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RAPID COMMUNICATION

Improvement of bacterial cellulose production in *Acetobacter xylinum* using byproduct produced by *Gluconacetobacter hansenii*

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Abstract–A single sugar α-linked glucuronic acid based oligosaccharide (SSGO) is water soluble oligosaccharides (WSOS) obtained by *Gluconacetobacter hansenii* PJK (KCTC 10505BP) as a byproduct during bacterial cellulose (BC) production. In this study, SSGO was used for the improvement of BC production by the vinegar bacterium, *Acetobacter xylinum*, which produces heteropolysaccharides as a byproduct. The addition of 1.0% SSGO to the chemically defined medium (CDM) resulted in an 89.3% increase in BC production by *A. xylinum* after 15 days of cultivation under static condition, and a 52.3% increase in BC production by *G. hansenii*. Both the dry cell weight and live cell density of *A. xylinum* increased 50% with the addition of 1.0% SSGO. SSGO successfully improved BC production by *A. xylinum*.

Key words: Bacterial Cellulose, *Acetobacter xylinum*, *Gluconacetobacter hansenii*, Water Soluble Oligosaccharides, Characterization

INTRODUCTION

Cellulose produced by some kinds of microbial cells such as *Acetobacter*, *Agrobacteria*, *Rhizobia*, and *Sarcina* strains is free from lignin and hemicelluloses [1]. The degree of polymerization (DP) and fiber thickness of bacterial cellulose (BC) are 2,000-6,000 and 0.1 μm, respectively [2,3]. Its high water holding capacity, high mechanical strength, elasticity, high crystallinity, *etc*. have increased its demand in various fields [4,5].

Most BC producing vinegar bacteria produce water soluble oligosaccharides (WSOS). The vinegar bacterium was able to synthesize different kinds of WSOS depending on the carbon source and the bacterial strain [1,7]. The *G. hansenii* strain produces a water soluble homopolymer, single sugar α-linked glucuronic acid based oligosaccharide (SSGO) composed of glucuronic acid and the typical chemical structure of SSGO can be found in the previous literature [6]. The WSOS produced by *A. xylinum* were composed of glucose, mannose, rhamnose, and glucuronic acid at a molar ratio 3 : 1 : 1 : 1 when the main carbon source was glucose [8] and at a molar ratio 4 : 1 : 1 : 1 when the carbon source was fructose [9].

The amount of heteropolysaccharide produced by *A. xylinum* increased as BC production decreased in an air lift reactor [10]. The proposed BC biosynthesis pathways show that some glucose is transformed into WSOS [11], and Chao et al. [12] suggested that byproduct formation should be minimized in order to increase BC production. In a recent study of BC production by *G. hansenii* [13], SSGO addition to the chemically defined medium resulted in an increase in BC production by inhibiting the synthesis of the byproduct, SSGO itself.

The improvement in BC production is necessary due to the increased use and potential applications of BC. It is necessary to develop a simple and reliable method in order to improve BC production by general vinegar bacteria. In this study, we investigated whether SSGO could improve BC production by *A. xylinum* whose byproduct is a heteropolysaccharide that is very different from SSGO. The effect of SSGO addition to the culture medium on the cell density and live cell population of *A. xylinum* was also investigated.

EXPERIMENTAL

G. hansenii PJK (KCTC 10505BP) and *A. xylinum* (ATCC 23769) were grown on a basal medium composed of glucose (10 g), yeast extract (10 g), peptone (7 g), acetic acid (1.5 mL), and succinate (0.2 g), dissolved in 1 L of distilled water. The agar plates used for keeping strains were prepared by dissolving agar (20 g/L) in the basal medium. The prepared basal medium was autoclaved for 15 min at 121 °C. Colonies of *G. hansenii* and *A. xylinum* were inoculated into 50 mL of medium in a 250 mL flask shaken at 150 rpm and cultured at 30 °C for 24 h.

From the basal medium, four types of media were prepared as described below:

Medium A: basal medium of pH 5.0; Medium B: basal medium of pH 5.0 with 1.0% SSGO; Medium C: basal medium of pH 3.5; Medium D: basal medium of pH 3.5 with 1.0% SSGO.

All the prepared media were autoclaved at 121 °C for 15 min and were fed with 0.5 mL (1.0%) of ethanol. Initially, the experiments were performed under shaking as well as under static conditions using *G. hansenii* and *A. xylinum* as culture strains and adding 0.50 g (1.0%) of SSGO into 50 mL of medium A. In the shaking culture, BC productions were performed by separately inoculating 5.0% of *G. hansenii* and 5.0% of *A. xylinum* culture broth into two sets of flasks at 150 rpm and 30 °C. All the media (A-D) mentioned above were employed for BC production. The harvest of BC and WSOS

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was done after 5, 10, and 15 days of cultivation. In the static culture, the same procedure was carried out. SSGO was produced by the method introduced by Khan et al. [14].

