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# Production of Bacterial Cellulose by *Gluconacetobacter xylinus* TCCC 10025 Isolated from Vinegar Culture

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**A cellulose-producing strain isolated from vinegar culture, preservation number TCCC 10025 in the Tianjin university of science & technology's center of culture collection, was identified as *Gluconacetobacter xylinus* based on morphological character, traditional physiological and biochemical method and 16S rDNA complete sequencing analysis. The composition of medium and fermentation conditions was studied. When the initial pH of the medium was 6.0, which contained 70g/L sucrose, 9g/L yeast extract, 9g/L tryptone, trace amount of $Ca^{2+}$, $SiO_3^{2-}$ and $Mg^{2+}$, and the strain was cultivated at 28°C, the amount of bacterial cellulose produced by *Gluconacetobacter xylinus* TCCC 10025 was the highest and reached to 9.214±0.053 g/L. The film produced by *Gluconacetobacter xylinus* TCCC 10025 was turn out to be bacterial cellulose by the SEM and FT-IR analysis.**

**Key words**: Bacterial cellulose, *Gluconacetobacter xylinus*, Identification, Fermentation conditions.

The bacterial cellulose (BC) is popular referred to microbial synthetic cellulose produced by *Acetobacter* sp., *Agrobacterium* sp., *Pseudomonas* sp., Achromobacter sp., *Alcaligenes* sp., *Aerobacter* sp., *Azotobacter* sp., *Rhizobium* sp. and *Sarcina* sp. [1]. As a tremendous producer of cellulose, *Acetobacter xylinum*,a Gram-negative, bacilliform bacteria, has been studied immensely and renamed as *Gluconacetobacter xylinus* subsp. *xylinus* in 1997 [2, 3], *Gluconacetobacter xylinus*, a micromicrobe widely distributed in nature, is a troublesome superfluity in the industrial production of vinegar. *Gluconacetobacter xylinum* has been isolated from rotting fruits [4, 5], vegetables, fermenting coconut water[6] and vinegar culture.

The BC is a glucan composed of straight-chain glucose molecule linked at the b-1,4 glycosidic bond. It is different from plant cellulose because of its high purity and high degree of polymerization. Besides, it is specifically characterized by its high water-holding capacity and mechanical strength [7, 8]. BC can be used for various applications including paper industry, adhesive-bonded fabric, fidelity acoustic materials, soft tissue engineering, drug controlled release carrier, artificial duramater, artificial skin, wound covers, membrane for separations [9-11] and so on.

With the increasing demand for BC, increase in the production yield of BC is very urgent. Therefore, there has been a recent increased interest in BC production by microbial cell. *Gluconacetobacter xylinum* is one of the best in these microbes. Cellulose produced by *Gluconacetobacter xylinum* has been reported both in static as well as agitated culture[6, 12, 13]. The production of cellulose is affected by the type and concentration of carbon source, nitrogen source,

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inorganic salt, trace element and pH[14]. The carbon sources involve glucose, sucrose, fructose, invert sugar, ethanol and glycerol[15] and nitrogen source involve yeast extract, beef extract, peptone, and tryptone. *Gluconacetobacter* xylinum is able to grow at PH as low as 3.5 owing to its distinct acid tolerable [16].

As mentioned above, production of BC by *Gluconacetobacter xylinum* has a high potential for commercialization. However, the yield of BC is not high enough to be industrialized. Accordingly, further research is required to isolate high-producing strain and optimize its culture medium. The effects of culture conditions and medium component on the yield have been studied by many researchers[17-19]. However there are few studies on the inorganic salt and trace element. In this investigation, *Gluconacetobacter xylinum* was used to produce BC and the effects of carbon source, nitrogen source, inorganic salt, trace element, pH and temperature on BC yield were studied.

