Recent Advances in Bacterial Cellulose Production

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Abstract Bacterial cellulose (BC), which is produced by some bacteria, has unique structural, functional, physical and chemical properties. Thus, the mass production of BC for industrial application has recently attracted considerable attention. To enhance BC production, two aspects have been considered, namely, the engineering and genetic viewpoints. The former includes the reactor design, nutrient selection, process control and optimization; and the latter the cloning of the BC synthesis gene, and the genetic modification of the speculated genes for higher BC production. In this review, recent advances in BC production from the two viewpoints mentioned above are described, mainly using the bacterium *Gluconacetobacter xylinus*.

Keywords: bacterial cellulose, *Gluconacetobacter xylinus*

Many species of bacteria, such as those in the genera *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Rhizobium, Sarcina, Salmonella* and *Escherichia,* have been reported to produce solid extracellular cellulose [1-3]. Cellulose produced by *Gluconacetobacter* is called bacterial cellulose (BC). The molecular formula of BC $(C_6H_{10}O_5)_n$, having a β-1,4 linkage between two glucose molecules, is the same as that of plant cellulose, but their physical and chemical features are different [4]. One of the differences lies in the highly order structure of these celluloses. For plant cellulose, a number of cellulose molecular chains accumulate, forming microfibrils, and subsequently bundles and cluster of high-order structures called fibril lamella and fiber cells. Plant cellulose usually exists in the cell wall, forming a complex structure with hemicellulose, lignin and other impurities. Conversely, the BC produced by *G. xylinus* is secreted in the form of a ribbon, composed of bundles of microfibrils [2]. Such a ribbon is very thin, with a width of only one-hundredth that of plant cellulose. Ribbon cellulose further grows as a visible reticular structure, forming a regular structure, unlike plant cellulose. BC exhibits the following unique properties:

(i) High purity, as neither hemicellulose nor lignin in plant cellulose is present.
(ii) High crystallinity.
(iii) BC-sheets form, high Young's modulus of 15~30 GPa, the highest of all the two-dimensional organic materials.
(iv) Excellent biodegradability.
(v) Large water holding capacity, upto one hundred times its weight.
(vi) Excellent biological affinity.

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With the above characteristics, BC is expected to have applications as an alternative of plant cellulose or as new biodegradable materials available in food and chemical industries and in medical field [5-7]. However, BC is still expensive compared with other popular commercial organic products. Therefore, it is important to develop economical BC production methods to eliminate such a disadvantage and for the promotion of BC use. In this review, the focus will be on recent research related to the improvement of BC production.

Biochemistry of Bacterial Cellulose Produced by *G. xylinus*

Species in the genus *Gluconacetobacter*, particularly *G. xylinus*, is the most extensively characterized microorganism with respect to cellulose synthesis. Initially, *G. xylinus* has been used as a model to clarify the mechanism of cellulose synthesis in plants [3,8,9]. During basic research on BC production by this bacterium, a BC biosynthesis system, composed of *bcs* or *acs* and *cdg* operons, which play important roles in cellulose synthesis and its activation, respectively, has been proposed [10-13]. The *bcs* operon is constructed of *bcsA, bcsB, bcsC* and *bcsD*. BcsA is thought to be responsible for the polymerization of uridine diphosphate glucose (UDP-Glc) (Fig. 1); BcsB a binding subunit of cyclic di-guanosine monophosphate (c-di-GMP), which is an activator of cellulose synthesis [14] and BcsC and BcsD are speculated to be located on the outer membrane, playing roles in the crystallization and/or extrusion of cellulose. The *acs* operon is composed of *acsAB, acsC* and *acsD,* and their translation products are considered to be similar to those of the *bcs* operon. Thus far, three types of *cdg* operon have been found, and one, the *cdg1* operon, is composed of *pdeA1* and *dgc1*, with two functional unknown open reading