BC was produced in pellet form from the shaking culture and in plate form from the static culture. In the shaking culture, BC was harvested by centrifuging the culture broth for 20 min at 3,580 g, and washing with distilled water. The process of washing with distilled water and centrifugation was repeated twice. The dry weight of BC with microbial cells was measured after freeze-drying at −50 °C. Simultaneously, the BC containing cells were treated with 20 mL of 0.3 N NaOH and treated at 121 °C for 15 min using an autoclave in order to disrupt and dissolve the microbial cells [15]; afterwards, the solution was filtered through Whatman filter paper (pore size: 8 μm) with an aspirator to remove the dissolved materials. The filter cake was rinsed repeatedly with distilled water until the pH of the filtrate became neutral. The dry weight of BC without any microbial cells was measured after freeze-drying at −50 °C. The dry cell weight was taken to be the difference between the weights of the dried BC containing cells and the dried BC after treatment with NaOH [16]. In the static culture, BC was harvested by removing the BC plate from the culture flask and washing it with distilled water more than twice. The BC plates were also treated with 0.3 N NaOH solution for 15 min and were then rinsed with distilled water. The dry cell weight was measured using the freeze-drying method as for the shaking culture.

For the measurement of colony forming units (CFU), the culture broth was diluted with saline solution and spread onto agar plates containing the basal medium. The plates were incubated at 30 °C until colonies formed on the agar plate. Thereafter, the CFU were counted [17].

RESULTS AND DISCUSSION

1. Effect of SSGO on BC Production under Different Culture Conditions

It is well known that BC production is very sensitive to pH, [18, 19]. Kouda et al. reported that BC can be produced within the pH range from 3.0 to 7.0 [20]. Tantratian et al. [21] reported that *Acetobacter* species produce maximal BC yields at pH values between 3.5 and 6. Hwang et al. reported that the optimal pH for the metabolism of glucose to gluconic acid was 4, but a pH of about 5 is preferable for cell growth and cellulose production by *A. xylinum* [18]. For maximal production of BC by *G. hansenii* pH 5 for the medium is preferable, and pH 6 is preferable for the maximal production of SSGO by *G hansenii* [14,22]. In the present study, the initial pH for BC production with 1.0% SSGO addition for *G. hansenii* and *A. xylinum* was adjusted to 3.5 and 5.0, respectively. Fig. 1 indicates that for *G hansenii*, higher BC production was obtained at pH 5.0 in both the static and shaking cultures. The effect of SSGO addition on the improvement of BC production was better at pH 3.5 than pH 5 in both the static and shaking cultures. In the shaking culture using *G. hansenii*, BC production increased 1.17 and 1.47 times with SSGO addition at pH 5.0 and 3.5, respectively, after 15 days of cultivation (Fig. 1(c)). Similarly, in the static culture the production of BC increased 1.52 and 4.68 times with SSGO addition at pH 5.0 and 3.5, respectively, after 15 days of cultivation (Fig. 1(d)).

The same study was done with *A. xylinum*; this study indicated that higher BC production with addition of SSGO was obtained at pH 5.0 and 3.5 in shaking and static cultures, respectively. The effect of SSGO addition on the improvement of BC production was better

