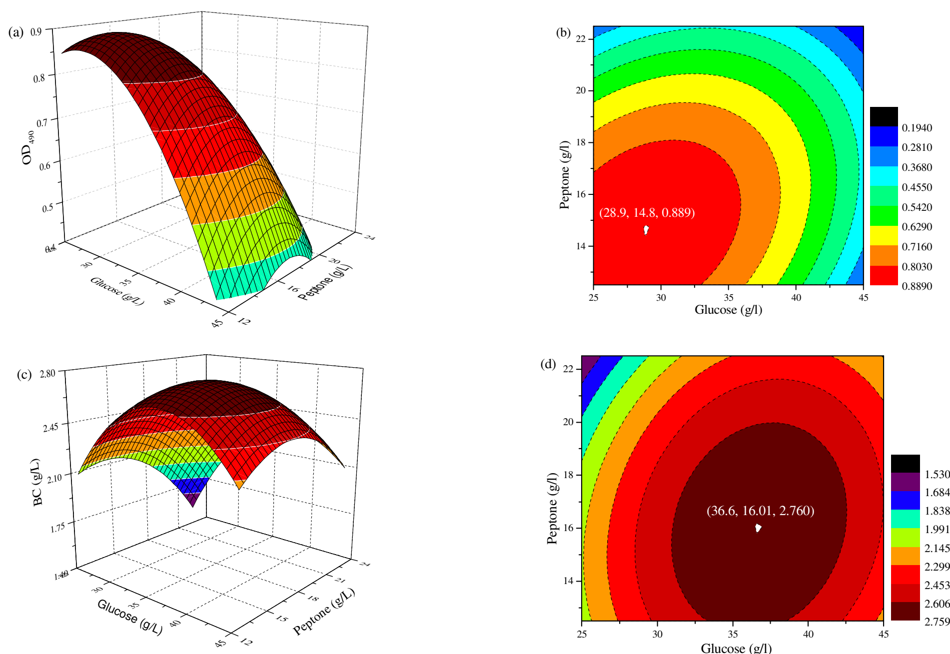

Figure 2: Response surface plots and contour diagrams of bacterial multiplication (a), (b); and BC production (c), (d) as functions of significant interactions between glucose and peptone

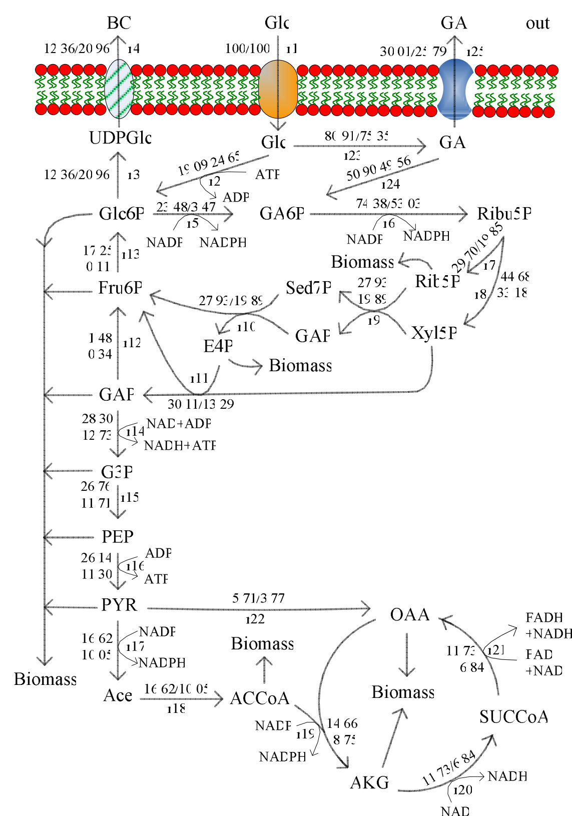

Figure 3: Metabolic network of *A. xylinum* cultured with different C/N-ratios. Network reactions of central carbon metabolism were constructed as described by Tonouchi and Zhong[18,19] (Glc: glucose; GA: gluconic acid; Glc6P: glucose-6-phosphate; UDPGlc: uridinediphophoglucose; BC: bacterial cellulose; GA6P: gluconate-6-phosphate; Ribu5p: ribulose-5-phosphate; Rib5P: ribose-5-phosphate; Xyl5P: xylulose-5-phosphate; Sed7P: sedoheptulose-7-phosphate; GAP: glyceraldehydes-3-phosphate; E4P: erythrose-4-phosphate; Fru6P: fructose-6-phosphate; G3P: glycerate-3-phosphate; PEP: phosphoenol pyruvate; PYR: pyruvate; Ace: acetic acid; ACCoA: acetyl-coenzyme-A; AKG: á-keto-gluterate; SUCCoA: succinyl-CoA; OAA: oxaloacetate)

At the end of the fermentation process, the metabolic flux into BC synthesis at a C/N-ratio of 6.31 was 1.70-fold that at a 5.39 C/N-ratio. Meanwhile, the cell growth consumption at a 5.39 C/N-ratio was 1.51-fold that at a 6.31 C/N-ratio. The fractions of the carbon source that ended in the TCA pathway were fairly similar for different C/N-ratios. Within the metabolic pathway of *A. xylinum* NUST4.2, 74.38% of the carbon source entered into the HMP pathway (r6) under 5.39 C/N-ratio and 53.03% under 6.31 C/N-ratio. The HMP is a crucial way that provides NADPH and different structure carbohydrates used for cell growth.[18] A slightly lower level of C/N-ratio induces a high cell proliferation, which makes a large portion of the carbon source to be consumed in this process. Cellulose is produced by *A. xylinum* from a metabolic pool of hexose phosphate, such as exogenous hexoses, the hexose monophosphate pathway and the gluconeogenic pathway. The determined fluxes (r1, r2, r3, r4) from glucose to cellulose are influenced by cell growth. Different C/N-ratios cause the metabolic flux shift.

Fermentation tank validation

In order to evaluate the validity of the results, further experiments using optimized and non-optimized C/N-ratios were performed. We used the C/N-ratio of 5.39 (glucose, 28.9 g/l, peptone, 14.8 g/l) in the seed culture stage, and the C/N-ratio of 6.31 in the fermentation stage. The results showed a maximal BC production of 1.57 g/l on day 3 and glucose was almost exhausted at this time. The yield was by 45% higher than that of the control, which was just 1.08 g/l. The result showed that optimal conditions can improve BC production effectively.

CONCLUSION

This work has demonstrated the use of Response Surface Methodology to determine the optimum C/N-ratio of cell growth and cellulose production from *A. xylinum*. Three-dimensional response surface and the contour line were efficient in visualizing the interactions of factors. According to the results, a C/N-ratio of 5.39 provided the largest cell growth. Meanwhile, the C/N-ratio of 6.31 gave the optimal bacterial cellulose production. Elsewhere, large scale fermentation in a 15 liter fermentator also verified the veracity of this outcome. The fundamental findings obtained in this study provide the basis for further investigation for enhancing BC production from *A. xylinum* NUST4.2, and may be helpful for the fermentation of bacterial cellulose by submerged cultivation on a large scale.

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