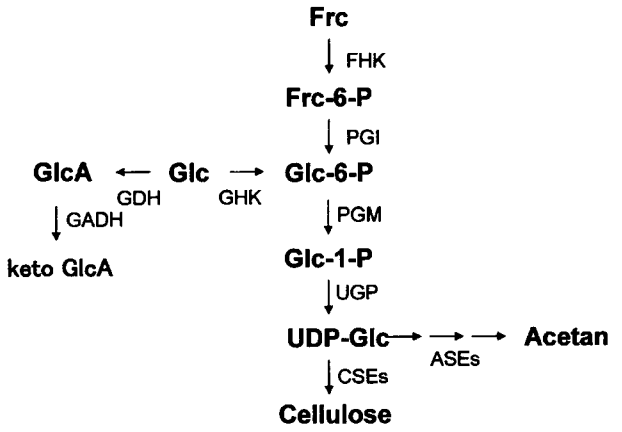

Fig. 1. Simplified scheme of BC and acetan production using glucose or fructose as the carbon source. FHK, fructose hexokinase; GHK, glucose hexokinase; GDH, glucose dehydrogenase; GADH, gluconate dehydorogenase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; UGP, UDP-glucose pyrophosphorylase; CSEs, cellulose synthesis enzymes; ASEs, acetan synthesis enzymes; Frc, fructose; Glc, glucose; GlcA, gluconate; keto GlcA, keto-gluconate; Frc-6-P, fructose-6-phosphate; Glc-6-P, glucose-6-phosphate; Glc-1-P, glucose-1-phosphate; UDP-Glc, uridine diphosphate glucose.

frames. PdeA1 is phosphodiesterase A, and degrades c-di-GMP, and Dgc1 is a di-guanilate cyclase that synthesizes c-di-GMP. Aside from these operons, there are several genes for BC synthesis, such as CMCase, β-glucosidase and ORF2 [15-17]. However, the entire BC synthesis gene remains to be scientifically confirmed, and thus no transformation of the gene into other bacteria has been successful.

The model of BC secretion to the outside of the cell has also been proposed as a model comprising a row of terminal complexes (TC) on the cell surface [18,19]. Recently, BcsB has been elucidated as part of the TC, using the sodium dodecyl sulfate freeze fracture labeling technique, under transmission electron microscopy [20]. Very few genetic studies of BC production were carried out before the mid 1990's; however, several trials to increase BC production have subsequently been performed by genetic modification as well as by improving culture conditions and strains.

Screening of Optimum Strain for BC Production

BC-producing bacteria are well-known to form cellulose pellicles on the surface of the medium in static cultures. Therefore, their BC production depends not on the volume, but on the surface area of the culture. This means that the wider the surface area, the higher the BC production, which is unsuitable for large-scale cultures. Conversely, in shaking flask cultures, BC production by BC-producing bacteria is restricted mainly by an insufficient oxygen supply and the suspension of irregular shaped BC, consisting of cells and cellulose. Another problem is that the non-BC-producing mutants often appear spontaneously in agitated culture of bacteria, particularly *Gluconacetobacter* strains [2]. In case of *G. hansenii*, the addition of ethanol to a medium was effective to repress the spontaneous mutation [21,22]. However, the productivity was still not satisfactory. Therefore, to raise BC production for large-scale production, the selection of stable BC-producing strains in agitated culture is indispensable. Toyosaki *et al.* extensively screened for BC-producing *Gluconacetobacter* strains suitable for agitated culture [23], and *G. xylinus* BPR2001 was selected as the most suitable strain for agitated culture from 2096 strains. Extensive studies from biochemical and engineering viewpoints have been accelerated by the use of this strain.

