Isolation and Culture Conditions Optimization of a New Bacterial Cellulose Producing Strain *Komagataeibacter intermedius* 6-5

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**Abstract.** A strain producing bacterial cellulose (BC) screened from Shanxi millet fermented vinegar was identified as *Komagataeibacter intermedius* 6-5 by the examination of taxonomic characteristics and 16S rDNA sequence analysis. Then, Fourier transform infrared (FT-IR) spectrum showed that pellicles produced by strain *K. intermedius* 6-5 had the same spectral characteristics as typical BC. Box-Behnken experiments studied used pear residue as a medium, the fermentation conditions of strain *K. intermedius* 6-5. The results showed that the optimal fermentation conditions were: glucose additive amount 3.62% (w/v), citric acid additive amount 0.45% (w/v), inoculation amount 9.39% (v/v). The BC yield was as high as (11.54 ± 0.42) g/L after seven days of culture at a natural pH and 30°C under static cultivation conditions.

1. Introduction

Bacterial Cellulose (BC) is a new kind of biomacromolecule material, which is formed by the connection of the glucosyl group through the β-(1-4)-glycosidic bond [1]. Compared with artificial cellulose and plant cellulose, bacterial cellulose has the following advantages: high purity, high water-content ability, high crystallinity, well mechanical strength, excellent biocompatibility, etc [2]. Based on the extraordinary properties of bacterial cellulose, it has a broad application prospect in food, paper making, biomedicine, photoelectric materials, and other fields [3, 4]. Previous studies have shown that the microorganisms used to produce bacterial cellulose include *Gluconacetobacter*, *Acetobacter*, *Pseudomonas*, *Sarcina*, *Komagataeibacter*, *Alcaligenes*, etc [5].

As a mostly agricultural country, China's utilization rate of crop by-products is less than 40%, which not only wastes resources but also pollutes the environment. At present, some studies have been carried out on the production of bacterial cellulose from the by-products of crop processing such as pericarp [6], olive mill residue [7], lentils waste liquid [8] and corn syrup [9].Pear residue is a by-product of pear processing, which contains a lot of sugar, protein, mineral elements, and other nutrients. It can be used as a cheap raw material for bacterial cellulose fermentation.
In this work, a cellulose producing bacterial strain *Komagataeibacter intermedius* (*K. intermedius*) 6-5 from Shanxi millet fermented vinegar was isolated, and its morphological, physiological, biochemical, and genic characteristics were investigated. Pear residue was used as the raw material for the culture medium for BC production. The optimal technological conditions for fermentation of pear residue with this strain to produce bacterial cellulose were explored, and the structural characteristics of the fermentation product BC were characterized.

2. Materials and methods

2.1. Microorganism and materials

*Bacillus subtilis* and *E. coli* strains were stored in the Laboratory of Biochemistry, School of Perfume and Aroma Technology, Shanghai Institute of Technology. Millet fermented vinegar was purchased from Shanxi, China. The pear was purchased from the Fengxian bay farmers market, Shanghai, China. All other reagents used in this study were of analytical grade unless otherwise stated.

2.2. Culture medium

The basic medium contained 1.0% (w/v) glucose, 1.0% (w/v) yeast extract in 1 L deionized water. The medium was mixed with 2.0% (v/v) ethanol before poured into Petri dishes.

The screening medium contained 1.0% (w/v) glucose, 1.0% (w/v) yeast extract, 0.5% (w/v) CaCO₃, 2.0% (w/v) agar in 1 L deionized water. The medium was mixed with 4.0% (v/v) ethanol before poured into Petri dishes.

The agar slant culture medium contained 1.0% (w/v) glucose, 0.5% (w/v) yeast extract, 2.0% (w/v) agar, 2.5% (v/v) glycerol in 1 L deionized water.

