Improved bacterial nanocellulose production from glucose without the loss of quality by evaluating thirteen agitator configurations at low speed

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Summary

Thirteen agitator configurations were investigated at low speed in stirred-tank reactors (STRs) to determine if improved crude bacterial nanocellulose (BNC) productivity can be achieved from glucose-based media while maintaining high BNC quality using *Komagataeibacter xylinus* ATCC 23770 as a model organism. A comparison of five single impellers showed the pitched blade (large) was the optimal impeller at 300 rpm. The BNC production was further increased by maintaining the pH at 5.0. Among the single helical ribbon and frame impellers and the combined impellers, the twin pitched blade provided the best results. The combined impellers at 150 rpm performed better than the single impellers, and after optimizing the agitation conditions, the twin pitched blade (large) and helical ribbon impellers performed the best at 100 rpm. The performances of different agitators at low speed during BNC production were related to how efficiently the agitators improved the oxygen mass transfer coefficient. The twin pitched blade (large) was verified as providing the optimum performance by an observed crude BNC production of 1.97 g $(L \times d)^{-1}$ and a BNC crude yield of consumed glucose of 0.41 g g^{-1}, which were 2.25 and 2.37 times higher than the initial values observed using the single impeller respectively. Further characterization indicated that the BNC obtained at 100 rpm from the STR equipped with the optimal agitator maintained high degree of polymerization and crystallinity.

Introduction

Bacterial nanocellulose (BNC) is a natural nanostructured biopolymer synthesized by many species of bacteria, including the widely studied *Komagataeibacter xylinus* (Ross *et al.*, 1991; Lin *et al.*, 2013; Lee *et al.*, 2014). BNC possesses many outstanding properties, such as a superfine diameter, a high degree of polymerization, and a high crystallinity, which endow BNC with great potential in the areas of food, biomaterial, textile, paper and advanced functional composites (Chawla *et al.*, 2009; Gatenholm and Klemm, 2010; Gama *et al.*, 2012; Lee *et al.*, 2014). However, the commercial application of BNC is heavily restricted due to its high price compared with other popular commercial bioproducts (Sani and Dahman, 2010). The high price of BNC is mainly attributed to the high cost of the carbon source, the low productivity and yield of BNC and the high labour intensity required for the traditional static production process.

Submerged cultivation, especially agitated cultivation, has been considered to be more suitable for the large scale production of BNC because of the typically higher mass transfer rate, better aeration and higher theoretical production rates, and this method is also less labour intensive (Toyosaki *et al.*, 1995; Yoshinaga *et al.*, 1997;

Huang *et al.*, 2014). In a stirred-tank reactor (STR), an agitator is the crucial structure that affects mixing, oxygen transfer and shear force, all of which greatly affect bacterial growth and bioconversion of sugar. However, little effort has been made to investigate the effect of agitators on BNC production (Kouda *et al.*, 1997; Jung *et al.*, 2007). Kouda *et al.* (1997) observed that the 'maxblend and gate with turbine' agitator was the most suitable after evaluating five agitator configurations for BNC production from fructose at extremely high agitation speeds (600–1200 rpm). This agitator performed best because the culture broth was well mixed, and a high oxygen mass transfer coefficient (K_{La}) was obtained.

An extra high agitation speed would damage the quality of BNC (for example, the degree of polymerization, DP) (Watanabe *et al.*, 1998; Chen *et al.*, 2017) and consume high amounts of energy, increasing the production cost. It is noteworthy that BNC is a unique material, and many applications greatly depend on its quality. In most cases if BNC is directly used as a functional material, as a raw material for regenerated cellulose material, or as an additive for enhancing composites, higher DP or crystallinity characteristics are preferred since BNC with a lower DP or crystallinity has reduced strength (Reiniati *et al.*, 2017a). To maintain the high quality of BNC obtained in traditional static culture systems, a low agitation speed would be favourable, but would result in relatively poor mixing and inefficient oxygen transfer. In addition to agitation speed, agitator configuration also significantly affects the oxygen transfer. Compared to high agitation speeds (such as 1200 rpm), the metabolism of *K. xylinus* in an STR with low agitation speed would be different. In this context, a new and comprehensive study on the comparison and selection of agitator configurations for use at low agitation speeds is highly needed.

In addition, most of the previous research on STRs have been conducted using fructose-based media. It is well-known that fructose is a very expensive sugar that is not as widely available as glucose. The high price and low availability of fructose make it difficult to be used in the industrial production of BNC at a large scale. By contrast, glucose is relatively inexpensive and widely available due to its many sources and common uses in industry, which would make it more easily used as a cost-effective carbon source to produce BNC at large scale. However, if glucose rather than fructose was used in an STR with a high agitation speed (600–1200 rpm) as suggested by Kouda *et al.* (1997), the microbial metabolism would be quite different from that observed using fructose, such as an easy consumption but with high gluconic acid productivity. Furthermore, because the formation of by-products of gluconic acid

would be greatly accelerated, low BNC productivity would be obtained (Tantratian *et al.*, 2005). As a result, the production of BNC from a glucose-based medium in an STR using a specific agitator would be very different from that obtained using fructose. Thus, a comprehensive comparison of STR agitator configurations and a study of the related conditions during BNC production from a glucose-based medium would also be required to achieve both high BNC productivity and high quality. For this reason, a suitable low agitation speed should be considered to ensure the high production and high quality of BNC. Importantly, the agitation speed should not be too low, since the extra low agitation speed would limit the oxygen transfer rate and decrease the BNC production. Speeds of 100–300 rpm have previously been reported to be appropriate for BNC production in agitated cultivation using glucose as carbon source (Tantratian *et al.*, 2005; Chen *et al.*, 2017; Singhsa *et al.*, 2018).

Several studies have described how the production of BNC is influenced by carbon sources (such as glucose and fructose) and by agitation conditions and the pH of the medium (Kouda *et al.*, 1997; Lee *et al.*, 2014; Reiniati *et al.*, 2017b). However, to the best of our knowledge, few studies have achieved high BNC productivity from glucose-based culture medium at low agitation speeds that did not come at the expense of the high quality. Thus, the selection of an appropriate combination of agitators at low speed is one of the most important issues for the large scale production of BNC. Importantly, enhancing the crude productivity and yield of BNC should not come at the cost of deteriorating high-quality BNC.

