Enhancement of bacterial cellulose production in *Bacillus amyloliquefaciens*

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Abstract. Recently, bacterial cellulose (BC) has become more commonly applied as a new nano-biological material within the food, paper manufacturing and pharmaceutical production industries. However, the current methods of BC production are not ideal because of their low productivity and large number of byproducts. To improve the yield of cellulose production in bacteria, various carbon and nitrogen sources for the fermentation conditions of BC by *Bacillus amyloliquefaciens* ZF-7 were investigated. The effects of D-glucose, yeast extract and ethanol concentration on BC production by strain ZF-7 were studied by single factor methods. Based on the above results, the effects of D-glucose, yeast extract and ethanol concentration on BC production were investigated by means of a five-level factor central composite design and response surface methodology. D-glucose and yeast extract concentration were found to have a significant linear effect on BC production, and the interaction between D-glucose concentration and yeast extract concentration also had significant influence on BC production. The obtained optimum culture medium contained 56.1 g L\(^{-1}\) of D-glucose, 9.9 g L\(^{-1}\) of yeast extract and 17.2 mL L\(^{-1}\) ethanol. Under these conditions, the BC yield of *B. amyloliquefaciens* ZF-7 reached 7.88 g L\(^{-1}\), which represents a 35.4 % increase compared to the initial yield before the optimization.

1. Introduction

Bacterial cellulose (BC) is an extracellular insoluble polysaccharide. It is a homopolymer of glucose with β-(1,4) glucan chains that can be produced by certain bacteria, including the genera *Gluconacetobacter*, *Rhizobium*, *Agrobacterium*, *Rhodobacter*, *Sarcina* and *Bacillus* [1-3]. BC is demonstrably more chemically pure than the cellulose from plants for BC without lignin or hemicellulose [4]. BC has been successfully used in pharmaceutics as a temporary pharmaceutical skin substitute called “Biofill” [5] food [6], paper [7], and many other products due to its extreme purity, high strength, excellent hydrophilic properties, degradation, and strong biological affinity, compatibility and adaptability [8].

Although BC has the potential to be useful in many industries, the current difficulty of producing BC has greatly limited its broader applications. Several researchers have explored methods of increasing the yield of BC production methods, such as screening of high-yield strains [9], optimization of medium composition [10], and selection of suitable cultivation methods [11] among others. However, almost all of these works were focused on *Acetobacter xylinum* or *Gluconacetobacter xylinus* [11, 12], which were rod-shaped, strictly aerobic gram-negative bacteria and produce pellicle of BC, while the BC production
of other strains has rarely been reported. In this paper, an effective cellulose producer was screened to develop an effective microbial production system for BC. This producer belonged to *Bacillus amyloliquefaciens* [3]. Strain ZF-7 produced a marked amount of cellulose (6.2 g\cdot L^{-1} and 6.6 g\cdot L^{-1} under static and shaking flasks respectively), which indicated that strain ZF-7 is an effective cellulose producer. Thus, the main objectives of this study was to optimize the composition of the medium to enhance BC production by *B. amyloliquefaciens* ZF-7.

2. Materials and Methods

2.1. Bacterial strain
The strain used is *B. amyloliquefaciens* ZF-7, which was isolated from vegetable samples and identified by using 16S rDNA sequence besides the morphological, physiological, and biochemical characteristics. The strain has been deposited in China General Microbiological Culture Collection Center, CGMCC with the registered number as CGMCC 6266.

2.2. Medium and cultivation
All the chemicals used were of analytical grade and commercially available, unless otherwise specified. The strain culture was maintained in a glucose medium consisting of (g\cdot L^{-1}): D-glucose 20, peptone 5, beef extract 5, citric acid 5, Na$_2$HPO$_4$·12H$_2$O 1, CaCO$_3$ 10 and agar 20. Agar slants were preserved at 4 °C until further subculturing. For seed culture, colonies of *B. amyloliquefaciens* ZF-7 were inoculated into 100 mL of Hestrin and Schramm (HS) medium containing (g\cdot L^{-1}): D-glucose 20, peptone 5, beef extract 5, Na$_2$HPO$_4$·12H$_2$O 5, and citric acid 1 in a 500 mL flask shaken at 160 rev/min and cultured at 30 °C for 2 days. Two milliliters of the prepared inoculum were added to 100 mL of a sterilized basic medium consisting of (g\cdot L^{-1} or mL\cdot L^{-1}): D-glucose 50, beef extract 8, and ethanol 14, with pH was adjusted to 6.5 in a 500 mL flask and statically cultivated at 30 °C for 5 days.