The pear residue medium contained 3.0% (w/v) glucose, 0.4% (w/v) citric acid, in 1 L pear residue water. Pear residue water was prepared by mixing pear residue and deionized water in the ratio of 1:6 (w/v).

2.3. Isolation of BC-producing strain

The fermentation broth of 1mL millet vinegar was transferred into the basic medium and oscillated at 30 °C for 180 r/min overnight. Then appropriate dilution gradient sample of 1mL was coated on the screening medium plate and cultured at 30 °C for 3 to 5 days. The single colony with a sizeable hydrolytic circle was inoculated in pear residue medium, cultured 30 °C for 3 to 5 days. Finally, the strain producing the white pellicle was transferred to the agar slant culture medium and preserved [10].

2.4. Identification of BC-producing strain

Morphological, physiological, and biochemical analyses were carried out according to the Berger Bacterial Identification Manual and the Common Bacterial System Identification Manual [11]. The colony configuration and cell morphology were evaluated through a microscope (BK1000, Chongqing Photoelectric Instrument Company, China). In the morphological analysis, *Bacillus subtilis* was used as a positive control and *E.coli* as the negative control. Physiological and biochemical characteristics such as oxidation reaction of ethanol, acetic acid and lactate, production of gluconic acid, cellulose, 5-ketogluconic acid, and γ-pyrone, glycerol ketogenic test, glucose fermentation test, starch hydrolysis test, were evaluated.

The selected strains were also identified by using 16s rDNA sequencing. The genomic DNA of bacterial strains was extracted with the SK8255 bacterial genome extraction kit. The F-primer was 5’-AGTTTGTATCMTGGCCTCAG-3’, and the R-primer was 5’-GGTTACCTTTGTTACGACTT-3’. The primers were subjected to polymerase chain reaction (PCR) amplification, and the amplified products were sent to Shanghai Biotech Co., Ltd. for sequencing. After sequencing results were obtained, the sequence was entered into the NCBI (www.ncbi.nlm.nih.gov), and BLAST analysis was performed in the GenBank database to find and download the sequences which were close to the strains. The phylogenetic tree was generated by MEGA X software to determine the taxonomic status of strains.
2.5. Cultivation and harvest of BC pellicles
A bacterial isolate was inoculated into the pear residue medium under static conditions. The bacterial strain was incubated in the pear residue medium at 30°C and natural pH for seven days. The pellicles were harvested and washed with distilled water thoroughly to remove residual medium and other impurities. Then the pellicles were rinsed in 0.1 N NaOH solution at 80°C for 30 min, washed with distilled water repeatedly until the pH was less than 7.0. Finally, the BC sample was dried at 60°C to obtain constant weight [2]. These pellicles were further used for FT-IR analysis.

2.6. Fourier transform infrared (FT-IR) spectroscopy
After the BC dry pellicles were crushed, it was mixed with KBr to be scanned by FT-IR. The investigated spectral range was from 4000 to 400 cm⁻¹ with a resolution of 4 cm⁻¹ for each measurement.

2.7. Optimization of the fermentation conditions
A 250-mL flask containing 50 mL of pear residue medium was inoculated with bacterial strain, cultivation at 30°C, and natural pH for seven days. The Box-Behnken method was performed to find the optimal culture conditions. The effects of glucose additive amount, citric acid additive amount, and inoculation amount on BC yield were investigated in the pear residue medium. Each significant variable was studied at three different levels (−1, 0, +1), where 0 represents the central value of each variable (Table 1) [1].

BC yield was taken as a response (Y), and culture conditions were taken as independent variables (X). The response function (Y) predicted by the use of a second-order polynomial regression equation is expressed by Equation (1),

\[
Y = \alpha_0 + \sum_{i=1}^{k} \alpha_i X_i + \sum_{i=1}^{k} \alpha_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \alpha_{ij} X_i X_j
\]

Where Y indicates the response obtained (% BC yield), \(\alpha_0\) represents a constant term for the reaction with \(X_i\) value zero for each variable. \(\alpha_i\) gives the linear effect of the input factor \(X_i\), \(\alpha_{ii}\) gives the square impact of the input factor \(X_i\), and \(\alpha_{ij}\) offers the quadratic effect of the input factor \(X_i\) and \(X_j\) that indicates the essential parameters affecting the characteristics of the process [12].