## MATERIALS AND METHODS

### Isolation and identification of cellulose-producing bacteria

Cellulose-producing bacteria were isolated using the modified method reported by Toyosaki[12]. Sample from vinegar culture was inoculated into the basic medium ( 30g/L glucose, 10g/L peptone, 6g/L yeast extract, 5g/L $Na_2HPO_4$, 1g/L sodium citrate, pH6.8, 121°C holding 20 min.) and cultured statically at 30°C. When the pellicle was formed on the surface of culture broth, it was selected out and eluted with sterile normal saline, and then the elution was coated onto an agar plate containing the basic medium. The plates were incubated at 30°C for 2 days until colonies formed. After that these colonies were inoculated into the basic medium respectively. Finally, the pure strains were isolated after repeating the static culture for 6 times, as described above.

The method of identifying cellulose-producing strain was formulated in the ninth edition of 'the Bergey's bacteria identification manual' and the second edition 'Bergey system bacteriology manual'. Reference to these manuals, the highest cellulose-producing strain was identified by Physiological and biochemical actions.

Screening the strain with the highest yield and identifying it using 16S rDNA complete sequence analysis were implemented with the following procedure suggested by Juke and Canter. The 16S rDNA complete sequence was submitted to the GeneBank and the percent similarity and phylogenetic tree were analyzed according to the method suggested by Saito and Nei.

### Optimition of medium

The basic medium was used as the initial medium for the cell culture. The strain was kept on 1% agar plates containing the basic medium, then inoculated into 50mL of medium in a 250mL flask and cultured at 30°C for 24h in a shaking condition of 120 rpm. The culture broth was then inoculated into 100mL of medium in a 250mL conical flask by 5% inoculums size and cultured at 30°C for 6days in static condition. To increase the yield of BC, the medium was optimized by the following steps. The glucose, fructose, sucrose, lactose and galactose were chose as carbon resources and the effect of different carbon resource on yield of BC was investigated. Under the condition of the concentration being 30g/L, what was the optimal carbon resource was verified. After that, the concentration of the optimal nitrogen resource was studied. The yeast extract, beef extract, peptone, and tryptone were selected as nitrogen resources and the effect of different nitrogen resource on the yield of BC was studied when the total concentration of nitrogen carbon resource was 10g/L. The content of the total nitrogen resource was studied after it was confirmed. Afterwards, the trace element was considered as an influence factor, and $Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$,$Cu^{2+}$, $Zn^{2+}$, $Na^{+}$, $Cl^{-}$ ,$K^{+}$, $SiO_3^{2-}$ and $SO_4^{2-}$were involved in this study.

### Optimition of fermentation conditions

The initial pH of broth could influence the growth of strain and further the production of BC in fermentation process. The pH from 5.5 to 6.5 with a 0.1 step was selected to study the effect of the initial pH on the yield of BC. *Gluconacetobacter xylinus* could grow lushly between 25°C and 35°C. Therefore, 25°C, 28°C, 30°C and 35°C were selected as testing point, and the impact of temperature on the production of BC was investigated.

### Analysis of BC

When the fermentation process was terminated, the BC containing cells was treated

with a lot of water to dislodge the impurities on the surface and weighed ($W_{wet}$), and then soaked into 1 mol/L sodium hydroxide solution for 24 h. After that, the material was put into 1 mol/L fresh sodium hydroxide solution boiled for 15-20min, flushed repeatedly, then placed in 1% acetic acid for 10min and washed with water again and again. The material was freeze-dried and weighed ($W_{dry}$). The yield of BC was calculated as $W_{dry}$. Water holding capacity was calculated as:

$$WHC(\%) = (W_{wet} - W_{dry}) / W_{dry} \times 100\%$$

The cellulose was cut into oblong pieces after freeze-drying. The samples were fixed on test board. A Tensile Strength Tester 062 (Lorentzen &Wettre, Sweden) was used to analyze the tensile strength of cellulose.

The cellulose was cut into small pieces after freeze-drying. Dried specimens were mounted on aluminum studs and coated with a gold/ palladium alloy under high vacuum conditions. Specimens were examined using a Quanta 200 scanning electron microscope (FEI, Netherlands) to observe the micro-structure of cellulose surfaces.