2.3. Carbon source optimization
The BC yield was determined for fermentations containing (g\cdot L^{-1} or mL\cdot L^{-1}): beef extract 8, ethanol 14, and 50 of one of the following carbon sources: D-glycerol, D-glucose, sucrose, D-fructose, maltose or inulin hydrolysate. Furthermore, the effect of the concentration of the carbon source on the BC yield was also investigated.

2.4. Nitrogen source optimization
The BC yield was determined for fermentations containing (g\cdot L^{-1} or mL\cdot L^{-1}): D-glucose 50, ethanol 14, and 8 of yeast extract, peptone, beef extract, corn steep liquor, (NH$_4$)$_2$SO$_4$ and NH$_4$Cl. The effects of the nitrogen source concentration on the BC yield were also investigated.

2.5. Ethanol optimization
The effects of the ethanol concentration on the BC yield were investigated. The medium for fermentations contained (g\cdot L^{-1} or mL\cdot L^{-1}): D-glucose 50, yeast extract 10, and an additional 8, 10, 12, 14, 16 or 18 ethanol, respectively.

2.6. Central composite rotatable design
Statistical optimization experiments were carried out according to a three-factor central composite rotatable design (CCRD) with either corner points, six axial points, or four repeats of the centre point and $\alpha=1.683$. The design matrix shown in Table 7 was obtained by means of the statistica 8.0 software. The encoded values of the design matrix were substituted with the values shown in Table 6, to obtained the medium composition for the 18 required BC fermentations. A second-order polynomial model was obtained by multiple regression analysis of the experimental data (Table 7). Analysis of variance and a lack of fit tests were applied to validate the regression model.
2.7. Analytical methods

2.7.1. Cell mass. The cell mass was estimated by measuring the optical density at 660 nm after treating the culture broth with 5% (v/v) cellulase at 50 °C for 2 h to release the cells from the bacterial cellulose membranes [13].

2.7.2. Porosity. Porosity was calculated using the equation [14]:

\[
\text{Porosity (\%) = \frac{\text{wet weight - dry weight}}{\text{wet weight - weight in water}} \times 100.}
\]

2.7.3. Quantification. For quantification, the cellulose matrix was isolated by filtration by using a nylon mesh. The matrix was successively washed by suspending in appropriate volumes of (1) deionized water, (2) 1 mol/L NaOH, (3) 0.5 mol/L acetic acid, and (4) deionized water. Each suspended sample was incubated at 80 °C for 20 min. The final preparation was dried by heating at 80 °C for 48 h and weighed.

3. Results and discussion

3.1. Effects of carbon sources on BC production of B. amyloliquefaciens ZF-7

This study examined the effect of media containing different carbon sources on B. amyloliquefaciens ZF-7 growth, BC yield and porosities (Table 1). The results showed that when D-glucose was used as the sole carbon source, the yield of bacterial cellulose membrane was up to 5.82 g•L⁻¹. This was the best result followed by D-glycerol and inulin hydrolysate. Additionally, the results also showed that the cell growth of ZF-7 was up to 3.99 when D-glycerol was used as the sole carbon source. If sucrose was used as the sole carbon source, limited growth of bacteria resulted, which was verified by cell mass measurement; the cell mass was only about 30% of the result when D-glycerol was used as the sole carbon source, and about 50% of the result when D-glucose was used as the sole carbon source. As a result, smaller amounts of BC were produced, which explained the least cell mass and the highest porosity. As shown in Table 1, the BC yield against unit cell mass from D-glucose as the sole carbon source was higher than the other carbon sources. When both production and porosity were considered, D-glucose was chosen as the sole carbon source to be used in following experiments.

