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Effect of Organic Acids on Bacterial Cellulose Produced by *Acetobacter xylinum*

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Research Article

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ABSTRACT

Based on the difference of bacterial cellulose production from rice saccharificate medium and chemical medium under static cultivation, effect of organic acids in the process of bacterial cellulose produced by *A. xylinum* was studied. The results showed that the kinds and contents of organic acids were different in both culture medium, in which accumulated oxalic acid and tartaric acid inhibited *A. xylinum* producing BC in chemical medium, while pyruvic acid, malic acid, lactic acid, acetic acid, citric acid and succinic acid, as ethanol, promoted *A. xylinum* to produce BC. Compared to the blank BC production 1.48 g/L, the optimum addition concentrations of pyruvic acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid, and ethanol in chemical medium were 0.15%, 0.1%, 0.3%, 0.4%, 0.1%, 0.2% , 4% and the BC productions were 2.49 g/L, 2.83 g/L, 2.12 g/L, 2.54 g/L, 2.27 g/L, 1.88 g/L , 2.63 g/L, respectively. The co-existence of above organic acids and ethanol increased BC production even further.

INTRODUCTION

Bacterial cellulose (BC) is kind of biofilm fermented by microorganisms, which demonstrates unique properties including strong hydrophilic, high viscosity, water binding capacity and gel-properties. Bacterial cellulose has been used in the food industry for applications such as low-calorie desserts, salads, and fabricated food, in the paper manufacturing industry to enhance paper strength, in acoustic diaphragms for audio speakers [1,2], in tissue engineering to prepare scaffolds for wound dressing, artificial cartilage, bone regeneration, vascular grafts, and dental implants [3], and in pharmaceutical industry to develop drug delivery systems [4]. With the widespread availability, researches on improvement of bacterial cellulose production are attracting a close attention of the world in recent years.

Acetobacter xylinum, mainly occurring as a contaminant in vinegar fermentation, is a highly investigated organism in terms of its biochemical pathways that lead to cellulose biosynthesis, utilizing a large variety of carbon and nitrogen sources [5-7]. The carbon source used for the culturing of cellulose producing bacteria is one of the most important factors affecting the BC yield. Various carbon sources including monosaccharides, oligosaccharides, organic acids, alcohols, and sugar alcohols have been studied to increase BC production up to now [8]. Among them, the research on organic acids is very limited. The studies made by Ma Xia [9] and WANG Zhi-guo [10] had shown that organic acids can increase the output of BC, but for citric acid, the additional effects were contradictory. Furthermore, species of organic acids were chosen without foundation. In our investigations, we have studied effect of organic acids on bacterial cellulose production by *A. xylinum*. This work consisted of the following objectives: (1) to evaluate the yield of BC production with different culture mediums; (2) to analyze changes of organic acids with different culture mediums in the process of BC producing; (3) to test affection on BC production of analyzed organic acids and ethanol; (4) to optimize the addition of synergistic organic acids and ethanol; (5) to test effect of organic acids and ethanol co-existing on BC production.

MATERIALS AND METHODS

Bacterial strains

The bacterial strain used in this study was *Acetobacter xylinum* maintained in Guizhou Province Key Laboratory of Fermentation Engineering and Biological Pharmacy.

Culture medium

Preculture medium: The preculture medium contained the following per liter: sucrose 20 g, yeast extracts 5 g, peptone 5 g, KH_2PO_4 2 g, and $MgSO_4$ 1 g. The pH value of the medium was in nature.

Chemical medium: The chemical medium contained the following constituents per liter: sucrose 50 g, yeast extracts 10 g, peptone 8 g, KH_2PO_4 4.3 g, and $MgSO_4$ 2.5 g. The pH value of the medium was in nature.

Rice saccharificate medium: Filter the rice saccharification liquid provided by Guizhou Province Guiyang City Weichunyuan Foodstuff Co. Ltd Division through four layers of gauze, then, dilutes the filtrate with distilled water by 1:4. Its main components were total sugar 49.85g/L, total acid 0.15 g/kg.