BC Production Using Shaking Flasks or Stirred-tank Reactors

In a shaking flask culture, BC accumulation in the culture broth causes an increase in the viscosity and a decrease in the homogeneity of the culture. This results in a reduced oxygen level, which affects BC production as well as cell growth. However, in an agitated culture, using a stirred-tank reactor, the vigorous mechanical agitation by an impeller increases the homogeneity of the broth. Kouda *et al.* investigated the mixing properties of BC cultures in an aerated and agitated vessel, introducing a power law model of a non-Newtonian BC suspension [24]. They further investigated the effect of the agitator configuration on BC production and concluded that impellers, such as Maxblend and gates with turbines, were suitable for BC production [25].

Hwang *et al.* studied the effects of pH and dissolved oxygen (DO) on BC production using a stirred-tank reactor [26]. When the pH was shifted to within the optimum range during culturing, the BC production increased and the culture time was shortened. When the level of DO was controlled at 10% saturation using pure oxygen in a DO stat and glucose was supplied in a fed-batch, the maximum BC yield (15.2 g/L) was obtained.

The production rate and yield of BC were dependent on the oxygen transfer coefficient, k_La. The oxygen consumption rate and the productivity in a continuous culture are generally better than those in batch cultures. In a continuous culture in an ethanol-supplemented medium [27], the BC production rate and yield were maintained at 0.95 $gL^{-1}h^{-1}$ and 46%, respectively. However, after 92 h, the enhanced adhesion of culture broth to the inside wall and the upper part of the reactor, decreased the cell concentration and BC production rate and yield. Ethanol addition is often effective at increasing the BC concentration, as ethanol is metabolized in the TCA cycle, thus increasing ATP production by the genus *Gluconacetobacter*. It is speculated that increased ATP production inhibits glucose-6-phosphate dehydrogenase (G6PD), resulting in a decrease in the amount of fructose flowing into the pentose phosphate pathway for energy production and assimilation, with an eventual increase in BC produc-

tion. Son *et al.* also reported that ethanol addition to the culture medium raised BC production in shaking flasks [28].

Improvement in media compositions is important in reducing the cost of BC production. Initially, Schramm and Hestrin medium (SH) was used for BC study [29], but it was unsuitable for commercial BC production, due to its high price, owing to the use of yeast extract as the nitrogen source. Alternatively, Toyosaki *et al.* used corn steep liquor (CSL) as the nitrogen source for shaken flask cultures [23], and obtained a high BC production in a CSL-fructose medium (CSL-Fru). In contrast, there was no difference in the BC productivity in static cultures between CSL-Fru and SH media. This result suggests that CSL-Fru contains factors for enhancing BC production in shaken cultures. Since then, CSL-Fru has been used as a basal medium by many researchers for BC production.

Fig. 1 shows a scheme for simplified BC and acetan syntheses from glucose and fructose, which are mainly used as the carbon sources for basic research. In practical BC production, purified sugar cannot be used. In the fermentation industry, molasses is the most popular carbon source used. Recently, a high BC production, at a low production cost, was achieved using molasses and CSL as the carbon and nitrogen sources, respectively [30]. The cost of producing one gram of BC using medium containing molasses and CSL was one-hundredth of that using conventional CSL-Fru medium. However, the high molasses concentration required at the start of culturing was inhibitory to BC production. Therefore, pre-treatment of molasses, by heat and acid, followed by a fed-batch supply of molasses were better than in batch cultures for avoiding the inhibitory effect of molasses. Particularly, in intermittent fed-batch cultures, the BC concentration was significantly higher than that in batch cultures [31].

Optimization in Batch Culture

BC productivity depends on the culture conditions, including the cultivation method, carbon and nitrogen sources, pH, temperature and dissolved oxygen (DO). To optimize BC production, the conventional method of medium optimization, one-factor-at-a-time, is time-consuming and expensive, often leading to misinterpretation of results when interactions between different components are present. Statistical experimental designs minimize the error in determining the effect of parameters, and allow simultaneous, systematic and efficient variation of all parameters [32]. These statistical experimental designs can be adopted for various optimization processes, such as comparative studies, screening experiments or determining optimal conditions. Response surface methodology (RSM) is commonly used to determine the optimal conditions, which is an efficient statistical technique for optimizing multiple variables with a minimum number of experiments [33,34]. Embuscado *et al.* selected four factors, namely the fructose and sucrose concentrations, pH and temperature, and obtained a predicted BC yield to consumed sugars of 13.2 g/L by RSM, which was close to the experimental yield, 12.7 g/L, in flask experiments [35]. The simple second-order polynomial model function was applied to the determination of the optimal medium composition using a multivariable linear regression analysis, with a maximum BC yield to consumed fructose of 5 g/L obtained in flask experiments [36].