The current study addressed the main bottleneck in the production of high-quality BNC, namely obtaining an optimal oxygen transfer rate in a complex system where the bacterial cells demand high oxygen availability, and the final product is damaged if the agitation rate is too high. Thirteen agitator configurations in STRs at low speed were investigated to assess the possibility of achieving enhanced crude BNC productivity and yield from glucose while maintaining high BNC quality compared to a static culture system. The investigation began with a simple STR system, that is BNC production was evaluated with five single impellers at 300 rpm without pH control. Subsequently, the single impellers that performed well were combined and compared together with helical ribbon and frame impellers at the half agitation speed of 300 rpm (150 rpm). After optimizing the agitation speed, additional impellers (including helical ribbon, frame and five twin impellers) were investigated for the production of BNC at the optimal agitation speed of 100 rpm. Finally, the most suitable agitator was selected and re-examined for BNC production. The corresponding

BNC was characterized by scanning electron microscopy (SEM), viscometry and X-ray diffraction (XRD). Using a low agitation speed, high BNC productivity is difficult to achieve due to a relatively low oxygen transfer and poor mixing, but high BNC quality can be attained. The current work is significantly different from previous works in that it was the first to investigate the optimization of BNC productivity while maintaining high BNC quality; the use of low agitation speed to maintain the high BNC quality using an optimized agitator configuration to obtain good oxygen transfer and nutrient mixing; the use of glucose, not fructose, as a cost-effective carbon source; the use of a different bacterial strain, *K. xylinus* ATCC 23770; and the performance of a comprehensive comparison of multiple agitator configurations.

Results and discussion

Effect of single impeller types on BNC production

In the first series of experiments, five single impellers (group I in Scheme 1) were studied with respect to BNC production, and no pH control was applied as described in a previous study (Kouda *et al.*, 1997). In all the cultures, the glucose concentration slowly decreased during the first day (Fig. 1A), which corresponded to the lag growth phase. During the next 3 days, the glucose was quickly consumed, while during the last 2 days the glucose concentration changed slowly or became stable. The dissolved oxygen (DO) content decreased the fastest in the STR with a pitched blade (small) and with a propeller, while the DO content decreased the slowest in the STR with a pitched blade (large) (Fig. 1B). It should
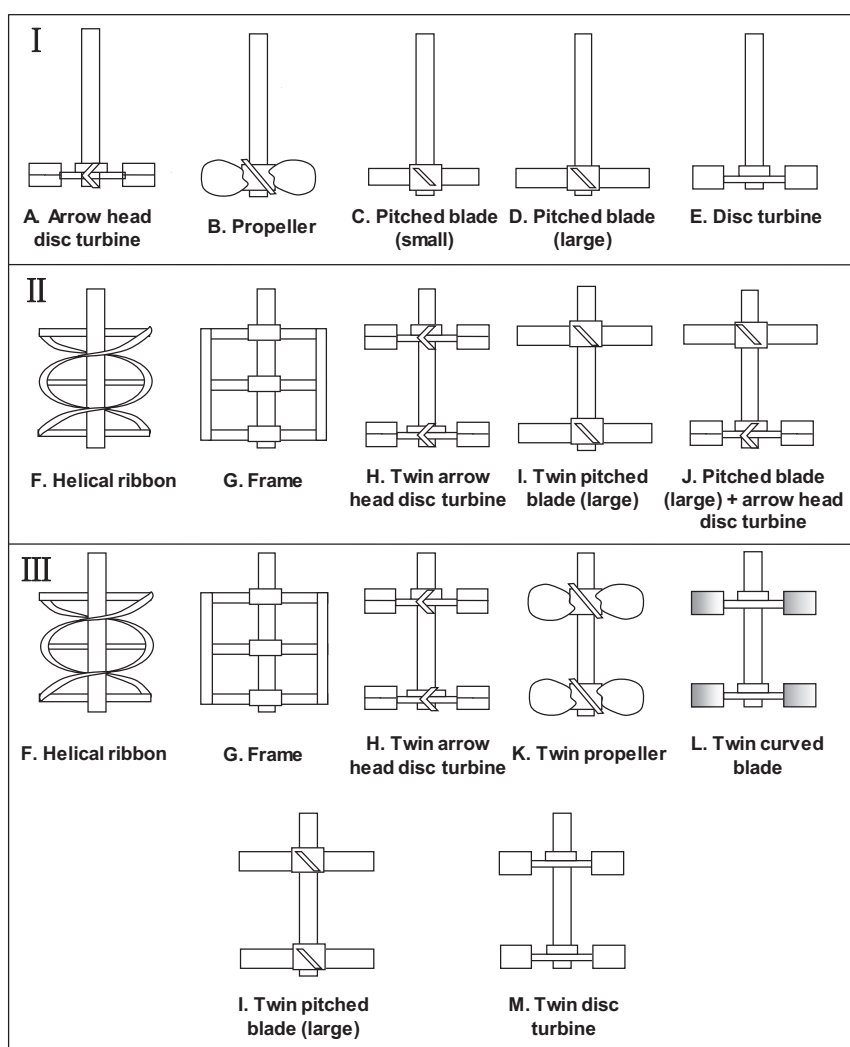
Scheme 1. Different agitator configurations. The agitators in group I were used at 300 rpm in comparison of single impellers during the BNC production without pH adjustment. The agitators in group II were used at 150 rpm in comparison of the helical ribbon and frame impellers with the combined impellers during the BNC production under pH control at 5.0. The agitators in group III were used at 100 rpm in comparison of the helical ribbon and frame impellers with the twin impellers without pH adjustment.
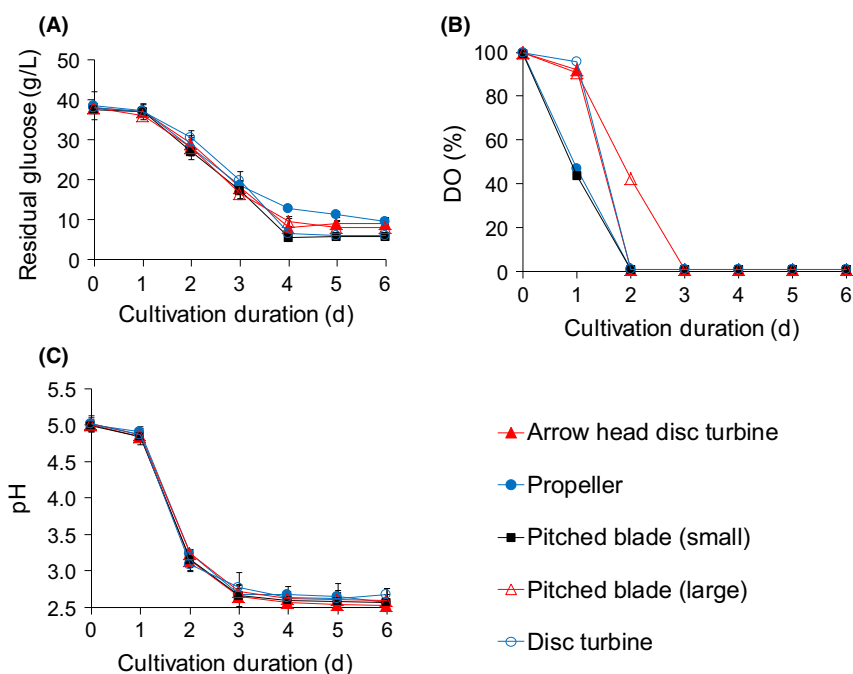
Fig. 1. Time-course of residual glucose (A), DO (B), and pH (C) in STRs equipped with the single impellers in group I of Scheme 1. The agitation speed was 300 rpm.