EXPERIMENTAL METHODS

Precultivation

Freshly cultivated *A. xylinum* was inoculated by using three loops of microbe and then shaking cultured at 28°C at 100 rpm for 24 h in a 250 mL flask containing 50 mL of sterilized chemical medium. This cultivated medium was used as inoculum for further culture, and 8% (v/v) of the inoculum was incubated into the following conditions.

Cultivation

Eight percent (4 mL) of the precultured inoculum was incubated into conical flasks (250 mL), which contained 50 mL of sterilized fermentation medium (chemical medium or rice saccharificate medium), and was incubated in static condition at 30°C for 7 days.

Treatment of bacterial cellulose

Collected bacterial cellulose with tweezers, washed repeatedly with distilled water to remove medium and impurity on the surface of the membrane, placed in 80°C, 0.5 mol/L NaOH, 30 min to remove the bacterial protein and the residual culture medium, until the membrane become milky white translucent, neutralized with 0.5% acetate buffer after cooling, washed thoroughly with distilled water, dried to constant weight at 105°C [11].

Analysis methods

Determination of bacterial cellulose production

After treatment, BC production can be calculated by the following formula:

$$\text{BC production(g/L)}=\frac{\text{Dry film quality}}{\text{The initial volume of fermentation liquid}}\times 1000$$

Determination of organic acids

The concentration of organic acids was determined by high-performance liquid chromatography (HPLC). First, broth samples were centrifuged at 3000 rpm for 10 min. Then, 1 mL supernatant with 0.2 mL.1 mol/L H_3PO_4 was diluted to 10 mL with deionized water. And then the diluted supernatant was filtrated through a 0.2 μm filter. Last, the concentration of organic acids was determined with analytical column C_{18}. The UV detection wave length was 210 nm, the mobile phase was KH_2PO_4 buffer solution at a temperature of 30°C, a flow rate of 0.5 ml/min, pH value 2.8, and the sample size was 10 μL.

RESULTS AND DISCUSSIONS

BC Production with different culture mediums

A. xylinum was cultured in rice saccharificate medium and chemical medium 7 days. Bacterial cellulose productions are shown in **Figure 1.**

Figure 1 shows that BC production in rice saccharificate medium was 1.64 g/L, which was higher (increase 10.66%) than that from chemical medium. This may be resulted from more complex composition of rice saccharificate medium, which was in favor of *A. xylinum* to produce BC. So researches on the differences metabolites in both culture mediums can provide relevant train of thought to improve BC production in future. Changes of organic acids in different culture mediums.

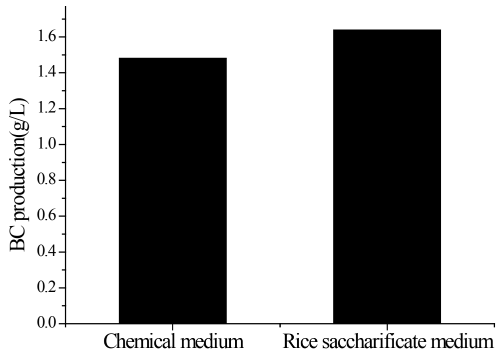

Figure 1. BC production in different culture mediums.

In static conditions, 7 kinds of organic acids were determined every day by HPLC in the process of fermentation. The results are shown in **Figures 2 and 3.**

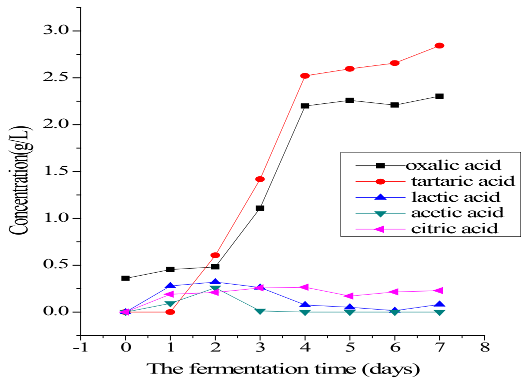

Figure 2. Changes of organic acid in chemical medium.