Culture conditions in a stirred-tank reactor for BC production by *A. xylinum* BPR2001 were optimized by statistical analysis using the Box-Behnken design [37,38]. In BC production by *A. xylinum* BPR2001, the optimal temperature and pH ranges have previously been determined [25,39]. Fructose and corn steep liquor (CSL) were the best carbon and nitrogen sources, respectively, among the tested materials for BC production [24]. The addition of water-soluble polysaccharides, like acetan and agar, enhanced BC productivity [40-42]. Based on these previously obtained data, RSM was used to predict the levels of the four factors, fructose, corn steep liquor (CSL), dissolved oxygen (DO) and agar concentrations. A total of 27 experimental runs, with combinations of each factor, were carried out in a 10-L stirred-tank reactor, and a three-dimensional response surface generated to determine the effect of the factors and the optimum concentrations of each factor for maximum BC production. The predicted results showed an optimum BC production of 14.3 g/L under the condition of 4.99% fructose, 2.85% CSL, 28.3% DO and 0.38% agar concentrations. Under the optimized culture conditions, improvement in BC production, 14.1 g/L, was experimentally confirmed [38].

BC Production Using an Airlift Reactor

When a high BC concentration is achieved with a high density of *G. xylinus*, the suspension of fibrous BC becomes a highly viscous non-Newtonian fluid. This causes difficulty in mixing the culture and limits oxygen transfer [24]. Therefore, a higher agitation power is necessary, which increases energy consumption. Another type of reactor for the consideration of low power consumption is that of an airlift reactor, which is mainly used for microbial wastewater treatment. This reactor has features of a simple mechanical structure and low energy consumption compared with stirred-tank reactors [43,44]. Chao *et al.* intensively investigated BC production using a 50-L internal-loop airlift reactor [40,45-47]. The reactor was equipped with a draft tube (134 mm inner diameter × 800 mm height), with a diameter ratio of the downcomer to the riser of 0.58. Air or oxygen-fortified air was supplied through the riser from gas spargers set at the bottom of the reactor, and the medium circulated by only the air pressure. These studies were performed from the following viewpoints:

(i) BC production by supplying oxygen-fortified air

When air was supplied to the reactor, the final BC concentration was only 3.8 g/L after 67 h with an initial 40

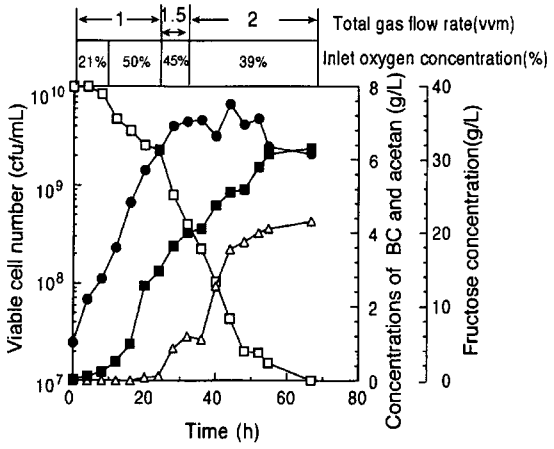

Fig. 2. Time courses of typical BC production by *G. xylinus* when oxygen-fortified gas was supplied to a batch cultivation in a 50-L internal-loop airlift reactor. Symbols: closed circles, cell number; open squares, fructose concentration; closed squares, BC concentration; open triangles, soluble polysaccharide, acetan concentration.