Table 1. Glucose consumption rate, BNC volumetric productivity and BNC yield (Product, P) from initial and consumed glucose (Glc)

Agitator number	Impeller	Glucose consumption rate [g (L×d)$^{-1}$]	Q [g (L×d)$^{-1}$]	$Y_{P/\text{initial Glc}}$ (g g^{-1})	$Y_{P/\text{consumed Glc}}$ (g g^{-1})
A	Arrowhead disc turbine	**4.82** $\pm$ 0.17	**0.45** $\pm$ 0.04	**0.071** $\pm$ 0.006	**0.093** $\pm$ 0.008
B	Propeller	4.83 $\pm$ 0.65	0.40 $\pm$ 0.05	0.063 $\pm$ 0.008	0.083 $\pm$ 0.009
C	Pitched blade (small)	5.29 $\pm$ 0.08	0.43 $\pm$ 0.02	0.068 $\pm$ 0.004	0.080 $\pm$ 0.003
D	Pitched blade (large)	**5.03** $\pm$ 0.13	**0.61** $\pm$ 0.02	**0.095** $\pm$ 0.004	**0.121** $\pm$ 0.005
E	Disc turbine	5.36 $\pm$ 0.02	0.38 $\pm$ 0.05	0.060 $\pm$ 0.008	0.072 $\pm$ 0.007

The impellers used are shown in group I of Scheme 1. The agitation speed was 300 rpm and pH was not controlled. The noticeable values are shown in bold.

be noted that although the DO content reached zero during days 2–3, the DO concentration did not reach absolute zero, rather it was nearly zero in the calibration curve (a percentage of the saturated DO concentration in the culture). When the DO content is shown near zero, the oxygen in the air supplied at speed of 1.5 vvm is almost consumed, and the oxygen consumption balances the oxygen supply, and the submerged cells are not deprived of oxygen. The consumption of oxygen occurred very quickly because the metabolism of the cells was vigorous. From Fig. 1, it can be seen that after the DO content reached close to 0%, glucose was still being rapidly consumed, indicating that the culture was still healthy and that there were few dead cells. If more dead cells were present, the DO would increase, indicating the loss of metabolic activity. The DO content in the *K. xylinus* culture is difficult to maintain at high levels,

and it has been repeatedly reported that the DO content decreases in glucose-based media after one or 2 days in an STR. Jung *et al.* (2005) studied BNC production in a 5-l STR with an airflow of 1 vvm at a stirring speed of 500 rpm and found that the DO decreased to zero in < 24 h. Park *et al.* (2004) used an aeration rate as high as 1.7 vvm at 100 rpm in a 5-l STR and found that the DO also decreased to approximately zero in 1 day. However, it should be noted that the bioconversion from glucose to BNC does not include any oxidation reaction and does not require much oxygen. Furthermore, DO content that is too high has been reported to primarily promote the production of by-products of gluconic acid, a metabolite of glucose, a process which requires oxygen (Tantratian *et al.*, 2005). Thus, it was not necessary for us to attempt to maintain the DO content at a high level in the current work. After the lag phase, the pH of

all the cultures rapidly decreased to 3.0–3.5 (Fig. 1C). During the final 3 days, the pH slowly decreased to 2.5–3.0. It was previously speculated that high levels of by-products, mainly gluconic acid and ketogluconic acids, are formed under these conditions (De Wulf *et al.*, 1996). However, because the proper pH for BNC synthesis is between 4 and 6 (Masaoka *et al.*, 1993; Hwang *et al.*, 1999), the low pH in the last 3 days would inhibit BNC production.

Among the single impellers, the pitched blade (large) generated the highest crude BNC volumetric productivity of 0.61 g (L×d)$^{-1}$ (significant difference, $P < 0.05$), the crude BNC yield from the initial glucose (0.095 g g^{-1}) (significant difference, $P < 0.05$) and the yield from the consumed glucose (0.12 g g^{-1}) (significant difference, $P < 0.05$) (Table 1). Following the pitched blade (large), the arrowhead disc turbine also offered better BNC volumetric productivity and BNC yield than the other single impellers.

The BNC yield from the consumed sugar with a single pitched blade (large) was comparable to that obtained using *K. xylinus* subsp. *sucrofermentans* BPR2001 in glucose-based media (0.12–0.14 g g^{-1}) (Toyosaki *et al.*, 1995). However, it is noteworthy that in the current study, the BNC was only washed with water, and the differences in washing procedures would affect yield values, making it is impossible to compare these results with those of the previous work. *K. xylinus* BPR2001 is believed to be suitable for agitated cultures and fructose-based medium, but it has sometimes been cultivated in glucose-based media (Toyosaki *et al.*, 1995). Bae and Shoda (2005) investigated the use of strain BPR2001 to produce BNC in a glucose-based medium. The BNC was treated with 0.1 M NaOH solution at 80°C for 20 min and then rinsed with deionized water. A BNC yield from consumed glucose of 0.134 g g^{-1} was previously reported (Bae and Shoda, 2005). Zuo *et al.* (2006) also used strain BPR2001 to produce BNC in a glucose-based medium,

but in an air-lift reactor. The BNC was washed with 0.1 M NaOH at 90°C for 30 min and then washed twice with deionized water, and the BNC yield from consumed glucose was reported to be 0.12–0.13 g g^{-1} (Zuo *et al.*, 2006).

BNC production under pH control

As described above, the pH quickly decreased in the glucose-based media (Fig. 1C), and because pH adjustment can significantly enhance the BNC productivity and yield (Lindsay, 1973; Chawla *et al.*, 2009), the BNC production while maintaining the pH at 5.0 was studied using two single impellers [arrowhead disc turbine and pitched blade (large)], and the BNC volumetric productivity was assessed. Under pH control, the volumetric productivities of crude BNC were enhanced from 0.45 to 0.84 g (L×d)$^{-1}$ using the arrowhead disc turbine and from 0.61 to 0.99 g (L×d)$^{-1}$ using the pitched blade (large). The increase in BNC productivity by controlling the pH was consistent with previous results under pH control in a 400-ml STR with twin disc turbine (Chen *et al.*, 2017).