FT-IR spectra of freeze-dried BC was recorded on a VECTOR 22 (Bruker, Germany) in absorption mode in the range of 4000–450 $cm^{-1}$. Twenty scans were performed to establish accuracy.

## RESULTS AND DISCUSSION

### Isolation and identification of the strain from vinegar culture

In the past studies, many strains had been isolated and identified as capable of producing cellulose[19, 20]. Eight strains producing BC were isolated from vinegar culture and the strain with the greatest production was preserved in Tianjin university of science & technology's center of culture collection(TCCC) and was indexed TCCC 10025. On the basis of its morphological observation and physiological and biochemical tests, the cellulose-producing strain was classified as the genus of *Gluconacetobacter* sp., the result was in Fig.1 and Table1. Afterwards it was identified by 16S rDNA complete sequence analysis. The base sequence submitted to GeneBank was 1377bp. The similarity was examined and a phylogenetic tree was created. The isolated strain exhibited a high similarity value (99.8%) and a closest distance to *Gluconacetobacter xylinus* in the phylogenetic tree. Therefore, the cellulose-producing strain isolated from vinegar culture was identified as *Gluconacetobacter xylinus*.

### Effect of carbon resource

The carbon resource is important for the growth of strain and the production of BC, which is composed of glucoses. In this study, glucose, fructose, sucrose, lactose and galactose were selected as carbon resource and the concentration was 30g/L. The other compositions in medium were 10g/L peptone, 6g/L yeast extract, 5g/L $Na_2HPO_4$, 1g/L sodium citrate, pH6.8, 121°C holding 20 min. The *Gluconacetobacter xylinus* TCCC 10025 was inoculated into 50mL of the basic medium in a 250mL flask at 28°C for 24h; 5% of broth was inoculated into 100mL of adjusted medium in a 250mL flask at 28°C for 6days.Obtained celluloses

**Table1.** Physiological and biochemical properties of Gluconacetobacter xylinus TCCC 10025

| Items | Result |
|---|---|
| Gram Faerbung | - |
| Catalase test | + |
| Formation of acetic acid from ethanol | + |
| Oxidation of acetate | - |
| Oxidation of lactate | - |
| Formation of ketone from glycerol | + |
| Formation of ketone from glucose | + |
| Metabolization of malonic acid | - |
| Formation of acid from sorbitol | - |
| Formation of acid from maltose | - |
| Formation of acid from mannitol | - |
| Metabolization of arabinose | + |
| Main ubiquinone type | Q10 |
| Growth in Frateu's Hoyer | - |

**Table 2.** Effect of different carbon resource

| Carbon resource | Yield of BC (g/L) | OD600 |
|---|---|---|
| Glucose | 1.662±0.051 | 0.235±0.011 |
| Sucrose | 2.284±0.033 | 0.403±0.012 |
| Lactose | 1.266±0.032 | 0.274±0.011 |
| Fructose | 1.099±0.022 | 0.321±0.009 |
| Galactose | 0.858±0.022 | 0.197±0.010 |

