EFFECT OF DIFFERENT CONDITIONS ON THE AVERAGE DEGREE OF POLYMERIZATION OF BACTERIAL CELLULOSE PRODUCED BY *GLUCONACETOBACTER INTERMEDIUS* BC-41

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The average degree of polymerization (DP) of bacterial cellulose (BC), which was secreted by selected *Gluconacetobacter intermedius* BC-41, was studied using the viscosity measurement method under five different sets of conditions, with varying media, cultivation modes, fermentation time, carbon sources and surfactants. The DP of BC produced in A9 medium was 22.9% higher than that produced in Schenk and Hildebrandt (SH) medium. Meanwhile, there was not a significantly different DP under static and shaking culture conditions. However, the DP was remarkably influenced by fermentation time. The BC produced with glycerin or xylose as carbon source exhibited lower DP than that produced with glucose. Adding Tween 20 or Tween 80 in A9 medium had little effect on the DP, while adding Triton X-100 decreased it. The results presented here would provide some experimental data for BC production with different DP in practical applications.

Keywords: *Gluconacetobacter intermedius* BC-41, average degree of polymerization, bacterial cellulose, viscosity method, different conditions

INTRODUCTION

The discovery of bacterial cellulose (BC) was accredited to A. J. Brown in 1886 for the synthesis of an extracellular gelatinous mat.[1] However, it was not until the 20th century that more intensive studies on BC were conducted.

Produced by some *Acetobacter* strains, the BC is identical with plant cellulose with respect to molecular structure.[2] However, BC is superior to plant cellulose in some physical and chemical properties, including high mechanical strength, porosity, purity, water-holding capacity and crystallinity, good biocompatibility and transparency, ultra-fine and finely pure fiber network structure.[2,3] These excellent properties have made BC a suitable biomedical and industrial raw material for applications where plant cellulose can hardly be used, such as conductive carbon film, products for artificial skin and tissue replacement, ultrafiltration membrane and calorie free food.[4-6]

It has been revealed that the characteristics of BC could be affected by many factors, such as media, fermentation modes and carbon sources. The BC produced in agitated culture exhibited a lower DP, crystallinity and Young's modulus of sheet, but a higher water-holding capacity and suspension viscosity in the disintegrated form than that produced in static culture.[7] In contrast, the BC harvested from stationary cultures demonstrated a much higher value of Young's modulus, but a much lower value of water-holding capacity.[8] In the presence of lignosulfonate for some *Gluconacetobacter xylinus* (namely *A. xylinum*), the BC displayed a higher crystallinity index and the amorphous region of the BC was relatively lower, meaning that lignosulfonate enhanced the crystallinity of BC.[9]

Some researchers have also proven that different carbon sources could influence the DP. Synthesized with glucose and mannitol, the DP of BC was approximately 1700, while it was approximately 1050 for BC synthesized with xylose.[10] Similar results were also found in *Acetobacter* sp. V6. The crystallinity index value, unlike the water-holding capacity and viscosity of the BC produced in glycerol medium, was higher than that obtained from glucose medium.[11]

The structure and properties of cellulose film produced by *A. xylinum* could also be modified by the addition of low molecular weight chitosan into the culture medium.[12] The controlled porosities of BC could be ensured by varying fermentation conditions and post-treatment methods.[13]

Although the factors that affect the properties of BC have been studied by many scientists, the data, especially about the DP of BC, seem rather scarce and unsystematic. So in this study, the DP of BC in *G. intermedius* BC-41 was investigated under different conditions using the viscosity method. The results would provide an experimental basis for obtaining specific BC with different DP.

EXPERIMENTAL

Microorganism and culture conditions

G. intermedius BC-41, stocked in our laboratory, was routinely grown in Schenk and Hildebrandt (SH) medium,[14] containing glucose 20 g/L, peptone 5 g/L, yeast 5 g/L, $Na_2HPO_4 \cdot 12H_2O$ 2.7 g/L, citric acid 1.5 g/L, with pH 5.0 at 28 °C, in a shaking incubator with 120 rotations per minute (rpm). Meanwhile A9 medium, consisting of glucose 40 g/L, yeast 1 g/L, peptone 7 g/L, $Na_2HPO_4 \cdot 12H_2O$ 8 g/L, corn syrup 6 g/L, acetic acid 1 ml/L, alcohol 14 ml/L, with pH 6.0, was used for the present investigation.