Comparison of helical ribbon and frame impellers with combined impellers under pH control

Since an STR is commonly equipped with more than one impeller (Kouda *et al.*, 1997; Bae and Shoda, 2005), an arrowhead disc turbine and pitched blade (large) impellers were doubled or used in combination, and the results were compared with those obtained using the helical ribbon and frame impellers (group II in Scheme 1). Since the number of impellers doubled or agitator size became larger, the agitation speed was halved to offset the effect caused by change in the agitator configuration. Due to the advantage of pH control as shown above, the pH was also manually controlled at 5.0.
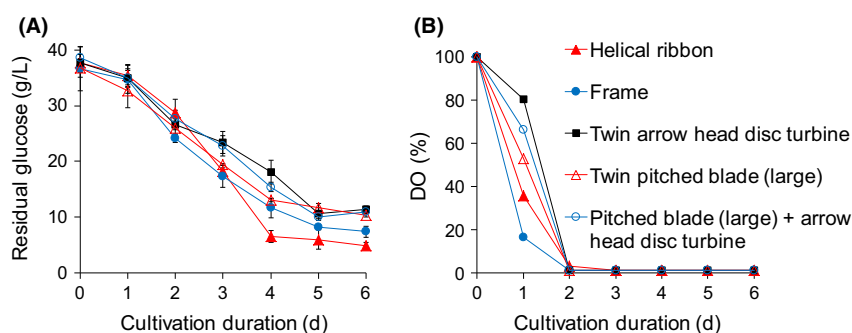

Fig. 2. Glucose consumption (A), and DO content (B) in the culture media agitated with different agitators in group II. The agitation speed was 150 rpm with manual pH adjustment.

Figure 2 shows the time-course of the residual glucose and DO contents in the STRs with the helical ribbon and frame impellers or with the combination of these impellers. Table 2 shows the glucose consumption rate, the BNC volumetric productivity and the yield. The observed glucose consumption rates with the combined impellers ranged from 4.38 to 4.61 g $(L{\times}d)^{-1}$, which were lower than those observed using a single pitched blade (large) without pH control [5.03 g $(L{\times}d)^{-1}$], the single arrowhead disc turbine without pH control [4.82 g $(L{\times}d)^{-1}$] (Table 1), and the helical ribbon [5.47 g $(L{\times}d)^{-1}$] and frame [4.87 g $(L{\times}d)^{-1}$] impellers. The DO content decreased more slowly in the STR agitated with the combination of impellers than in the STR with the helical ribbon and frame impellers (Fig. 2B). The volumetric productivities ranged from 1.02 to 1.71 g $(L{\times}d)^{-1}$, while the crude BNC yields from consumed glucose ranged from 0.20 to 0.37 g g^{-1} (Table 2). Compared with the volumetric productivity using single impellers at 300 rpm and under pH control [0.84 g $(L{\times}d)^{-1}$ and 0.99 g $(L{\times}d)^{-1}$, respectively, in section 3.2], all of the helical ribbon and frame impellers and the combined impeller had higher values [1.02–1.71 g $(L{\times}d)^{-1}$, Table 2]. Among the five agitators, the twin pitched blade (large) gave significantly ($P < 0.05$) higher values with regard to the BNC volumetric productivity, crude BNC yield from initial glucose and from consumed glucose [1.71 g $(L{\times}d)^{-1}$, 0.27 and 0.37 g g^{-1} respectively]. It is notable that the use of the helical ribbon and frame impellers resulted in higher glucose consumption rates but smaller BNC volumetric productivity and yields than the combined impellers. It is possible

Table 2. Glucose consumption rate, BNC volumetric productivity and BNC yield (Product, P) from initial and consumed glucose (Glc)

Agitator number	Impeller	Glucose consumption rate [g $(L{\times}d)^{-1}$]	Q [g $(L{\times}d)^{-1}$]	$Y_{P/\text{initial Glc}}$ (g g^{-1})	$Y_{P/\text{consumed Glc}}$ (g g^{-1})
F	Helical ribbon	5.47 ± 0.25	1.07 ± 0.09	0.17 ± 0.01	0.20 ± 0.01
G	Frame	4.87 ± 0.69	1.02 ± 0.13	0.17 ± 0.02	0.21 ± 0.03
H	Twin arrowhead disc turbine	4.38 ± 0.30	1.20 ± 0.05	0.19 ± 0.00	0.27 ± 0.01
I	Twin pitched blade (large)	4.61 ± 0.28	$\mathbf{1.71 \pm 0.06}$	$\mathbf{0.27 \pm 0.01}$	$\mathbf{0.37 \pm 0.01}$
J	Pitched blade (large) + arrowhead disc turbine	4.41 ± 0.37	1.44 ± 0.11	0.24 ± 0.01	0.33 ± 0.02

The STRs were equipped with the helical ribbon and frame impellers, as well as the combined impellers (group II in Scheme 1). The agitation speed was 150 rpm and pH was manually controlled at 5.0. The noticeable values are shown in bold.
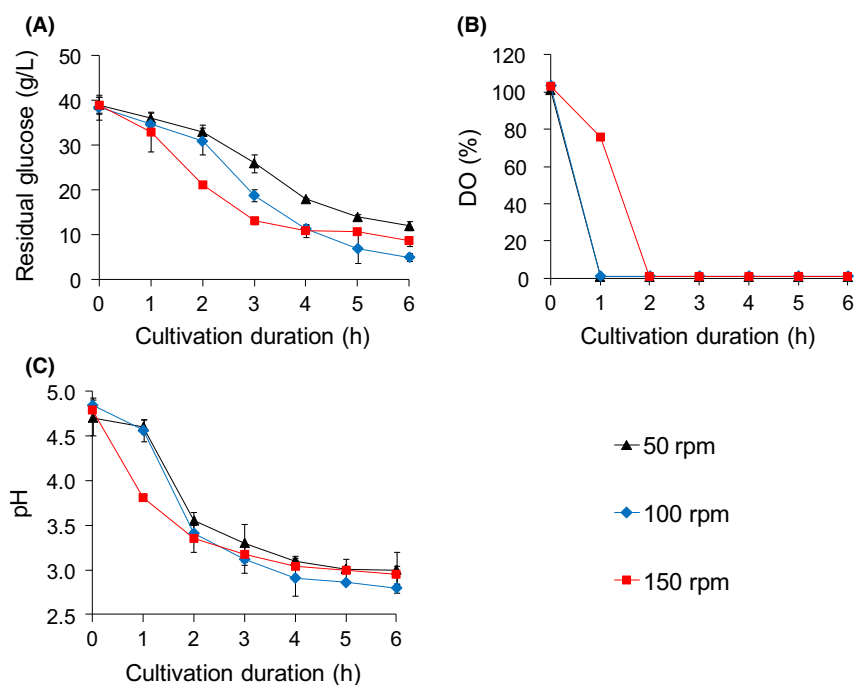

Fig. 3. Glucose consumption (A), DO content (B) and pH value (C) in the culture media agitated with the twin pitched blade (large) at speed of 50, 100 and 150 rpm, respectively.