The influence of variations in D-glucose concentrations on bacterial growth and BC production was also studied (Table 2). As the concentration of D-glucose increased, the bacterial growth initially increased and then decreased. The cell growth was almost inhibited when the D-glucose concentration increased to 80 g•L⁻¹, which indicates that the normal growth of bacteria was inhibited by high concentrations of D-glucose. Because the BC yield peaked at a D-glucose concentration of 50 g•L⁻¹, that concentration was selected as the optimal carbon source concentration.

| Carbon source          | OD₆₆₀ | BC yield (g•L⁻¹) | Porosities (%) | BC yield against unit cell mass (g BC produced/OD₆₆₀) |
|------------------------|-------|-----------------|----------------|---------------------------------------------------|
| D-glycerol             | 3.99  | 4.81            | 92.57          | 1.20                                              |
| D-glucose              | 2.51  | 5.82            | 92.26          | 2.31                                              |
| Sucrose                | 1.26  | 2.44            | 96.32          | 1.90                                              |
| D-fructose             | 2.97  | 3.91            | 94.90          | 1.31                                              |
| Maltose                | 1.49  | 3.22            | 95.64          | 2.14                                              |
| Inulin hydrolysate     | 2.88  | 4.13            | 94.50          | 1.42                                              |
Table 2. Effect of D-glucose concentration on BC yield and *B. amyloliquefaciens* ZF-7 growth

| D-glucose (g•L\(^{-1}\)) | OD\(_{660}\) | BC yield (g•L\(^{-1}\)) | Porosities (%) | BC yield against unit cell mass (g BC produced/OD\(_{660}\)) |
|---------------------------|------------|-----------------|---------------|----------------------------------|
| 20                        | 1.74       | 2.31            | 96.72         | 1.33                              |
| 30                        | 2.52       | 2.89            | 95.38         | 1.14                              |
| 40                        | 3.53       | 4.01            | 95.01         | 1.14                              |
| 50                        | 2.62       | 5.92            | 92.90         | 2.26                              |
| 60                        | 2.51       | 4.27            | 94.37         | 1.70                              |
| 80                        | 1.08       | 0.95            | 98.52         | 0.88                              |

3.2. Effects of nitrogen sources on BC production of *B. amyloliquefaciens* ZF-7

As an essential component of nucleic acids and proteins in microbial cells, nitrogen sources affect the growth and metabolites of microorganisms. In this study, the effects of yeast extract, peptone, beef extract, corn steep liquor, \((\text{NH}_4)\_2\text{SO}_4\) and \(\text{NH}_4\text{Cl}\) on the growth and BC production of *B. amyloliquefaciens* ZF-7 were investigated (Table 3).

The results listed in the table suggest that organic nitrogen sources promoted bacterial growth, while the cell growth was almost inhibited when inorganic nitrogen sources were used as the sole nitrogen sources in the medium. Use of yeast extract as the nitrogen source in the culture medium almost doubled the BC yield compared to the use of peptone as the sole nitrogen source in the medium, although the BC yield against unit cell mass was higher in the presence of peptone as the sole nitrogen source than it was in the presence of yeast extract as the sole nitrogen source. Because cell growth was inhibited when inorganic nitrogen source were added to the culture medium, the BC yield was also inhibited. Therefore, yeast extract, an organic nitrogen source, was selected as the nitrogen source to evaluate the effects of its concentration on bacterial growth and BC yield (Table 4).

As the concentration of yeast extract increased, bacterial growth was promoted. However, the BC yield was unlike the cell growth, it increased as the nitrogen concentration increased, and reached 7.36 g•L\(^{-1}\) when 10 g•L\(^{-1}\) yeast extract was added to the fermentation medium. After this point, the BC yield decreased rapidly as the nitrogen concentration increased. As shown in Table 4, the BC yield against unit cell mass decreased slightly from 2.74 to 2.61 when the yeast extract increased from 5 g•L\(^{-1}\) to 10 g•L\(^{-1}\) and then the BC yield against unit cell mass declined quickly to 0.89 when the yeast extract concentration increased to 20 g•L\(^{-1}\). Therefore, 10 g•L\(^{-1}\) yeast extract was selected as the optimum concentration.