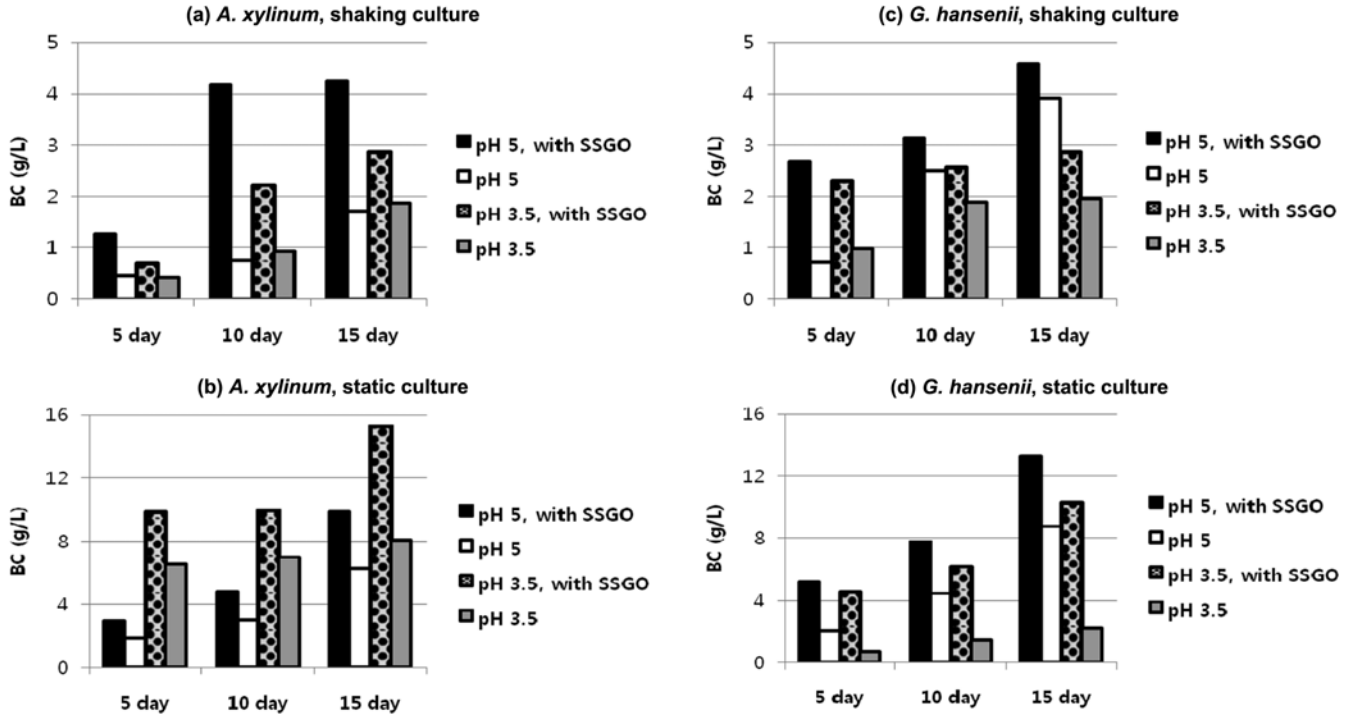

Fig. 1. Time course of BC production in the medium with and without the 1% SSGO addition at pH 5 and pH 3.5 by *Acetobacter xylinum* in shaking culture (a) and static culture (b) and also by *Ghensenii* PJK in shaking culture (c) and static culture (d).

Table 1. BC was produced by *G. hansenii* PJK. BC production was done at 30 °C in basal medium with and without 1.0% SSGO, in shaking and static cultures, at pH 5.0 and 3.5. CFU, dry cell weight were measured at the time interval of 5 days during BC production

	Culture time (day)	*G. hansenii* PJK							
		Shaking culture				Static culture			
		Initial pH 5.0		Initial pH 3.5		Initial pH 5.0		Initial pH 3.5	
		With SSGO	Without SSGO	With SSGO	Without SSGO	With SSGO	Without SSGO	With SSGO	Without SSGO
BC (g/L)	5	2.68	0.72	2.31	0.99	5.15	2.03	4.58	0.68
	10	3.14	2.50	2.57	1.89	7.77	4.44	6.17	1.45
	15	4.58	3.92	2.88	1.96	13.31	8.74	10.3	2.20
CFU	5	4.8×10^5	2.6×10^5	8.0×10^5	9.2×10^5	2.5×10^5	1.3×10^5	2.5×10^5	1.0×10^5
	10	3.0×10^3	2.0×10^3	6.0×10^3	1.0×10^3	1.2×10^5	7.4×10^4	3.7×10^4	3.5×10^4
	15	0	2.0×10^3	0	0	0	0	8.0×10^3	0
Dry cell weight (g/L)	5	1.25	0.21	0.75	0.83	1.94	1.01	1.53	0.68
	10	1.91	0.59	1.47	1.06	3.25	2.00	2.06	1.45
	15	3.25	0.80	2.39	1.31	3.43	2.22	4.75	2.25

at pH 5 in the shaking culture and pH 3.5 in the static culture. BC production with SSGO addition increased 2.48 and 1.54 times at pH 5.0 and 3.5, respectively, after 15 days of shaking culture (Fig. 1(a)). However, the increase in the static culture was 1.57 and 1.89 times, respectively (Fig. 1(b)).

Table 1 also indicates that in the case of *G hansenii*, the maximum productivity of BC with SSGO addition was achieved after 5 days of cultivation in both the static and shaking cultures. However, for *A. xylinum* the maximum productivity in the shaking culture was obtained after 10 days for both pH ranges. In the static culture, the maximum productivity was achieved after 15 days at pH 5.0 and after 5 days at pH 3.5.