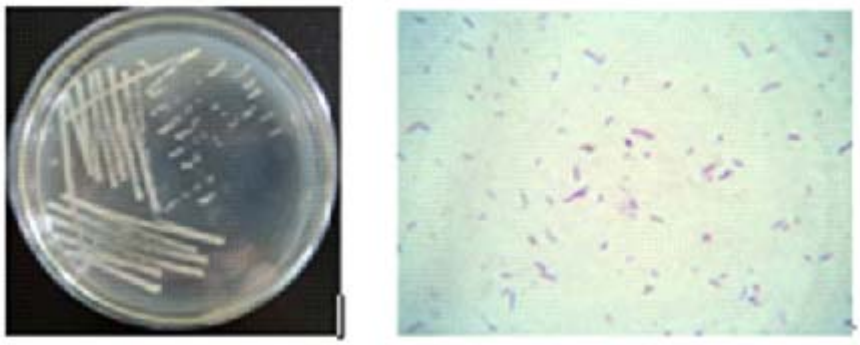

**Fig.1.** Feature of *Gluconacetobacter xylinus* TCCC 10025

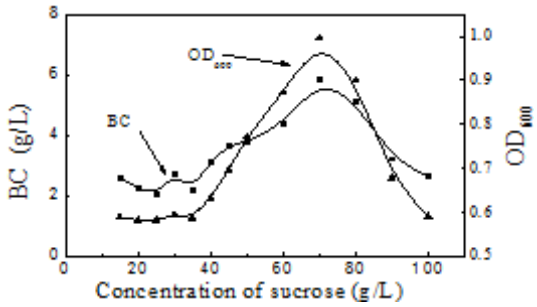


**Fig.2.** Effect of concentration of sucrose on yield of BC

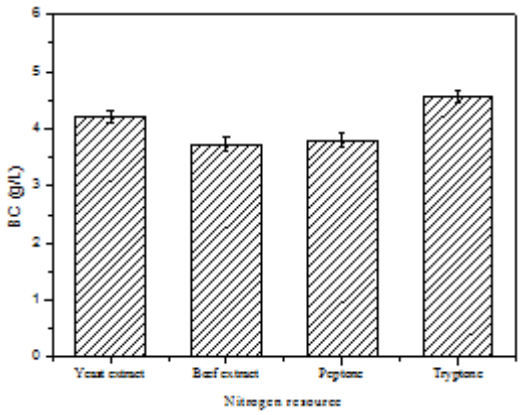


**Fig.3.** Effect of different nitrogen resource on yield of BC

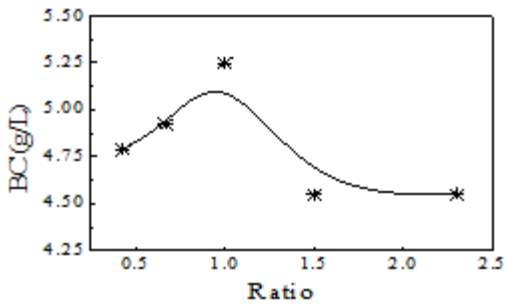


**Fig. 4.** Effect of ratio of yeast extract and tryptone on yield of BC

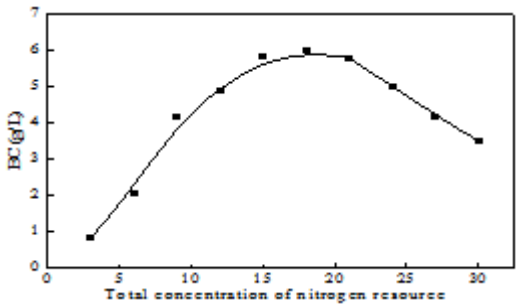


**Fig.5.** Effect of the total concentration of nitrogen resource on yield of BC

were handled, freeze-dried and weighted. As shown in Table2, the yield was the highest when the sucrose was selected as the carbon resource and reached 2.284±0.033g/L. The sucrose was decomposed into glucose and fructose in microbial cells, and the producing fructoside was utilized by microbial cells, however, the producing glucoside was used to assemble BC on the cell membrane. This process was more conductive to produce cellulose, because the activation reaction of glucose was needless. The lactose was decomposed into glucoside and the activation reaction of glucose did not come in the assembling process. Nevertheless, the yield of BC was lower using lactose than sucrose as carbon resource. The cells were assemblers and the cell number revealed the assembling speed to some extent and this is consistent with Table2. It is evident that the