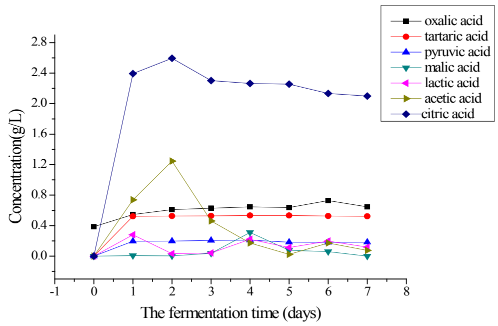

Figure 3. Changes of organic acid in rice saccharificate medium.

Figure 2 shows that 5 kinds of organic acids were tested in the process of BC produced with *A. xylinum* in chemical medium. Oxalic acid and tartaric acid were the major parts and increased with the fermentation period. At the end of fermentation, the concentrations of oxalic acid and tartaric acid were 2.30 g/L and 2.84 g/L. On the contrary, the concentrations of citric acid, acetic acid and lactic acid were much lower than oxalic acid and tartaric acid, which was below 0.30 g/L and kept within certain level in the fermentation process. In general, the kinds of organic acid were few and oxalic acid and tartaric acid accumulated a lot in the fermentation process in chemical medium.

Figure 3 shows that 7 kinds of organic acids were tested in the process of BC produced with *A. xylinum* in rice saccharificate medium. Citric acid and acetic acid were the major parts. Citric acid kept much more higher (around 2.20 g/L) than others, acetic acid reached a peak (1.25 g/L) at the next fermentation day and then decreased rapidly within the fermentation period. The contents of oxalic acid, tartaric acid, lactic acid, malic acid and pyruvic acid didn't change too much, the range of oxalic acid and tartaric acid concentration was 0.4 g/L -0.7 g/L, while the concentrations of lactic acid, malic acid and pyruvic acid kept below 0.2 g/L in the process of fermentation.

Making a comparison between **Figures 2 and 3**, the result showed that different kinds and concentrations of organic acids existed in chemical medium and rice saccharificate medium. It may be speculated that the higher BC production in rice saccharificate medium may be relevant to organic acids metabolic pattern, in which some organic acids, such as citric acid and acetic acid, can promote *A. xylinum* to produce BC, while oxalic acid and tartaric acid may decrease BC production. In order to verify this speculation, different kinds of organic acids were added to culture mediums. Chemical medium was selected as culture mediums due to its definite compositions.

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Effect of organic acids on BC production

0.1% of oxalic acid, tartaric acid, pyruvic acid, malic acid, lactic acid, acetic acid, citric acid and succinic acid were added into chemical medium to check effect of organic acids on BC production. The addition of ethanol into the culture medium was found to be beneficial for the production of BC [8]. In order to compare the effect of organic acids, 1% ethanol was added into chemical medium also. Blank medium was chemical medium without anything addition. At the end of fermentation (7 days), BC productions were shown in **Figure 4.**

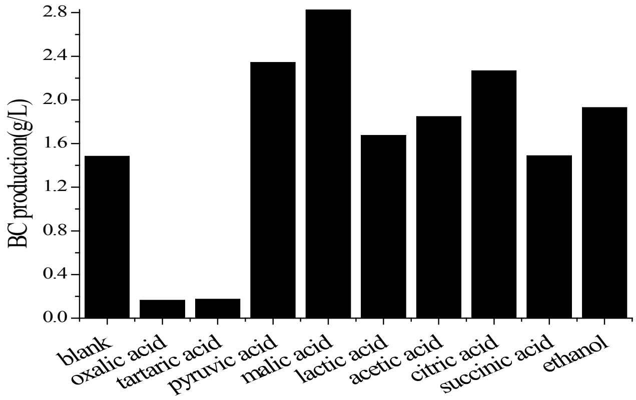

Figure 4. Effect of organic acids on BC production.

Figure 4 shows that oxalic acid and tartaric acid had obvious inhibition of BC synthesis. With the addition of oxalic acid and tartaric acid, BC production were 0.16 g/L and 0.17 g/L, much lower than blank (1.48 g/L). The accumulation of oxalic acid and tartaric acid might be the reason to reduce BC production in chemical medium **(Figure 1).** Synergistic effect of malic acid, pyruvic acid and citric acid on *A. xylinum* was obvious and BC productions were 2.83 g/L, 2.34 g/L, 2.27 g/L, respectively, higher than ethanol additional sample (1.93 g/L). Synergistic effects of lactic acid, acetic acid and succinic acid were not obvious and BC productions were 1.67 g/L, 1.85 g/L, and 1.49 g/L, respectively.