In order to clarify the influence of different cultures on the DP of BC, aliquots of 10% SH medium culture with *G. intermedius* BC-41 were transferred into 100 ml SH medium and A9 medium in 500 ml flasks and then cultured in a static incubator at 28 °C for 14 days.

Also, another A9 medium, which was also inoculated with aliquots of 10% SH medium culture, was cultivated in a shaking incubator for 15 days at 28 °C and the DP of BC was measured every three days to evaluate the change trend of DP with increasing time.

At the same time, two other A9 media were also cultured in a shaking incubator with 80 rpm or under static conditions at 28 ºC for 6 days to test the impact of different cultivation modes on the DP.

Furthermore, the glucose in A9 medium was replaced by the same amount of glycerin or xylose and cultured in a static incubator at 28 ºC for 14 days to analyze the effect of different carbon sources on the DP. 14 days later, the pH value of each culture broth was determined using a pH meter. Similarly, 1 ml/L Tween-20, Tween-80 or Triton X-100 was added into the A9 medium respectively and then the mixed medium was cultured in a static incubator at 28 ºC for 14 days to evaluate the influence of different surfactants on the DP.

Purification of cellulose

The harvest and preliminary treatment of BC were conducted according to previously reported methods[15] with slight modifications. Briefly, the BC sheets from every different culture broth were collected and flushed for various times with deionized water until all impurities and residual medium were thoroughly removed from the membrane surface. Then, the BC sheets were dipped into 0.1 M NaOH solution and heated in 100 °C water bath, until the gel became milky translucent. Subsequently, the gel was repeatedly washed with deionized water to make sure that the pH value of the washing water became 7.0. Finally, the gel was dried at 105 °C until constant weight.

Determination of DP of BC

Dried BC was weighed and dissolved in copper-ethylenediamine solution and the specific concentration (c) was determined. The relative viscosity (η_r) and specific viscosity (η_{sp}) of BC in copper-ethylenediamine solution were measured using a Ubbelohde viscometer (Sunlex Glass Instrument sales Co., LTD, Shanghai, China) in a thermostatic chamber at 25 °C and then calculated with equations 1 and 2, respectively:[16]

$$\eta_r = t / t_0 \tag{1}$$

$$\eta_{sp} = \eta_r - 1 \tag{2}$$

where t_0 is the flow time of solvent, t is the flow time of solution.

According to the single point method, the polymer solution intrinsic viscosity (η) was determined using the general formula (equation 3):[16]

$$\eta = \frac{\sqrt{2(\eta_{sp} - \ln \eta_r)}}{c} \tag{3}$$

At the same time, a viscosity average molecular weight M was also determined using the Mark-Houwink empirical equation 4:[17]

$$\eta = KM^{\alpha} \tag{4}$$

The constant value of K and α can be obtained from the Polymer Data Handbook.[18]

Finally, the DP of BC was ultimately determined using equation 5:[19]

$$DP = \frac{M}{162} \quad (5)$$

where 162 equals the molecular weight of an anhydroglucose unit. The DP values were calculated by three independent experiments.

Data analysis

To evaluate the differences among groups, all the results of DP obtained from the above experiments were recorded and analyzed with DPS software using Student's *t* tests or one-way analysis of variance (ANOVA).[20]

RESULTS AND DISCUSSION

Influence of different culture conditions on DP

In order to measure the influence of different culture conditions on the DP of BC, the *G. intermedius* BC-41 culture broth, cultivated initially in SH medium, was transferred into A9 and SH media and incubated for 14 days at 28 °C in a static constant incubator. The DP of BC collected from A9 medium was 2301.7, which was significantly higher (by 29.9%) than that of BC harvested from SH medium – 1613.3 (Fig. 1a) ($p < 0.01$).

It was also found that the average viscosity value of the produced sheets from HSL medium (namely SH medium with addition of lignosulfonate) was 76.98 cP, whereas it was 36.48 cP for HS medium (namely SH medium), which indicated a high degree of polymerization.[21] In this study, the A9 medium was found more suitable for producing cellulose with high DP than the SH medium. The reasons may be explained as follows. As mentioned in Experimental, alcohol and corn syrup, which contain lactic acid and other growth factors, were added to the A9 medium. The results of the batch culture experiment on *A. xylinum* subsp. *sucrofermentans* BPR3001A, using ethanol as the main carbon source, suggested that ethanol may function as an energy source for ATP generation and not as a substrate for BC biosynthesis.[22] Meanwhile, in *A. xylinum* subsp. sucrofermentans BPR 2001, the researchers speculated that the lactate in corn steep liquor (CSL) acted as an accelerator driving the Tricarboxylic Acid (TCA) cycle, as well as an energy producer, which resulted in high cellulose production and rapid cell growth.[23] To sum up, nutrition-rich conditions may be the most important factor in producing BC with high DP.