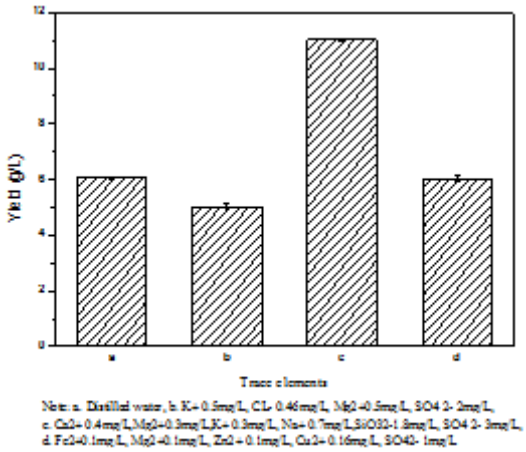


**Fig.6.** Effect of trace elements on the yield of BC

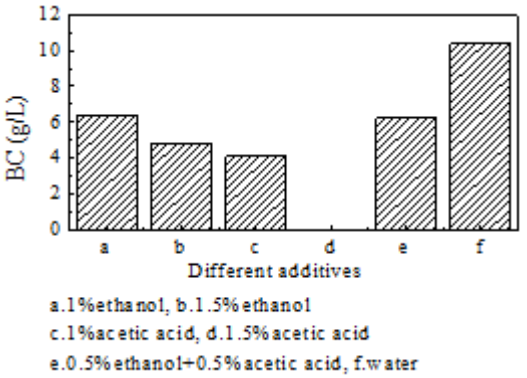


**Fig.7.** Effect of adding ethanol and/or acetic acid

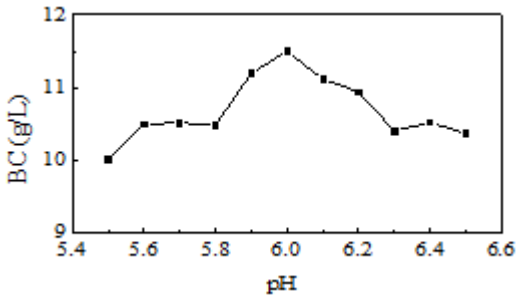


**Fig.8.** Effect of pH on the yield of BC

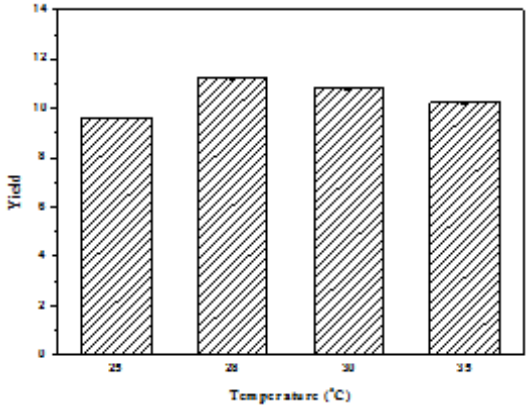


**Fig.9.** Effect of temperature on the yield of BC

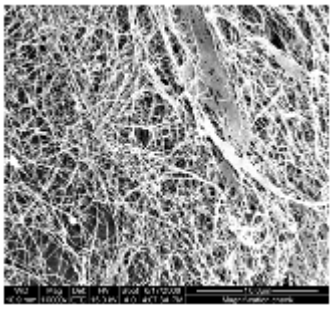

**Fig.10.** SEM images of BC(10000×)

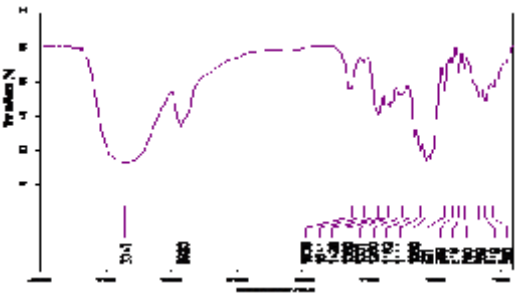

**Fig.11.** FTIR spectra of BC

fructose was better metabolized than glucose by *Gluconacetobacter xylinus* TCCC 10025.

After sucrose was selected as carbon resource, the effect of its concentration on yield of BC was studied. The concentration of sucrose was from 15 to 100g/L, with the interval of 5g/L between 15 and 50g/L, and with the step size of 10g/L between 50 and 100g/L, the other fermentation conditions were the same with described above.