g/L fructose concentration. When oxygen-fortified air was supplied, the concentration was enhanced to 8 g/L after 67 h. The BC production rate was 0.116 $gL^{-1}h^{-1}$, which was two times higher than that of the air-supplied culture, and the BC yield was 25%. This suggests that dissolved oxygen (DO) is limited when only air is supplied, mainly due to the mild mixing in an airlift reactor. A typical pattern of BC production with oxygen-fortified air supply is shown in Fig. 2 [48]. After 67 h, the fructose had been completely consumed. This suggests that the fructose concentration of the medium can be increased for higher BC production. Therefore, the effect of the fructose concentration in the medium on BC production was investigated [47]. When the initial fructose concentration was increased to 70 g/L, the BC yield and production rate were enhanced to 35% and 0.22 $gL^{-1}h^{-1}$, respectively. The enhanced BC production was reflected as a decrease in the production of CO_2 and other unknown substances. However, no further increase in BC production was observed when the fructose concentration was increased still further, mainly because the culture broth in the reactor became stagnant and the flow practically nonexistent after the BC concentration exceeded 10 g/L. The BC productivities in the airlift reactor were almost equivalent to those in the stirred-tank reactors. Therefore, an airlift reactor can maintain a BC production similar to that in a stirred-tank reactor when supplied with oxygen-fortified air.

(ii) Effects of addition of water-soluble polysaccharides on BC production

BC production in a reactor is complex, mainly because the culture used consists of solid BC and cells, liquid medium and air gas. The heterogeneous coagulation of BC forms a large clump of BC on the surface, with adhered cells and the subsequent deterioration of BC production. Therefore, a homogeneous and small BC suspension is essential for sufficient agitation and oxygen transfer for enhanced BC production. The BC produced by the airlift reactor formed unique ellipse pellets (BC pellets), which were also much larger and different from the fibrous BC form produced in the stirred-tank reactor. However, large pellets are not advantageous for the fluidity of a culture broth due to the weak agitation power of an airlift reactor compared with that of a stirred-tank reactor. Therefore, to minimize the pellet size, several viscous water-soluble polysaccharides were supplemented to the medium. The BC pellets then became smaller in medium supplemented with agar or acetan [40]. Since smaller pellets are advantageous in the transfer of nutrients and oxygen to bacterial cells located inside and on the surface of the cellulose matrix, faster growth rates of BPR2001 would be expected. This effect was also confirmed by the addition of polyacrylamide-co-acrylic acid, which is not polysaccharide, but a viscous synthetic polymer [49]. Even in the stirred-rank reactor, with the addition of 0.4% agar to the medium, the BC production was increased by 50%, suggesting that the role of water-soluble polysaccharides in BC production is universal to all reactors [41].

(iii) Buffering capacity for BC production

The optimum pH of the culture medium for BC production was in the range 4.5 to 5.5, but variation occurs because of the accumulation of secondary metabolites due to the consumption of sugars or nitrogen sources. Therefore, it is important to control the pH within the optimal range. It is actually difficult to maintain an optimal pH by auto regulation using a pH sensor as viscous BC often attaches to the sensor, causing inaccurate pH readings. Even in a stirred-tank reactor, a high BC concentration causes inhomogeneous mixing, which results in the adsorption of BC onto the pH sensor. Noro *et al.* proposed one promising method to solve this problem, which uses the buffering capacity of CSL to maintain the pH, as CSL contains various buffering substances [39]. The value, β, shown in Eq. (1) was introduced to evaluate the buffering capacity of CSL when added to the medium. When a CSL-Fru medium with a buffering capacity was used, high BC production was achieved and the pH maintained within the optimal range, without any pH control using pH sensors.

$$\beta = d[\mathrm{OH}]/d\mathrm{pH} \quad (1)$$

Where β is the buffering capacity, dOH] the change in the molarity of the CSL-Fru medium on the addition of 0.2 M NaOH or 0.1 M H_2SO_4 and dpH the change in pH.