Pyruvic acid as the premise substance of TCA cycle and gluconeogenesis had an obviously synergistic effect on bacterial cellulose. Malic acid, citric acid and succinic acid were intermediates of TCA cycle [10]. Ethanol, acetic acid and lactic acid could be consumed quickly to provide energy in the fermentation process [12]. So, the addition of malic acid, pyruvic acid, citric acid, lactic acid, acetic acid, succinic acid and ethanol to culture medium had synergistic effect, and the optimum addition concentrations for BC yields needed test further.

Effect of organic acids and ethanol on BC production

Organic acids and ethanol were tested optimum concentrations for BC yields except oxalic acid and tartaric acid. **Figures 5-8** show BC production changes with different concentrations of pyruvic acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid and ethanol. Too much or too little organic acids and ethanol concentrations led to reduced BC production. The optimum concentrations of pyruvic acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid, ethanol were 0.15%, 0.1%, 0.3%, 0.4%, 0.1%, 0.2%, 4% and the corresponding BC productions were 2.49 g/L, 2.83g/L, 2.12 g/L, 2.54 g/L, 2.27 g/L, 1.88 g/L, 2.63 g/L, respectively. Compared to the blank BC production 1.48 g/L, organic acids and ethanol could increase BC production obviously.

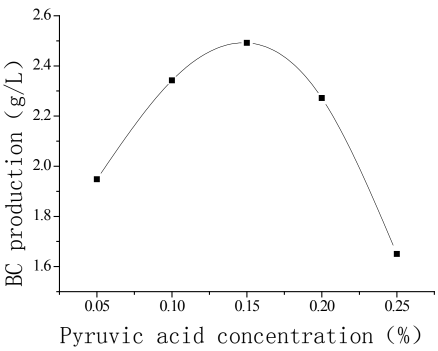

Figure 5. Effect of pyruvic acid concentration on BC production.

When 0.4% acetic acid, 0.3% lactic acid and 0.4% pyruvic acid coexisted in the medium, the yield increased significantly as reported by WANG [10]. So, effect of organic acids and ethanol coexisted on BC production was tested follow.

Effect of organic acids and ethanol mixing on BC production

In order to test effect of organic acids and ethanol coexisted on BC production, orthogonal test was selected. Pyruvic acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid and ethanol were as factors, and orthogonal design experiments were studied by means of 7 factors and 3 levels (L18 (37)). Through extreme difference analysis of **Table 1**, the order of affecting factors to BC production was ethanol, pyruvic acid, malic acid, succinic acid, citric acid, acetic acid, lactic acid. Optimum combination was A1B3C1D1E3F2G1, which meant 0.1% pyruvic acid, 0.12% malic acid, 0.2% succinic acid, 0.12% citric acid, 0.3% acetic acid, 0.2% lactic acid and 3% ethanol. Because the optimum combination was A1B3C1D1E3F2G1 did not appear in **Table 2**, three parallel experiments had been carried out, and BC production from the average optimal output was 3.012 g/L, which was higher than single organic acid or ethanol additional sample.

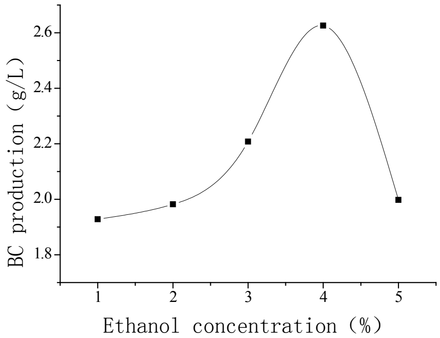

Figure 6. Effect of ethanol concentration on BC production.