As shown in Fig.2, when the concentration of sucrose was low (10-40g/L), the output of BC and the cell concentration almost had no change. However, when the concentration of sucrose exceeded 40g/L, the output of BC and

cell concentration increased while sucrose concentration raised. Then the output of BC maximized 5.858±0.029g/L when the concentration of sucrose was 70g/L, after that it went down quickly with the concentration of sucrose raising continually. One possible reason could be thae the strain was intolerance to high sucrose concentration. The result indicated that the harvest of BC was related to the concentration of cell.

**Effect of nitrogen resource**

The nitrogen resource is important for the growth of cells. For different nitrogen sources, the effect of the concentration on yield of BC was investigated. The medium contained 70g/L sucrose, 5g/L $Na_2HPO_4$, 1g/L sodium citrate and 10g/L nitrogen resource (Yeast extract, beef extract, peptone, tryptone) and pH6.8, 121°C holding 20 min. The *Gluconacetobacter xylinus* TCCC 10025 was inoculated into 50mL of the basic medium in a 250mL flask at 30°C for 24h; 5% --of broth was inoculated into 100mL of adjusted medium in a 250mL flask at28°C for 6days; obtained celluloses were handled, freeze-dried and weighted.

As shown in Fig.3, when yeast extract or tryptone were chose as nitrogen resource, the outputs of BC were 4.211±0.115 g/L and 4.567±0.120g/L respectively which are higher than others. In order to maintain the diversity of nitrogen source, tryptone and yeast extract were selected as nitrogen sources and the total concentration of nitrogen resource was kept at 10g/L, however the ratio of tryptone and yeast extract was changed. The result (Fig.4) indicated that the output was highest when the ratio of tryptone and yeast extract was 1.

After tryptone and yeast extract with the ratio 1 were selected as nitrogen resources, the effect of the total concentration of nitrogen resources on the yield of BC was considered. The total nitrogen concentration was from 3g/L to 30 g/L with 3g/L step value. The other compositions in medium were 70g/L sucrose , 5g/L $Na_2HPO_4$, 1g/L sodium citrate, pH6.8, 121°C holding 20 min. As shown in Fig.5, the yield of BC was highest reaching 6.014±0.15 g/L when the total concentration of nitrogen resource was 18g/L.

**Effect of trace elements**

It has been demonstrated that the trace elements play an important role in the fermentation process of the lipase (M.M.D Maia et al.,2001) and the poly(²-hydroxybutyric acid) ( Enrico Grothe et al.,1999). In an occasional experiment, the obvious increase in the yield of BC was observed when the tap water was chose as diluents. Therefore the effect of trace elements which was considered as an important factor on the yield of BC was discussed. Three groups of trace elements were selected to study the performance and the medium was 70g/L sucrose, 5g/L $Na_2HPO_4$, 1g/L sodium citrate, 9g/L tryptone, 9g/L Yeast extract, and 1mL trace elements solution, pH6.8, 121°C holding 20 min. As shown in Fig.6, the output of BC changed when the trace elements were added to the medium, the production of group a decreased group b almost did not change, and group c gave the highest result.

The result indicated that $Ca^{2+}$ and $SiO_3^{2-}$ played the role of promoting production of BC and $Fe^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Cu^{2+}$, and $SO_4^{2-}$ had almost no influence on the production of BC, but $Cl^-$ maybe restrain the production of BC. When 0.9% NaCl was added into medium, the membrane of BC did not form though cultivated statically for 10days and the output was very low (the result was not shown). The results (the production was increased when adding $Ca^{2+}$ and $SiO_3^{2-}$ into medium) in this study will transform the other fermentation process and improve the production. It has a certain significance.

**Effect of adding ethanol and/or acetic acid**

It was reported that the output of BC would increase if the ethanol and/or acetic acid were added into the medium[4, 21]. As shown in Fig. 7, the yield of BC reduced when the ethanol and/or acetic acid were added into medium. This result was different with other researchers[22].