(iv) Comparison of energy consumption between an airlift reactor and a stirred-tank reactor.

The energy consumption was compared between the airlift reactor supplied with both 0.1% agar and oxygen-fortified air and a stirred-tank reactor. The same CSL-Fru medium and the strain *A. xylinum* BPR2001 were

used in the two reactors. In the airlift reactor, the total energy was estimated as 43 kW, with energy efficiency for 1 g/L BC production of 0.11 kW/h. Conversely, the energy efficiency for 1 g/L BC production in the cultivation of the same strain in the same medium in a stirred-tank reactor was 0.66 kW/h. This indicates that only approximately one-sixth the energy used for a stirred-tank reactor is needed for an airlift reactor [40,46].

BC Production Using Rotating Disk Reactor

In stirred-tank or airlift reactors, the adhesion of BC culture broth to the inside wall and the upper part of the apparatus causes problems. However, the use of a rotating disk reactor solves this problem. The rotating disk reactor is designed such that the half surface of its disks is submerged in the medium broth with the other half exposed to the atmosphere. As the disks rotate continuously, part of the surface of the disks is alternately located between the medium area and the atmosphere [50]. When this reactor is used in BC production, cells stuck to the disk surface take nutrients when they are immersed in the medium and exposed to O_2 in the atmosphere. Thus, the produced BC also sticks to the disks. Krystynowicz *et al.* investigated the optimum condition for BC production by changing the medium volume, rotation speed and number of disks, using a similar apparatus [51]. The BC production was maximized when the rotation speed and the ratio of surface area to medium volume (S/V) were 4 rotations per minute (rpm) and 0.71, respectively.

Genetic Modification for BC Production

As yet, no total BC synthesis operons have been cloned or no cells transformed with the BC synthesis gene produced BC [3,52]. In this sense, genetic modification other than the direct self amplification of BC genes has been conducted in relation to BC production.

In *G. xylinus*, when glucose or sucrose is used as the carbon source, the main product is not cellulose, but keto-gluconate, as shown in Fig. 1, which decreases the pH, cell growth and BC production. Therefore, the first attempt to improve BC productivity genetically was aimed at generating a mutant with restricted keto-gluconate synthesis [53]. Using UV mutagenesis, a non-keto-gluconate-producing mutant was derived from a parent strain, and its BC production increased from 1.8 g/L for the parent strain to 3.3 g/L after 10 days of shaking culturing.

One of the problems raised regarding BC production is that the decreased fluidity of the culture broth adversely affects BC production. *G. xylinus* secretes not only water-insoluble cellulose, but acetan into the culture broth also, which is a viscous water-soluble polysaccharide. Acetan production is in parallel to BC production. Acetan consumes uridine diphosphate glucose (UDP-Glc) for self-synthesis, which is also the starting material for cellulose synthesis, as shown in Fig. 1. If acetan is not synthesized, the amount of UDP-Glc used for BC synthesis is expected to increase, resulting in an increase in BC production. Based on this hypothesis, Ishida *et al.* generated the acetan-non-producing mutant strain, EP1, from the parental strain *G. xylinus* BPR2001 [54]. However, against expectation, the BC production by EP1 decreased in the shaken flask culture, although the productivity was maintained at the same level as that of the parent strain BPR2001 in a static culture. The culture broth of EP1 became a heterogeneous suspension, containing large flocks formed by the aggregation of cells and BC, compared with that of the parent strain in the shaken flask. This suggests that acetan raises the viscosity of the culture, preventing the coagulation of cells and BC, resulting in an increase in BC production. When EP1 was cultivated in the medium supplemented with acetan, its BC production recovered to the same level as that of the parent strain. When agar was added to the medium, the same effect as with acetan was shown toward BC production. Acetan and BC were produced by the parent strain almost simultaneously. Therefore, when agar was added from the start of cultivation, BC production was observed from the start of the experiments, and the cultivation time was reduced to two-thirds that without agar addition.