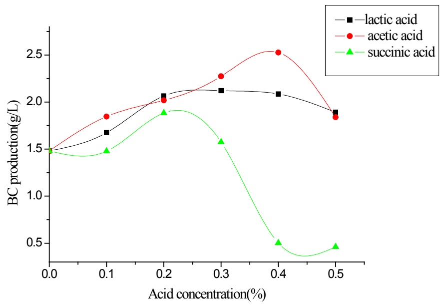

Figure 7. Effect of lactic acid, acetic acid and succinic acid concentration on BC production.

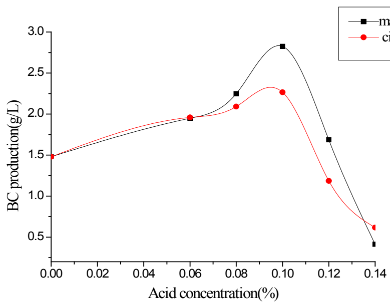

Figure 8. Effect of malic acid and citric acid concentration on BC production.

Table 1. Orthogonal factor level table of organic acids and ethanol.

Factor	A Pyruvic acid (%)	B Malic acid (%)	C Lactic acid (%)	D Acetic acid (%)	E Citric acid (%)	F succinic acid (%)	G Ethanol (%)
level1	0.10	0.08	0.2	0.3	0.08	0.1	3
level2	0.15	0.10	0.3	0.4	0.10	0.2	4
level3	0.20	0.12	0.4	0.5	0.12	0.3	5

Table 2. Orthogonal design and direct-vision analysis of organic acids and ethanol.

Test number	A	B	C	D	E	F	G	BC production(g/L)
1	0.10	0.08	0.2	0.3	0.08	0.1	3	2.499
2	0.10	0.10	0.3	0.4	0.10	0.2	4	2.295
3	0.10	0.12	0.4	0.5	0.12	0.3	5	1.638
4	0.15	0.08	0.2	0.4	0.10	0.3	5	0.843
5	0.15	0.10	0.3	0.5	0.12	0.1	3	2.379
6	0.15	0.12	0.4	0.3	0.08	0.2	4	2.34
7	0.20	0.08	0.3	0.3	0.12	0.2	5	1.155
8	0.20	0.10	0.4	0.4	0.08	0.3	3	2.112
9	0.20	0.12	0.2	0.5	0.10	0.1	4	2.103

10	0.10	0.08	0.4	0.5	0.10	0.2	3	2.595
11	0.10	0.10	0.2	0.3	0.12	0.3	4	2.394
12	0.10	0.12	0.3	0.4	0.08	0.1	5	1.188
13	0.15	0.08	0.3	0.5	0.08	0.3	4	1.632
14	0.15	0.10	0.4	0.3	0.10	0.1	5	0.885
15	0.15	0.12	0.2	0.4	0.12	0.2	3	2.733
16	0.20	0.08	0.4	0.4	0.12	0.1	4	1.524
17	0.20	0.10	0.2	0.5	0.08	0.2	5	0.9
18	0.20	0.12	0.3	0.3	0.10	0.3	3	2.511
K1	2.102	1.708	1.912	1.964	1.799	1.763	2.472	
K2	1.802	1.827	1.860	1.782	1.872	2.003	2.048	
K3	1.718	2.085	1.849	1.875	1.971	1.855	1.101	
range	0.384	0.377	0.063	0.182	0.192	0.240	1.371	

CONCLUSION

BC production in rice saccharificate medium was higher than in chemical medium by *A. xylinum*. Meanwhile, the kinds and concentrations of the organic acids were different in both culture mediums. The result maybe derives from different metabolic modes of organic acids. Accumulation of oxalic acid and tartaric acid might suppress BC production, while accumulation of citric acid and acetic acid could improve BC production. The experiment confirmed that, just like ethanol, a normal additive to increase BC yield, pyruvic acid, malic acid, succinic acid, citric acid, acetic acid, lactic acid could improve BC production, and the co-existence of organic acids and ethanol increase BC production even further. The formation of oxalic acid and tartaric acid in chemical medium and the metabolic pathway of pyruvic acid, malic acid, lactic acid, acetic acid, citric acid and succinic acid need be continued to study in subsequent experiments.

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