**Effect of the initial pH**

*Gluconacetobacter xylinus* had been shown acid tolerance and could grow at pH as low as 3.5[5]. Verschuren et al.reported that it was ideal for the development of cellulose under pH 4.0 to pH5.0 [16]. In this study, pH from 5.5 to 6.5 was selected as condition for considering production of BC, and there was no BC formation at pH5.4 and the lower even after cultured for 10 days. The result in Fig.8 indicated that the initial pH 6.0 in the medium was in favor of the yield of BC. The *Gluconacetobacter xylinus* TCCC 10025 developed fast at pH6.0; the pH declined sharply with the growing of cell and the BC began spawning at pH

5.0, therefore a large number of cells were generated firstly and then the BC production occurred in the fermentation process. The cells grew fast at pH6.0, however the cellulose began to boom at pH5.0 and the lower.

**Effect of temperature**

The temperature was an important factor is the growth of cell and production of metabolin. In this study, different temperature (25 °C, 28 °C, 30 °C and 35 °C ) were selected as different culture temperature for cultivating the strain. The medium was 70g/L sucrose, 5g/L $Na_2HPO_4$, 1g/L sodium citrate, 9g/L tryptone, 9g/L Yeast extract, and trace element c, pH6.8, 121°C holding 20 min. As shown in Fig.9, the strain can produce more cellulose at 28 °C than other temperature. The laboratory finding was different with other researchers[4, 17, 23].

**Characteristic of BC**

The cellulosic film produced by *Gluconacetobacter xylinus* TCCC 10025 in a static culture was located on the surface of the culture broth and became thicker with the increased culture time[24] According to the method in 2.4, the wet and dry films of BC were obtained; the water holding capacity and the tensile strength were tested. The water binding capacity of wet film was 138±31% and the strength of dry film was 1.91±3MPa at 10mm/min tensile speed.

Morris discussed the combinative gelation mechanism of two component systems, and envisioned three possible models for the combinative gelation of active polymers able to form a network structure. Model I was an interpenetrating network, formed by active polymers that were structurally cooperative because of the weaving of the two networks. Model II was a phase-separated network in which two parallel networks existed due to the incompatibility of the two active polymers. Model III was a coupled network cross-linked between two active polymers. A cellulose network consisting of nano-scale cellulose was surveyed in the scanning electron micrograph of the BC produced by *Gluconacetobacter xylinus* TCCC 10025. As shown in Fig. 10, the SEM micrograph was typical of BC. The cellulose ribbons and network were similar to that had reported[23]. The FT-IR was employed to further evaluate the microstructure of BC produced by *Gluconacetobacter xylinus* TCCC 10025. As shown in Fig.11, almost all functional groups and bonds were same with Huang H.C.[21] in the FTIR spectra of BC. The main peakes in the fingerprint region were almost the same as BC. The region 3600-3000$cm^{-1}$ was correspond to O-H stretching frequencies and the peak was 3347$cm^{-1}$ in the spectrum. Therefore, the above-mentioned SEM micrograph and FT-IR spectrum confirmed that the cellulose produced by *Gluconacetobacter xylinus* TCCC 10025 was pure cellulose and free of any other impurities.

## CONCLUSION

A strain, named *Gluconacetobacter xylinus* and numbered TCCC 10025, that can produce BC in quantity was isolated from vinegar culture and identified by physiological and biochemical test and 16S rDNA complete sequencing analysis. When cultivated in a static culture, the medium was optimized to improve the yield of BC. The sucrose as carbon resource was more adaptive than other saccharide and the optimal nitrogen resource was 9g/Lyeast extract and 9g/L tryptone. When the trace elements were added into the medium, the yield of BC was increased; adding $Ca^{2+}$, $SiO_3^{2-}$ and $Mg^{2+}$ into medium were thought to be beneficial to the production of BC, however the mechanism involved in increasing BC producing still needs to be elucidated. The yield of BC was not increased when ethanol and/or acetic acid were added into medium. It is advantageous for cell growing and BC producing when the initial pH was adjusted to 6.0 and the culture temperature was 28°C in fermentation process. The film produced by *Gluconacetobacter xylinus* TCCC 10025 was proved to be bacterium cellulose with the SEM and FT-IR methods analysis.

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