Another interesting result of the genetic modification was obtained from the *dgc1*-disrupted mutant, DD, generated from the parent strain BPR2001. The gene, *dgc1* is known to be an important gene for activating BC synthesis. Therefore, *dgc1* disruption is expected to decrease BC production. Unexpectedly, the BC production by DD was almost the same as that of the parent strain in static and shake flask cultivations. Moreover, when the mutant DD was cultivated in the stirred-tank reactor, the BC production increased by 36% compared with that of the parent strain [53]. However, Tal *et al.* reported that BC production decreased with *dgc1* disruption [13]. Al though these two results seem to be inconsistent, the cul tivation time used by Tal *et al.* was too short to evaluate the final BC production, as the growth rate of DD is slower than that of the parental strain. It is speculated that although *dgc1* was disrupted, BC production rather increased, because other genes, *dgc2* and *dgc3*, which are functionally similar to those of *dgc1*, worked complementarily or were more stimulatory for BC synthesis. Therefore, *dgc1* disruption is not critical for total BC production.

Perspective

Economical feasibility of BC is primarily dependent on BC productivity. Published research data on BC productions are summarized in Table 1. It is clear that culture using a stirred-tank or an airlift reactor is effective for large-scale BC production compared with traditional static culturing. The merit of the stirred-tank reactor is its ability to prevent the inhomogeneity of the culture broth by strong mechanical agitation, while the demerit is its high energy cost for generating the mechanical power. Conversely, the energy cost of an airlift reactor is one-

Table 1. Comparison of bacterial cellulose (BC) production under various culture conditions

Apparatus	Culture method	Carbon source	Supplement	BC production rate ($gL^{-1}h^{-1}$)	Culture time (h)	BC concentration (g/L)	References
Shaking flask	Batch	Glucose	Ethanol	0.08	8 (days)	15.2	[28]
Stirred-tank	Continuous	Fructose	Ethanol	0.95[1)]	-	-	[27]
reactor	Fed-batch	Glucose	Ethanol, oxygen	0.31	50	15.3	[26]
	Fed-batch	Molasses	No	0.11	72	7.8	[31]
	Batch	Fructose	No	0.074	72	5.3	[41]
	Batch	Fructose	Agar, oxygen	0.20	72	14.1	[41]
	Batch	Fructose	Agar	0.21	56	12	[41]
Airlift reactor	Batch	Fructose	Oxygen	0.20	52	10.4	[46]
	Batch	Fructose	Agar, oxygen	0.20	44	8.7	[40]
Rotating disk	Batch	Glucose	No	0.02	7 (days)	3.5	[51]

[1)] At the steady state when the dilution rate was 0.07 h^{-1}

sixth that of a stirred-tank reactor. However, the agitation power of an airlift reactor is limited, resulting in low fluidity of the culture broth, especially at high BC concentrations. To meet the different demands for BC production, namely high BC concentration at a decreased energy cost and a stable fluidity of the culture broth, the combined use of an airlift and a stirred-tank reactors, or continuous cultivation, may be one possible solution

Another approach to raise the BC production is to apply genetic modification to bacteria. *G. xylinus* has a long doubling time compare to most other bacteria, such as *Eshcerichia coli* and *Bacillus subtilis*. A faster growth rate will reduce the BC production time. Recently, Gram-negative *E. coli* and *Salmonella* have been reported to produce cellulose, although the amounts produced by these bacteria were significantly smaller than that from *G. xylinus* [56]. As their cellulose synthetic genes are very similar to those of *G. xylinus* [3,52], the expression mechanism of cellulose genes is expected to be similar to that of *G. xylinus*. Since their growth rates are relatively faster than that of *G. xylinus*, genetic modification of these bacteria will also be one possible means of increasing BC production.

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