Effect of different cultivation strategies on DP

The DP of BC harvested from A9 medium cultured under static or shaking conditions for 6 days was determined. The DP value of 2257.3 was obtained for shaking conditions, and of 2189.4 for static conditions (Fig. 1b), which suggested that there was no significant difference between the two cultivation methods ($p>0.05$).

Previous researches reported by other authors revealed that there was almost no difference between reticulated structures of bacterial cellulose fibrils produced in agitated culture and in static culture. Nevertheless, bacterial cellulose produced in agitated culture exhibited microstructural changes, namely, a low degree of polymerization and crystallinity index.[8] Whereas in this study, we found that the DP in shaking medium was slightly higher than that in static medium, with no great difference between them. This may be explained by the fact that the rotating speed was only 80 rpm in this study, whereas it was 180 rpm in the previous report.

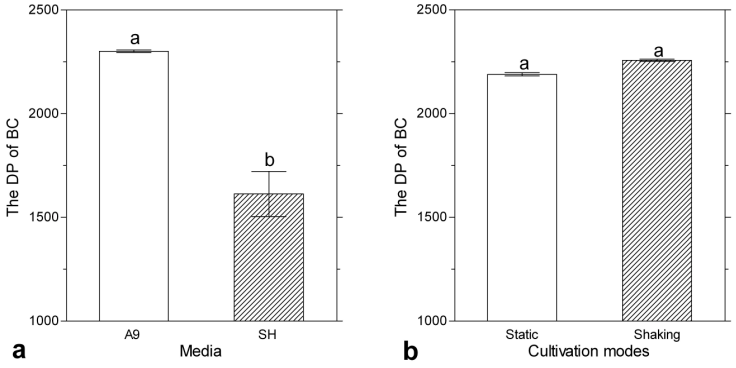

Figure 1: Effects of different culture media (a) and cultivation modes (b) on the average degree of polymerization of bacterial cellulose. Note: Lowercase letters above the bars of each group indicate statistically significant differences (Tukey's HSD, $P<0.05$). Error bars: standard deviation (SD) of three replicates. The note and error bars of latter figures are the same as in Figure 1

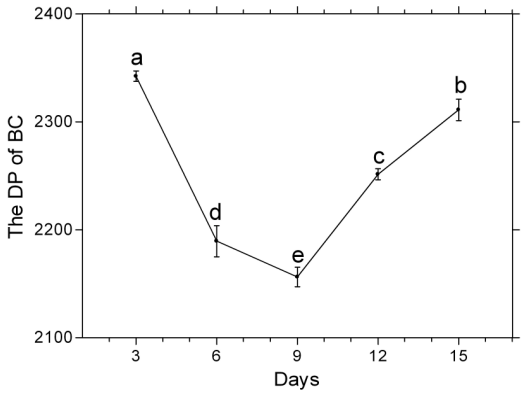

Figure 2: Effect of breeding time on the average degree of polymerization of bacterial cellulose

Effect of cultivation time on DP

In order to clarify the change trend with culture time, the DP of BC harvested from A9 medium was measured every 3 days. The range of average DP remarkably changed from 3-day to 15-day culture time ($p < 0.01$), with the highest DP of 2342.2, appearing on the third day and the lowest, of 2156.2, on the ninth day (Fig. 2). As shown in Fig. 2, there was a declining trend of the DP values from the third to the ninth day, and then the values ascended until the fifteenth day. Overall, the average value of DP was 2250.0. From the above results, it is speculated that the DP could be significantly influenced by fermentation time.

Some scientists have found that even for small inoculation volume, culture time could significantly influence porosity of the bacterial cellulose membrane,[13] but they have not yet managed to fully understand the controlling mechanism of bacterial cellulose porosity and to determine the DP of BC. In the present investigation, we proved that the DP could be significantly affected by fermentation time, but no evidence supported that fermentation time could also influence the porosity of BC.