The comparative analysis of the BC results obtained with two different species indicated that under both the shaking and static conditions over two different pH ranges the SSGO showed a positive effect on the increase in BC production and productivity. Moreover, SSGO improved BC production at an earlier stage of BC production by *G hansenii* and a little more time is required for the unique effect of SSGO addition on BC production by *A. xylinum*. This may be due to the fact that SSGO is the byproduct produced by *G hansenii* and is different in the structure from the byproduct produced by *A. xylinum*. The structure of the repeat units of the byproduct produced by *A. xylinum* can be found in the literature [23]. In other words, glucuronyl transferase catalyzes the addition of glucuronic acid to an intermediate of SSGO. However, glucuronyl transferase catalyzes the selective and competitive addition of glucuronic acid to an intermediate of the byproduct produced by *A. xylinum* because it is composed of glucose, mannose, rhamnose, and glucuronic acid. There should be more investigation in the future into why the effect of SSGO addition on BC production varied with culture mode and cell strains.

2. Dry Cell Weight and CFU

The addition of SSGO caused an increase in dry cell density. In the case of *G hansenii*, a higher dry cell density was observed at

Table 2. BC was produced by *A. xylinum*. BC production was done at 30 °C in basal medium with and without 1.0% SSGO, in shaking and static cultures, at pH 5.0 and 3.5. CFU, dry cell weight were measured at the time interval of 5 days during BC production

	Culture time (day)	*A. xylinum*							
		Shaking culture				Static culture			
		Initial pH 5.0		Initial pH 3.5		Initial pH 5.0		Initial pH 3.5	
		With SSGO	Without SSGO	With SSGO	Without SSGO	With SSGO	Without SSGO	With SSGO	Without SSGO
BC (g/L)	5	1.28	0.46	0.72	0.42	2.98	1.87	9.86	6.56
	10	4.17	0.76	2.23	0.94	4.8	2.99	10	7
	15	4.25	1.71	2.88	1.87	9.86	6.28	15.28	8.07
CFU	5	2.8×10^5	1.2×10^5	1.3×10^5	1.0×10^5	1.7×10^6	4.3×10^5	1.5×10^4	9.9×10^4
	10	4.7×10^4	1.1×10^4	3.4×10^4	1.3×10^4	2.9×10^5	1.1×10^4	2.1×10^3	7.5×10^4
	15	8.6×10^3	1.6×10^3	8.6×10^3	4.5×10^2	1.8×10^3	1.6×10^2	5.1×10^2	3.4×10^2
Dry cell weight (g/L)	5	1.57	0.37	1.5	0.95	1.49	1.87	2.49	2.33
	10	2.07	1.35	2.27	1.32	2.99	2.40	3.06	2.7
	15	3.37	2.12	3.21	2.08	3.29	3.14	4.93	3.28

pH 5.0 in both the shaking and static cultures. With SSGO addition to the shaking culture, the dry cell density increased 4.06 times at pH 5.0 after 15 days of cultivation. Similarly, in the static culture, the dry cell density increased 1.55 times at pH 5.0.

For *A. xylinum*, a higher dry cell density was found at pH 5.0 in the shaking culture and 3.5 in the static culture, respectively. For 15 days of cultivation in the shaking culture, the dry cell density increased 1.59 times at pH 5.0 with SSGO. In the static culture, the dry cell density increased 1.50 times at pH 3.5 with SSGO. SSGO addition caused an increase in cell growth although the effect varies with pH for *G. hansenii* and *A. xylinum*.

Addition of SSGO to the medium also affected the population of live cells, CFU values. As shown in Table 1, SSGO caused an increase in the CFU of *G. hansenii* for both the shaking and static cultures. The increase in cell mass and live cell population with SSGO addition can be partially explained by the report that the *G. hansenii* strain could grow and produce BC in the medium containing SSGO instead of glucose [13]. For *G. hansenii* the values of the CFU in the shaking and static conditions were higher at the earlier stages of cultivation and then decreased with cultivation time. The CFU became almost absent at 15 days of cultivation. The effect of SSGO addition on the cell growth of the *G. hansenii* strain was found at an earlier stage of cultivation because SSGO addition to the medium inhibited the enzyme that produces SSGO during BC production by *G. hansenii* [13]. Similarly, in the case of *A. xylinum* almost the same trend was observed except that CFU were found even at 15 days of cultivation. Nevertheless, for *A. xylinum*, the positive effect of SSGO addition on the cell growth, cell density and BC production was remarkable, although it seemed to be induced at a later stage of cultivation and required a longer culture time.

CONCLUSION

The role of SSGO as an inhibitor for byproduct production during BC production by *G. hansenii* might be applied to BC production by *A. xylinum* and thus resulted in an increase in BC production by *A. xylinum*. However, the optimum pH for the increase in BC production with SSGO addition varied with strain, and the effect of SSGO addition on BC production might require more time for some other species. The effect of SSGO addition seems to be enormous for the vinegar strains, but more study should be done for more strains in order to make it a firm and general phenomenon.

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