Table 3. Effect of agitation speed on glucose consumption rate, BNC volumetric productivity and BNC yield (Product, P) from initial and consumed glucose (Glc)

Agitation speed (rpm)	Glucose consumption rate [g (L×d)$^{-1}$]	Q [g (L×d)$^{-1}$]	$Y_{P/initial\ Glc}$ (g g^{-1})	$Y_{P/consumed\ Glc}$ (g g^{-1})
50	4.50 ± 0.46	0.85 ± 0.20	0.13 ± 0.02	0.18 ± 0.02
100	**5.57 ± 0.31**[a]	**1.15 ± 0.05**[a]	**0.18 ± 0.01**[a]	**0.21 ± 0.00**[a]
150	5.05 ± 0.30	0.85 ± 0.06	0.13 ± 0.01	0.17 ± 0.01

The twin pitched blade (large) impeller was used. The pH was not controlled.
[a]Significant difference with $P < 0.05$. The noticeable values are shown in bold.

that the volumetric productivity and yield were more dependent on the DO level rather than the glucose consumption rate. As the DO in the STRs with the helical ribbon and frame impellers was depleted faster than in the STRs with combined impellers (Fig. 2B), the crude BNC productivity and yield in the STRs with the helical ribbon and frame impellers were lower. Although the helical ribbon and frame impellers could promote glucose consumption, much of the glucose was converted to by-products. The STR with the twin pitched blade (large) generated the highest crude BNC productivity and yield, but the decrease in the DO content in this STR was not the slowest, since it occurred more rapidly than the other two combined impellers (Fig. 2B). It is possible that the culture broth became very thick, since the BNC in the STR with twin pitched blade (large) was produced the fastest and with the highest productivity, limiting the DO transfer and causing the DO content to rapidly decrease.

Optimization of agitation speed

As agitation speed could also affect BNC production (Chen *et al.*, 2017), the speed was further optimized without pH control using the twin pitched blade (large) impeller, since the best performance was observed using this configuration. Figure 3 shows the residual glucose, DO and pH changes during the cultivation process. At the highest agitation speed of 150 rpm, glucose was first consumed rapidly, but in the last 2 days, glucose was consumed at a very low rate, with an agitation speed of 100 rpm giving the lowest final glucose concentration. The decrease in the DO content at 150 rpm was the slowest during the first 2 days, suggesting that a better oxygen transfer rate occurred. Corresponding to the glucose consumption, the pH value at 150 rpm decreased the fastest in the first 2 days, but the lowest final pH was observed at 100 rpm.

Table 3 shows that among the three agitation speeds, 100 rpm resulted in the highest glucose consumption of 5.57 g (L×d)$^{-1}$ (significant difference, $P < 0.05$), a crude BNC volumetric productivity of 1.15 g (L×d)$^{-1}$ (significant difference, $P < 0.05$), and a crude BNC yield

from the initial glucose of 0.18 g g^{-1} (significant difference with $P < 0.05$) and from the consumed glucose of 0.21 g g^{-1} (significant difference, $P < 0.05$). Compared with the single pitched blade (large) at 300 rpm (Table 1), all volumetric productivities and yields increased significantly ($P < 0.05$) with the twin pitched blade (large). The lower BNC volumetric productivity and yield observed at an agitation speed of 50 rpm compared to those observed at 100 rpm could be due to the lower oxygen transfer, as described by Tantratian *et al.* (2005). The lower BNC volumetric productivity and yield at an agitation speed of 150 rpm compared to 100 rpm could be related to the higher DO content (Fig. 3B), which should increase the accumulation of the by-product gluconic acid rather than BNC resulting in reduced the cellulose production (Tantratian *et al.*, 2005). In addition, the inconsistency that the highest crude BNC productivity using the optimized agitation speed [1.15 g (L×d)$^{-1}$, Table 3] was even lower than that obtained with the twin pitched blade (large) impeller at 150 rpm in last experiment series [1.71 g (L×d)$^{-1}$, Table 2] could be attributed to the lack of pH control.

As the study on the effects of agitation shown in Table 3 was a single factor optimization and not a multiple factor optimization, the optimal agitation speed should not be affected in the culture without pH control. To assess the effect of pH control on the final optimization, an experiment was performed at the end of the study with manual pH adjustment after all the optimization experiments.

Comparison of the helical ribbon and frame impellers with twin impellers at 100 rpm

The effects of using helical ribbon and frame impellers and five twin impellers (group III in Scheme 1) were compared at 100 rpm. Glucose was consumed fastest during the first 4 days when the twin pitched blade (large) impeller was used. At the end of cultivation (6 days), the final glucose concentration (6.9 g l^{-1}) was also among the lowest observed (Fig. 4A), which was consistent with the glucose consumption rate shown in Table 4. In contrast, in the STR equipped with the helical
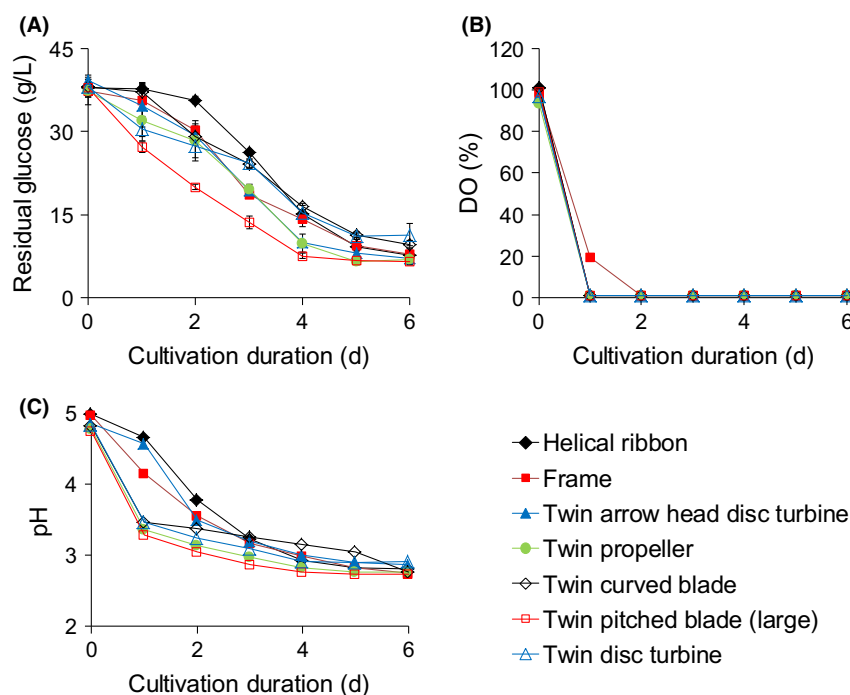
Fig. 4. Glucose consumption (A), DO content (B) and pH value (C) in the culture media agitated with different agitators in group III. The agitation speed was 100 rpm.