Table 1. Response surface test design factors and levels.

| Symbols     | Parameters          | Actual levels of coded factors |
|-------------|---------------------|--------------------------------|
|             |                     | -1    | 0    | 1    |
| X₁          | Glucose/%           | 3.00  | 3.50 | 4.00 |
| X₂          | Citric acid/%       | 0.40  | 0.45 | 0.50 |
| X₃          | Inoculation amount/%| 6.00  | 9.00 | 12.0 |

2.8. Statistical analysis
Statistical evaluation of all experimental data (variation from basal values) was performed using ANOVA. Mega X software was used to construct the phylogenetic tree, and Design-Expert 8.0.6, Excel and Origin 8.6 software was used to analyze the data.

3. Results and discussion

3.1. Isolation and identification of cellulose-producing strain
There was one strain screened from Shanxi millet fermented vinegar that produced significant amounts of cellulose. The morphological, molecular, and biochemical analyses were carried out to identify the strain. Colonies configurations were shown in Figure 1(a), and cell morphology was
shown in (b). As shown in Figure 1(a), the colonies were mainly milky white with a rounded shape and smooth surface. The strain was negative for Gram staining, and the cells were short rod-shaped or elliptical, growing singly or in pairs or chains (in Figure 1(b)).

![Colony morphology and optical micrograph](image)

**Figure 1.** Morphological observation of strain

The strain was subjected to relevant tests such as acetic acid oxidation, lactate oxidation, contact enzyme, and ethanol oxidation, etc. Results except for glucose fermentation test, oxidase assay, starch hydrolysis test and γ-pyrrrolidone test, the other tests were positive. Combining the Berger Bacterial Identification Manual and the Common Bacterial System Identification Manual, it can be preliminarily determined that the strain is *Komagataeibacter intermedius*. Regarding the 16S rDNA sequence analysis, the results revealed that the strain had 99.93% homology with a *K. intermedius* (Figure 2). Thus, the strain was identified as *K. intermedius* based on its taxonomic characteristics and 16S rDNA sequence analysis. Finally, we named it as *K. intermedius* 6-5.

![Phylogenetic tree](image)

**Figure 2.** Phylogenetic tree based on strain 16S rDNA sequence

### 3.2. Isolation and identification of cellulose-producing strain

The FT-IR spectrum of BC produced by *K. intermedius* 6-5 in the pear residue medium is shown in Figure 3. There was an absorption peak at 3346 cm\(^{-1}\), which is caused by the O-H stretching vibration, and a C-H stretching vibration peak at 2896 cm\(^{-1}\). Besides, the absorption peaks at 1645 cm\(^{-1}\), 1315 cm\(^{-1}\), and 1059 cm\(^{-1}\) were caused by the stretching vibration of the cellulose terminal semi-carboxyl aldehyde group, the shaking vibration of CH\(_2\) bond and the stretching vibration of straight-chain C-O-C, respectively. According to the above spectral information, the product membrane accorded with the characteristic absorption of bacterial cellulose, which proved that the product was bacterial cellulose (BC).
3.3. Establishment of the regression model and significance test

The yield of BC (g/L) was used as the response value, and the fermentation process was optimized by the Box-Behnken response surface method. The experimental design and the observed and predicted values were shown in Table 2.