Table 3. Effect of nitrogen source on BC yield and *B. amyloliquefaciens* ZF-7 growth

| Nitrogen source          | OD\(_{660}\) | BC yield (g•L\(^{-1}\)) | Porosities (%) | BC yield against unit cell mass (g BC produced/OD\(_{660}\)) |
|--------------------------|------------|-----------------|---------------|----------------------------------|
| Yeast extract            | 2.58       | 6.81            | 92.62         | 2.64                              |
| Peptone                  | 1.28       | 3.82            | 96.32         | 2.98                              |
| Beef extract             | 2.44       | 5.84            | 93.32         | 2.39                              |
| Corn steep liquor        | 3.07       | 4.98            | 95.90         | 1.62                              |
| \((\text{NH}_4)\_2\text{SO}_4\) | 0.31       | 0.21            | 97.64         | 0.68                              |
| \(\text{NH}_4\text{Cl}\)  | 0.45       | 0.32            | 97.50         | 0.71                              |

Table 4. Effect of yeast extract concentration on BC yield and *B. amyloliquefaciens* ZF-7 growth

| Yeast extract (g•L\(^{-1}\)) | OD\(_{660}\) | BC yield (g•L\(^{-1}\)) | Porosities (%) | BC yield against unit cell mass (g BC produced/OD\(_{660}\)) |
|-----------------------------|------------|-----------------|---------------|----------------------------------|
| 5                           | 1.74       | 4.76            | 95.33         | 2.74                              |
| 8                           | 2.52       | 6.89            | 92.29         | 2.73                              |
| 10                          | 2.83       | 7.36            | 92.04         | 2.61                              |
| 15                          | 3.22       | 5.49            | 93.73         | 1.71                              |
| 20                          | 3.51       | 3.12            | 96.51         | 0.89                              |
3.3. Effects of ethanol on BC production of B. amyloliquefaciens ZF-7

Ethanol has received much attention as a stimulatory factor, and previous research has indicated that it has a significant effect on BC production [15]. The influence of ethanol concentrations on bacterial growth and BC production were also studied (Table 5). As the table shows, the cell growth declined as the ethanol concentration increased. However, the BC yield increased and reached its maximum concentration, 7.64 g•L⁻¹, when 16 ml/l ethanol was added to the fermentation condition. Therefore, 16 mL•L⁻¹ ethanol was chosen as the optimum concentration. Under this condition, BC yield against unit cell mass reached 3.14 g BC produced/OD₆₆₀.

| Ethanol (mL•L⁻¹) | OD₆₆₀ | BC yield (g•L⁻¹) | Porosities (%) | BC yield against unit cell mass (g BC produced/OD₆₆₀) |
|------------------|-------|------------------|----------------|---------------------------------------------------|
| 8                | 5.27  | 4.33             | 95.99          | 0.82                                              |
| 10               | 4.72  | 5.91             | 94.48          | 1.25                                              |
| 12               | 3.28  | 6.34             | 93.76          | 1.93                                              |
| 14               | 2.86  | 7.47             | 93.21          | 2.61                                              |
| 16               | 2.43  | 7.64             | 92.95          | 3.14                                              |
| 18               | 2.21  | 6.05             | 93.87          | 2.74                                              |

3.4. Central composite rotatable design

A central composite rotatable design was applied to derive a statistical model for the effects of the D-glucose, yeast extract and ethanol concentrations on BC production of B. amyloliquefaciens ZF-7 and to identify the combination of factors that would lead to an optimal BC yield. Preliminary experiments were carried out to determine the parameter ranges for the three independent variables (Table 6).

| Variable          | Code | -α | -1  | 0   | 1   | +α |
|-------------------|------|-----|-----|-----|-----|-----|
| D-glucose (g•L⁻¹) | X₁   | 33.17 | 40  | 50  | 60  | 66.83 |
| Yeast extract (g•L⁻¹) | X₂   | 6.63  | 8   | 10  | 12  | 13.37 |
| Ethanol (mL•L⁻¹)  | X₃   | 12.63 | 14  | 16  | 18  | 19.37 |