Table 4. Glucose consumption rate, BNC volumetric productivity, and BNC yield (Product, P) from initial and consumed glucose (Glc) and oxygen mass transfer coefficient (K_{La})

Agitator number	Impeller	Glucose consumption rate [g (L×d)$^{-1}$]	Q [g (L×d)$^{-1}$]	$Y_{P/initial\ Glc}$ (g g^{-1})	$Y_{P/consumed\ Glc}$ (g g^{-1})	K_{La}[a]
G	Frame	4.90 ± 0.07	0.90 ± 0.05	0.15 ± 0.00	0.18 ± 0.00	19.84 ± 0.60
M	Twin disc turbine	4.46 ± 0.58	0.98 ± 0.05	0.15 ± 0.01	0.22 ± 0.02	20.58 ± 0.45
K	Twin propeller	5.04 ± 0.52	1.00 ± 0.08	0.16 ± 0.01	0.20 ± 0.02	23.35 ± 0.74
L	Twin curved blade	4.73 ± 0.09	1.03 ± 0.05	0.16 ± 0.00	0.22 ± 0.00	17.15 ± 0.32
H	Twin arrowhead disc turbine	5.37 ± 0.05	1.10 ± 0.12	0.17 ± 0.00	0.20 ± 0.00	18.84 ± 0.68
F	Helical ribbon	5.02 ± 0.19	1.23 ± 0.05	0.20 ± 0.01	0.24 ± 0.01	19.46 ± 0.60
I	Twin pitched blade (large)	**5.20 ± 0.20**	**1.25 ± 0.08**	**0.20 ± 0.00**	**0.24 ± 0.00**	**23.53 ± 1.06**

The STRs were equipped with the helical ribbon and frame impellers, as well as the twin impellers at the optimized agitation speed of 100 rpm (group III in Scheme 1). The pH of culture media was not controlled. The results are listed from small to big according to the values of volumetric productivity.
a. K_{La} was measured before inoculation. The noticeable values are shown in bold.

ribbon impeller, glucose was consumed the slowest during the first day before increasing with a final observed glucose concentration of 7.7 g l^{-1}. The DO content decreased during the first day of cultivation in cultures with each of the seven agitators (Fig. 4B). Among these agitators, the DO decreased slower than others within 2 days when the frame impeller was used. Figure 4C shows that the pH decreased the fastest in the STR with twin pitched blade (large) impeller, while the pH in the STR with the helical ribbon decreased the slowest during

the first 3 days. The highest BNC volumetric productivities and yields were obtained in the STR with the twin pitched blade (large) impeller, followed by the STR with the helical ribbon impeller. By contrast, the culture with the frame impeller offered the lowest BNC volumetric productivity and yield (Table 4). In a study using fructose as carbon source and without pH adjustment at 600–1200 rpm (Kouda *et al.*, 1997), the optimum agitator for BNC production was the 'maxblend and gate with turbine', which is similar to the frame impeller. The results
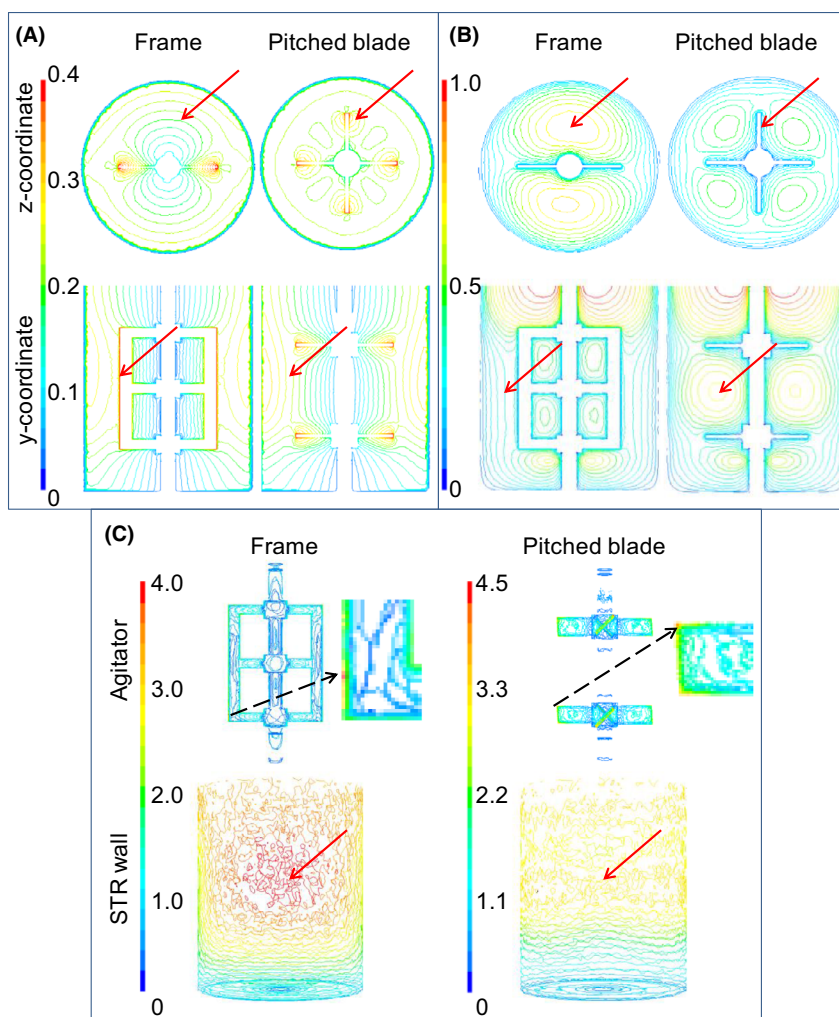
Fig. 5. Comparison of contours of velocity magnitude (A), contours of turbulent kinetic energy (B), and contours of wall shear stress (C) in the STRs equipped with impellers of frame and twin pitched blade (large). The agitation speed was 100 rpm.

obtained with this configuration were different from those in the current study, which could be attributed to the differences in agitation speed and the carbon source of the culture medium.