Table 2. Response surface analysis test design and results.

| Run | X1 glucose additive amount | X2 citric acid additive amount | X3 inoculation amount | Measured value | Predictive value |
|-----|----------------------------|-------------------------------|----------------------|----------------|-----------------|
| 1   | -1                         | -1                            | 0                    | 5.28 ± 0.14   | 5.63            |
| 2   | 1                          | -1                            | 0                    | 8.70 ± 0.08   | 8.75            |
| 3   | -1                         | 1                             | 0                    | 7.01 ± 0.07   | 6.96            |
| 4   | 1                          | 1                             | 0                    | 8.40 ± 0.12   | 8.05            |
| 5   | -1                         | 0                             | -1                   | 5.85 ± 0.11   | 5.81            |
| 6   | 1                          | 0                             | -1                   | 8.12 ± 0.07   | 8.39            |
| 7   | -1                         | 0                             | 1                    | 7.50 ± 0.14   | 7.23            |
| 8   | 1                          | 0                             | 1                    | 8.81 ± 0.17   | 8.84            |
| 9   | 0                          | -1                            | -1                   | 6.61 ± 0.05   | 6.29            |
| 10  | 0                          | 1                             | -1                   | 8.42 ± 0.11   | 8.50            |
| 11  | 0                          | -1                            | 1                    | 9.20 ± 0.02   | 9.12            |
| 12  | 0                          | 1                             | 1                    | 7.22 ± 0.07   | 7.54            |
| 13  | 0                          | 0                             | 0                    | 11.50 ± 0.07  | 11.38           |
| 14  | 0                          | 0                             | 0                    | 11.32 ± 0.04  | 11.38           |
| 15  | 0                          | 0                             | 0                    | 11.05 ± 0.14  | 11.38           |
| 16  | 0                          | 0                             | 0                    | 11.80 ± 0.02  | 11.38           |
| 17  | 0                          | 0                             | 0                    | 11.25 ± 0.26  | 11.38           |

The second-order polynomial equation defining the BC yield can be described as follows (Equation (2)):

\[ Y=11.38+1.05X_1+0.16X_2+0.47X_3-0.51X_1X_2-0.24X_1X_3-0.95X_2X_3+2.16X_1^2-1.87X_2^2-1.65X_3^2 \]  

where \( Y \) represents the predicted response for BC yield, and \( X_1 \), \( X_2 \), and \( X_3 \) gives the coded values of the experimental variables, glucose additive amount (% w/v), citric acid additive amount (% w/v), and inoculation amount (% v/v), respectively.
The analysis of variance (ANOVA) is used to predict the fitness and significance of the regression model established (Table 3). The model equation for BC yield was found in the range of independent variable parameters. As can be seen from Table 3, the F value of the model is 55.18 (P < 0.0001), indicating that the model reached a very significant level. The F value of the lack of fit is 2.59 (P = 0.1902 > 0.05), meaning that the difference is not substantial. The coefficient (R²) is 0.9861, which suggests that there is a good correlation between the predicted value and the measured value. Comprehensive analysis shows that the regression equation can be used to analyze and predict the BC yield test [13]. The significance test of the regression coefficient in the quadratic model is that the P values of X₁ and X₃ in the monomial ratio are <0.0001 and 0.0088, respectively, which are less than 0.05, indicating that the linear effects of factors X₁ and X₃ on BC yield are significant; The P values of X₁X₂ and X₂X₃ in the formula are 0.0278 and 0.0013, respectively, both of which are less than 0.05. Therefore, the interaction of X₁X₂ and X₂X₃ on BC yield is significant, while the P-value of X₁X₃ is higher than 0.05. Thus, the interaction effect on BC yield is not substantial; the P values of the quadratic coefficient factors X₁², X₂², and X₃² are all less than 0.05, so the surface effects of X₁², X₂², and X₃² on BC yield are significant. The value of F showed the primary and secondary impact of the factors, and the contribution rate of the elements in this experiment: the amount of glucose additive amount > the inoculation amount > the citric acid additive amount.