Effect of carbon source and pH value on DP

Under static conditions, the glucose in A9 medium was replaced by the same amount of glycerin or xylose, and the DP in different carbon media was determined. The DP of BC was significantly influenced by the carbon resources (Fig. 3) ($p < 0.01$). The DP of BC cultured with glycerol was 1380.5, while that of BC with xylose was 1361.2, both being lower by 40.03% and 40.85%, respectively, than 2301.7 – the DP for glucose medium. However, there was no significant difference between DPs of BC harvested from glycerol medium and the one from xylose medium ($p > 0.5$). Furthermore, the pH values of different media cultured for 14 days were also measured and the final values were of 3.2, 6.4 and 3.5 for glucose, glycerin and xylose, respectively (Fig. 3) ($p < 0.01$).

The production rate of bacterial cellulose in *G. xylinus* (ATCC 53524) was influenced by different carbon sources, such as mannitol, glucose, glycerol, fructose, sucrose or galactose, but the formed product was indistinguishable in molecular and microscopic features.[24] Cellulose is synthesized with cellulose synthase.[25] The DP of BC produced from glycerol and xylose was lower which may due to the series enzymes in gluconeogenesis and the Hexose Monophophate Pathway (HMP) pathway show lower activity so that the synthetic cellulose substrate concentration is low and finally affects the DP.

In this study, we found that cellulose synthesis can also be affected by pH values. The pH values of the fermentation broths with different carbon sources were different (Fig. 3). Glucose and xylose used as carbon sources showed lower pH values, while glycerol for carbon source, the pH value was higher. These results suggested that the

effect of different carbon sources on the DP was more important than pH value.

The relationship between final pH values and cellulose membrane yield from various carbon substrates had been studied. And the authors found that the glucose culture gave the lowest pH followed by xylose and ethanol. And they speculated that the pH changes of the culture might be the indicator of the side reactions taking place in the cellulose production culture.[26] Here, we obtained similar results but the glycerin culture exhibited the highest pH value.

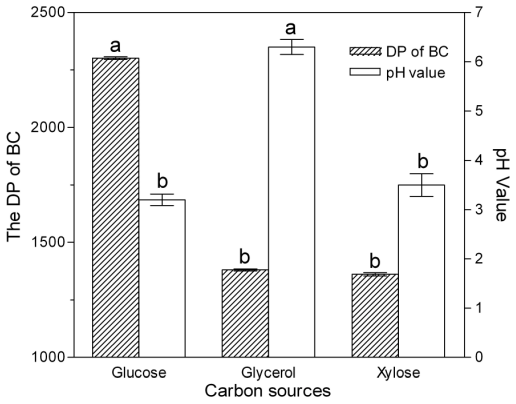

Figure 3: Influence of different carbon sources on the average degree of polymerization of bacterial cellulose and the pH values of the final broth media

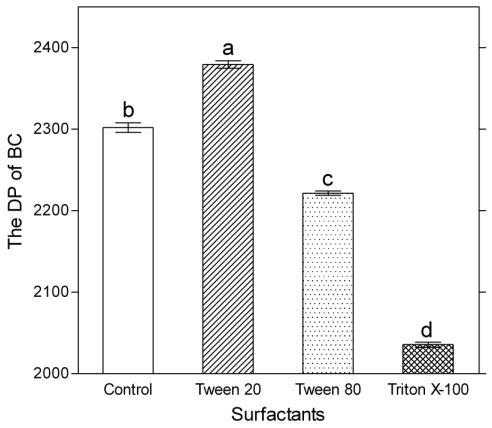

Figure 4: Effect of different surfactants on the average degree of polymerization of bacterial cellulose

Effect of different surfactants on the DP

As we all know, surface tension could be influenced by surfactants. So we hypothesized that surfactants could also affect the cellulose polymerization. Different surfactants were added into the A9 medium. Cultured for 14-day, the cellulose was harvested and its DP was also determined respectively. The DP of BC from Tween-20 and Tween-80 media was 2379.2 and 2221.4 respectively which showed a remarkable difference from the control (Fig. 4) ($p < 0.01$). While DP values, obtained from the Triton X-100 medium, was 2035.5 which reduced approximately by 11.5% compared to the control glucose medium (Fig. 4) ($p < 0.01$).

The Hydrophilic-Lipophilic Balance (HBL) values of Tween 20, Tween 80 and Triton X-100 were 16.7, 15 and 13.9 respectively. With the decrease of HBL of surfactants which added in A9 medium, the DP of BC decreased.

In addition, BC was not a part of the cell wall but be secreted out of the bacteria through the cell membrane microporous. Meanwhile, the bacterial cellulose secretion process is simultaneously with its biological synthesis.[27] And surfactants showed a certain effect on the membrane of bacteria which affected the synthesis process of cellulose and ultimately changed the DP.