To interpret the results, the oxygen K_{La} in the STRs was measured for each of the seven agitator configurations, since oxygen transfer can affect BNC production during submerged cultivation (Kouda *et al.*, 1997; Tantratian *et al.*, 2005) and K_{La} is possibly related to agitator configuration (Kouda *et al.*, 1997; Bae *et al.*, 2004). Table 4 shows that the STR equipped with twin pitched blade (large) impeller had the highest K_{La} value, whereas the STR with the twin curved blade had the lowest K_{La} value. In most cases, the K_{La} value correlated with the glucose consumption rate, pH change and BNC production. For example, the K_{La} values in the STRs with the frame impeller, twin disc turbine, and twin propeller positively correlated with their volumetric

productivity of BNC (Table 4, above the dashed line). In addition, the K_{La} values for the other four agitators [twin curved blade, twin arrowhead disc, helical ribbon and twin pitched blade (large)] also positively correlated with the volumetric productivity of BNC (Table 4, below the dashed line). These results are consistent with the findings of Kouda *et al.*, who previously reported that the BNC production rate was dependent on K_{La} (Kouda *et al.*, 1997). However, the glucose consumption, DO and pH change, and BNC production were not always dependent on K_{La}. For example, the STR with the twin arrowhead disc turbine had a significantly lower K_{La} value than that of the twin propeller STR ($P < 0.05$), although the STR with twin arrowhead disc turbine had higher BNC volumetric productivity (Table 4). This inconsistency may be related to the production of by-products, such as gluconic acid (Tantratian *et al.*, 2005).

In a subsequent investigation, a preliminary trial of computational fluid dynamics in STR is currently being performed in our laboratory. Mixing and shear force are believed to play a role in determining the BNC production in the STRs (Park *et al*., 2004). The Fluent software (version 15.0; Ansys, Inc., Canonsburg, PA, USA) was used to perform the computational fluid dynamics simulation in the STRs with the impellers producing the highest and lowest BNC volumetric productivity at 100 rpm [STR with the frame and twin pitched blade (large) impellers respectively]. In the z-coordinate graphic in Fig. 5A, it is shown that the liquid flow velocity was lower in some areas near the impeller (indicated with an arrow) in the STR with twin pitched blade (large) impeller. In contrast, in the y-coordinate (Fig. 5A), it could be observed that there were some areas in the middle of the STR near the frame impeller with a higher liquid flow velocity than was observed with the twin pitched blade (indicated with an arrow). Figure 5B shows that the turbulent kinetic energy in some areas near the impeller was lower in the STR with the twin pitched blade (large) impeller than that observed with the frame impeller (observed in the z-coordinate direction, indicated with an arrow), but some areas in the middle had higher turbulent kinetic energy (observed in the y-coordinate direction, indicated with an arrow). Figure 5C shows that the tips of the twin pitched blade (large) impeller contributed to a slightly higher wall shear force (enlarged parts of Fig. 5C) than was observed with the frame impeller, but much of the wall area of the STR with the twin pitched blade (large) impeller exhibited a much lower wall shear force (indicated with an arrow).

Despite exhibiting similar average liquid flow velocities and turbulences, the average wall shear force observed in the STR with the twin pitched blade (large) impeller was much lower than that observed in the STR with the frame impeller. The higher glucose consumption rate, volumetric productivity and yield of BNC in the STR with twin pitched blade (large) impeller are believed to be related to the lower shear force, as Bae *et al*. (2004) previously postulated. Bae *et al*. (2004) speculated that high agitation speeds in STRs can cause high shear forces, causing bacteria to mutate and produce less BNC. After adding agar in culture medium, a previous study showed that the BNC production increased, which was ascribed to the decreased shear force (Bae *et al*., 2004). Kralisch and Hessler (2012) also reported that the shear force in submerged cultivation would negatively affect BNC production.

Verification of the twin pitched blade (large) as the optimum agitator

Due to the high oxygen K_{La} value, the twin pitched blade (large) impeller at 100 rpm was anticipated to promote an even higher BNC productivity and yield under pH control. With twin pitched blade (large) impeller at 100 rpm and under pH control, there was a glucose consumption rate of 4.83 g $(L \times d)^{-1}$, a crude BNC volumetric productivity of 1.97 g $(L \times d)^{-1}$, a BNC yield from the initial glucose of 0.30 g g^{-1} and a crude yield from the consumed glucose of 0.41 g g^{-1}, all of which were higher than those values obtained with agitation speed of 150 rpm and pH control (Table 2). These results confirmed that the twin pitched blade (large) impeller was the most suitable for BNC production among these agitator configurations used. Compared with the values obtained in the first experiment in this study [from the single pitched blade (large) at 300 rpm and without pH control, Table 1], the volumetric productivity was enhanced 2.25 times and the yield from the consumed glucose was increased 2.37 times. The volumetric productivity of 1.97 g $(L \times d)^{-1}$ and the yield from the consumed glucose of 0.41 g g^{-1} observed in this study is comparable to that observed in a previous study, where BNC was produced from glucose in an STR by a mutant of *K. xylinus* ATCC 23770 strain that was hypothesized to be better for BNC production than *K. xylinus* ATCC 23770 (Chen *et al*., 2018). The crude BNC was observed to have a volumetric productivity of 0.43–0.55 g $(L \times d)^{-1}$ and a yield from consumed glucose of 0.085–0.15 g g^{-1} (Chen *et al*., 2018). It is not easy to compare the current BNC yields with those other works since different strains, purification processes and drying conditions were applied. Heo and Son (2002) produced BNC from *Acetobacter* sp. A9 using a shaking culture. The highest volumetric productivity and highest BNC yield from the initial glucose were observed to be 0.89 g $(L \times d)^{-1}$ and 0.20 g g^{-1}, respectively, after 20 min of purification in a 2% NaOH solution followed by washing with deionized water and drying overnight at 95°C (Heo and Son, 2002). Hungund and Gupta (2010) produced BNC using *K. persimmonics* GH-2 in a 5-lSTR. Using a constant pH of 5.5 and a DO of 20%, the highest BNC yield from the consumed glucose was 0.26 g g^{-1} after purification in a 2% NaOH solution for 30 min, thorough washing with deionized water, and then drying at 70°C for 6 h. Using the same *K. xylinus* ATCC 23770 strain under static cultivation conditions and using the same washing scheme as that described in the current work, a crude BNC volumetric productivity of 0.50–0.91 g $(L \times d)^{-1}$ was obtained (Cavka *et al*., 2013; Guo *et al*., 2013, 2016; Zhang *et al*., 2014a,b). In the current study using agitated cultivation, a crude BNC volumetric productivity of 1.97 g $(L \times d)^{-1}$ was obtained, which was even higher than that described in previous works using static cultivation.