Table 3. ANOVA results of the regression coefficient.

| Source      | Sum of squares | df | Mean square | F value | P value | Significance |
|-------------|----------------|----|-------------|---------|---------|--------------|
| Model       | 66.84          | 9  | 7.43        | 55.18   | <0.0001 | *            |
| X₁          | 8.80           | 1  | 8.80        | 65.38   | <0.0001 | *            |
| X₂          | 0.20           | 1  | 0.20        | 1.47    | 0.2640  |              |
| X₃          | 1.74           | 1  | 1.74        | 12.92   | 0.0088  | *            |
| X₁X₂        | 1.03           | 1  | 1.03        | 7.66    | 0.0278  | *            |
| X₁X₃        | 0.23           | 1  | 0.23        | 1.71    | 0.2320  |              |
| X₂X₃        | 3.59           | 1  | 3.59        | 26.68   | 0.0013  | *            |
| X₁²         | 19.73          | 1  | 19.73       | 146.58  | <0.0001 | *            |
| X₂²         | 14.76          | 1  | 14.76       | 109.64  | <0.0001 | *            |
| X₃²         | 11.46          | 1  | 11.46       | 85.13   | <0.0001 | *            |
| Residual    | 0.94           | 7  | 0.13        |         |         |              |
| Lack of fit | 0.62           | 3  | 0.21        | 2.59    | 0.1902  | not significant |
| Pure error  | 0.32           | 4  | 0.080       |         |         |              |
| Sum         | 67.78          | 16 |             |         |         |              |

R²=0.9861; R²Adj=0.9682; R²Pred=0.8458; C.V.%=4.21

Note: *: Significant differences (P < 0.05).

3.4. Analysis of response surface and contour map
The steepness of the response surface can reflect the influence of the interaction of various factors on the response value. The steeper the surface, the more significant the effect, and the gentler the surface, the opposite. The contour plot corresponds to the response surface plot, and the contour plot can reflect the interaction strength, the ellipse represents the significant influence, the circle represents the insignificant influence [14]. Figure 4 shows the Interactive effects of various factors on bacterial cellulose production. The Response Surface of glucose additive amount and citric acid additive amount, citric acid additive amount, and inoculation amount is steep, and the contour map is an ellipse. Their interaction effect on BC production is significant.
Figure 4. Interactive effects of various factors on bacterial cellulose production

3.5. Model validation and confirmation
The maximum correlation between process variables was verified by testing the predictive stability. The optimum conditions of strain *K. intermedius* 6-5 in Pear residue fermentation medium: glucose additive amount 3.62% (w/v), citric acid additive amount 0.45% (w/v), inoculation amount 9.39% (v/v). The BC yield was as high as 11.5373 g/L after seven days of culture at a natural pH and 30°C under static cultivation conditions. The accuracy of the model was tested by the optimized combination, and the actual yield of BC was (11.54 ± 0.42) g/L. The relative error was 0.09%. In this study, BC was produced by fermentation of pear residue. Compared with previous studies, the fermentation medium was simple, low cost and high yield of BC.

4. Conclusions
New bacterial cellulose (BC) producing strain 6-5 was screened from Shanxi millet fermented vinegar. It was identified as *Komagataeibacter intermedius* by the examination of taxonomic characteristics and 16S rDNA. The fermentation products of *K. intermedius* 6-5 in the pear residue medium were characterized by FT-IR. The results showed that the strain could produce BC in the pear residue medium. The Box-Behnken experiments were used to optimize the fermentation conditions of *K. intermedius* 6-5 in the pear residue medium. The optimal fermentation conditions were determined as follows: glucose additive amount 3.62% (w/v), citric acid additive amount 0.45% (w/v), inoculation amount 9.39% (v/v). The production of bacterial cellulose (BC) was (11.54 ± 0.42) g/L, which was 2.2 times that before optimization (5.28 ± 0.14) g/L. In this study, the material is cheap and easy to get; the fermentation process is simple and controllable; the yield is high and pollution-free.

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