CONCLUSION

Using the viscosity measure method, we attempted to elucidate which factors or conditions could affect the DP of BC in *G. intermedius* BC-41. Fortunately, some valuable data were obtained in this study under laboratory conditions. A9 medium was more suitable for higher DP of BC production than SH medium. Meanwhile, the DP could be influenced by fermentation time and surfactants but not cultivation modes. In addition, the BC, produced in A9 medium containing glycerin or xylose or Trition X-100, presented lower DP.

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REFERENCES

[1] A. J. Brown, *J. Chem. Soc*., **49**, 432 (1886).
[2] F. Yoshinaga, N. Tonouchi and K. Watanabe, *Biosci. Biotech. Biochem*., **61**, 219 (1997).
[3] E. J. Vandamme, S. De Baets, A. Vanbaelen, K. Joris and P. De Wulf, *Polym. Degra*d. *Stabil*., **59**, 93 (1998).
[4] M. Iguchi, S. Yamanaka and A. Budhiono, *J. Mater. Sci*., **35**, 261 (2000).
[5] R. Jonas and L. F. Farah, *Polym. Degrad. Stabil*., **59**, 101 (1998).
[6] A. Svensson, E. Nicklasson, T. Harrah, B. Panilaitis, D. L. Kaplan, M. Brittberg and P. Gatenholm, *Biomaterials*, **26**, 419 (2005).
[7] K. Watanabe, M. Tabuchi, Y. Morinaga and F. Yoshinaga, *Cellulose*, **5**, 187 (1998).
[8] A. Krystynowicz, W. Czaja, A. W. Jezierska, M. G. Miśkiewicz, M. Turkiewicz and S. Bielecki, *J. Ind. Microbiol. Biotechnol*., **29**, 189 (2002).
[9] S. Keshk and K. Sameshima, *Enzyme Microb. Technol*., **40**, 4 (2006).
[10] B. Surma-Ślusarska, S. Presler and D. Danielewicz, *Fibres Text. East. Eur*., **16**, 69 (2008).
[11] H. I. Jung, J. H. Jeong, O. M. Lee, G. T. Park, K. K. Kim *et al*., *Bioresource Technol*., **101**, 3602 (2010).
[12] M. Phisalaphong and N. Jatupaiboon, *Carbohyd. Polym*., **74**, 482 (2008).
[13] W. Tang, S. Jia, Y. Jia and H. Yang, *World J. Microbiol. Biotechnol*., **26**, 125 (2009).
[14] R. U. Schenk and A. C. Hildebrandt, *Can. J. Bot*., **50**, 199 (1972).
[15] O. Shezad, S. Khan, T. Khan and J. K. Park, *Korean J. Chem. Eng*., **26**, 1689 (2009).
[16] A. Boztuğ and S. Basan, *J. Mater. Sci*., **39**, 6843 (2004).
[17] I. L. Mello, M. C. Delpech, F. M. B. Coutinho, F. F. M. Albino and S. M. Santos, *Polym. Bull*., **55**, 115 (2005).
[18] R. Mansencal, in "Polymer Data Handbook", edited by J. E. Mark, Oxford University Press, Inc., 1999, pp. 39-48.
[19] D. D. Y. Ryu, S. B. Lee, T. Tassinari and C. Macy, *Biotechnol. Bioeng*., **24**, 1047 (1982).
[20] Q. Y. Tang and M. G. Feng, in "DPS Data Processing System: Experimental Design, Statistical Analysis and Data Mining", edited by D. P. Yan, Y. C. Zhao and R. Yang, Science Press, 2007, pp. 59-142.
[21] S. Keshk, *Enzyme Microb. Technol*., **40**, 9 (2006).
[22] T. Naritomi, T. Kouda, H. Yano and F. Yoshinaga, *J. Ferment. Bioeng*., **85**, 598 (1998).
[23] M. Matsuoka, T. Tsuchida, K. Matsushita, O. Adachi and F. Yoshinaga, *Biosci. Biotechnol. Biochem*., **60**, 575 (1996).
[24] D. Mikkelsen, B. M. Flanagan, G. A. Dykes and M. J. Gidley, *J. Appl. Microbiol*., **107**, 576 (2009).
[25] M. Shoda and Y. Sugano, *Biotechnol. Bioprocess. Eng*., **10**, 1 (2005).
[26] S. Keshk and K. Sameshima, *Afr. J. Biotechnol*., **4**, 478 (2005).
[27] R. M. Brown Jr. and I. M. Saxena, *Plant Physiol. Biochem*., **38**, 57 (2000).