| Treatment | Experimental values | BC yield (g/l) |
|-----------|---------------------|----------------|
| 1         | -1 -1 -1            | 6.62           |
| 2         | -1 -1 +1            | 6.48           |
| 3         | -1 +1 -1            | 5.89           |
| 4         | -1 +1 +1            | 5.86           |
| 5         | +1 -1 -1            | 6.42           |
| 6         | +1 -1 +1            | 7.05           |
| 7         | +1 +1 -1            | 6.89           |
| 8         | +1 +1 +1            | 7.31           |
| 9         | -α 0 0              | 5.32           |
| 10        | +α 0 0              | 7.41           |
| 11        | 0 -α 0              | 6.76           |
| 12        | 0 +α 0              | 5.49           |
| 13        | 0 0 -α              | 7.11           |
| 14        | 0 0 +α              | 7.31           |
| 15        | 0 0 0               | 7.66           |
| 16        | 0 0 0               | 7.63           |
| 17        | 0 0 0               | 7.71           |
| 18        | 0 0 0               | 7.61           |
Multiple regression analysis of the experimental data shown in Table 7 resulted in the following second-order polynomial model:

\[
Y = 0.9568X_1 - 0.8936X_1^2 - 0.4328X_2 - 1.0606X_2^2 + 0.2072X_3 - 0.2963X_3^2 + 0.5700X_1X_2 + 0.2550X_1X_3 + 0.0250X_2X_3 + 7.6509 \quad R^2=0.9505
\]  

Where \(Y\) is the BC yield (g•L\(^{-1}\)), \(X_1\) the D-glucose concentration (g•L\(^{-1}\)), \(X_2\) the yeast extract concentration (g•L\(^{-1}\)) and \(X_3\) the ethanol concentration (mL•L\(^{-1}\)).

**Figure 1.** Response surface plot and corresponding contour plot. a Response surface plot and corresponding contour plot showing the effects of D-glucose and yeast extract on BC production of *B. amyloliquefaciens* ZF-7; b Response surface plot and corresponding contour plot showing the effects of D-glucose and ethanol on BC production of *B. amyloliquefaciens* ZF-7; c Response surface plot and corresponding contour plot showing the effects of yeast extract and ethanol on BC production of *B. amyloliquefaciens* ZF-7.
The data in Table 8 shows that the linear main effect of the D-glucose and yeast extract concentrations had a significant influence on the BC yield. BC production during fermentation was also affected by negative quadratic effects ($X_1^2$ and $X_2^2$). The interaction between the D-glucose concentration and the yeast extract concentration in the BC fermentation medium was also significant. The remaining factor coefficients were statistically unimportant, and no significant interaction effects were observed between $X_iX_j$ and $X_jX_i$. Analysis of variance indicated that the regression model was adequate to represent the relationship between BC yield and fermentation medium composition, with an acceptable coefficient of determination ($R^2 = 0.87$).

The parameter combination that resulted in an optimal BC yield was obtained by solving the system of partial derivatives for the different independent variables. The resulting optimal values were as follows: D-glucose at 56.1 g•L$^{-1}$, yeast extract at 9.9 g•L$^{-1}$ and ethanol at 17.2 mL•L$^{-1}$. The optimal point was located inside the experimental region. An additional independent fermentation with suggested optimal medium composition was used to examine the adequacy of the model derived here. The predicted BC yield was 7.83 g•L$^{-1}$ and the actual experimental value was 7.88 g•L$^{-1}$ as determined by triplicate experiments. This study, which successfully predicted an effective culture medium for increasing BC yield, is of great practical significance.

The interactions of the three components and their optimal levels in the inulinase production were further analyzed by the response surface methodology. The contour plots (Figure 1) graphically confirm the computed optimum values for the three independent variables. The results showed that the response surface was convex, which suggests that the optimal conditions were well-defined and there existed a maximum for each variable.

4. Conclusion
In conclusion, conventional and statistical optimization methods were used efficiently and successfully to improve BC production by *B. amyloliquefaciens* ZF-7. The optimum culture medium contained D-glucose 56.1 g/l, yeast extract 9.9 g/l and ethanol 17.2 ml/l. In this environment, the BC yield peaked at 7.88 g/l.

As a naturally occurring wild strain, *B. amyloliquefaciens* ZF-7 failed to meet the requirements of BC industrial production even after the optimization of its fermentation conditions. Therefore, additional industrially applicable strains should be screened based on the mutation breeding of ZF-7. Further research is required to investigate the use of less expensive materials for BC production.

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