The BNC was subsequently characterized using SEM, viscometry and XRD. As previously reported, the BNC sample exhibited a reticulated structure consisting of
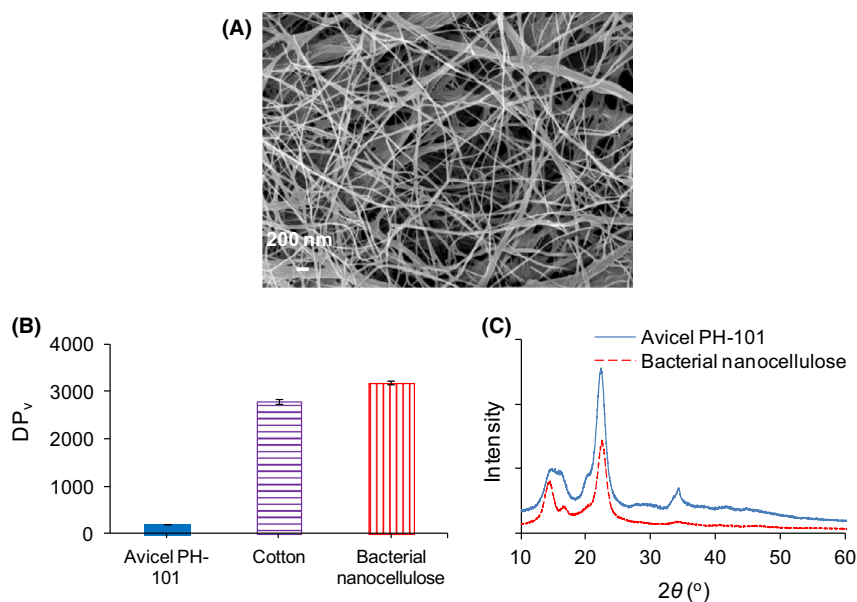
Fig. 6. SEM image (A), DP$_v$ value (B) and XRD spectrum (C) of the bacterial nanocellulose from cultivation in an STR with the optimal agitator [twin pitched blade (large)] at 100 rpm and with manual pH adjustment.

ultrafine cellulose fibres with diameters much smaller than 100 nm (Fig. 6A) (Watanabe *et al.*, 1998). The average viscometric degree of polymerization (DP$_v$) of Avicel PH-101, cotton cellulose and the BNC sample obtained from cultivation in the STR with the optimal agitator [twin pitched blade (large)] at 100 rpm and with manual pH adjustment was characterized, and the results are shown in Fig. 6B. The DP$_v$ of BNC (3190) was significantly higher ($P < 0.05$) than that of cotton cellulose (DP$_v$ = 2800). The DP value depends on how DP is calculated from the viscosity measurements, the strain used, etc. *K. xylinus* ATCC 23770 is a widely studied and used strain that is appropriate for determining the optimal impeller among the 13 agitator configurations in the current study. Thus, a DP$_v$ of 3190 could be considered to be good compared with the results of some other studies. By using a similar method to measure the DP$_v$ as the American National Standard of ASTM D 4243-16 (Institute, A.N.S, 2016) used in the current study, a DP$_v$ of 2000 for the BNC from *K. xylinus* ATCC 23769 under static cultivation and a DP$_v$ of 2280 for cotton linter previously observed (Shibazaki *et al.*, 1997). The retained high DP$_v$ of BNC, which was same as or better than that obtained from static cultivation, could be related to the low agitation speed (Chen *et al.*, 2017). However, it should be noted that BNC with much higher DP$_v$ of over 6000 could be obtained using the bacterial mutant *K. xylinus* DHU-ZGD-1186 (Chen *et al.*, 2017). Furthermore, the DP$_v$ would vary when the agitator type or cultivation conditions, such as agitation speed, is changed (Chen *et al.*, 2017, 2018). The results

of our previous work (Chen *et al.*, 2017) showed that agitation speed negatively affected BNC DP$_v$. Even though the bacterial strain used in that work was *K. xylinus* DHU-ZGD-1186, it has already showed that a low stirring speed promotes the production of high BNC with a DP$_v$ and that the DP$_v$ would decrease after the stirring speed exceeded a certain value. With the strain *K. xylinus* ATCC 23770, it is believed that the effect of agitation speed on the BNC DP$_v$ would be similar, and the agitation speed will be optimized in a future study.

The XRD spectra of the BNC sample and Avicel PH-101 are shown in Fig. 6C. After performing calculations using Segal's method (Segal *et al.*, 1959), the crystallinity index of BNC was 80%, comparable to the 79% value observed for Avicel PH-101. The crystallinity index of 80% is similar to that of BNC (78%) produced by the same strain cultivated using glucose under static cultivation in our previous study (Cavka *et al.*, 2013). However, this finding was different from another report in which agitation could result in decreased crystallinity of BNC (Watanabe *et al.*, 1998). In that study, the agitation speed used was high enough to maintain a DO concentration of above 1.0 ppm (DO of approximately 13% (Watanabe *et al.*, 1998)) to produce BNC in a 1-l STR, whereas in the current study, the DO content reached zero after 1–2 days. The crystallinity index of the BNC from the STR cultivation was 63%, which was much lower than the 71% observed from static cultivation (Watanabe *et al.*, 1998). In contrast, the current work showed that the submerged culture in the STR at 100 rpm could achieve improved crude BNC productivity

Table 5. Parameters of different agitator configurations

Number	Agitator	Number of blades	d/D[a]	L[b] or L'[c] (mm)	H[d] (mm)
A	Arrowhead disc turbine	6	0.48	14	30
B	Propeller	3	0.48	26	30
C	Pitched blade (small)	4	0.39	12	30
D	Pitched blade (large)	4	0.57	16	30
E	Disc turbine	6	0.42	12	30
F	Helical ribbon	2	0.51	108	40
G	Frame	2	0.58	100	40
H	Twin arrowhead disc turbine	2 × 6	0.48	70	30
I	Twin pitched blade (large)	2 × 4	0.57	70	30
J	Pitched blade (large) + arrowhead disc turbine	4 + 6	0.57, 0.48	70	30
K	Twin propeller	2 × 3	0.48	70	30
L	Twin curved blade	2 × 6	0.37	70	30
M	Twin disc turbine	2 × 6	0.42	70	30

a. d/D: impeller diameter (d) divided by tank diameter (D).
b. L: height of impeller.
c. L': distance between two impellers.
d. H: distance between tank bottom and impeller.

and yield compared to the static culture with *K. xylinus* ATCC 23770 and maintain the high crystallinity of BNC. These good results could be due to the better air supply and nutrient mixing compared to that attained in a static culture, as well as the low agitation speed when compared to the typical high agitation speed of 600–1200 rpm used in STR cultivation.

In conclusion, this work succeeded in identifying the most suitable agitator configuration for greatly enhanced crude BNC productivity without sacrificing high BNC quality using glucose as the carbon source in STR cultivation. The results indicated that a single pitched blade (large) impeller performed best among the single impellers assayed, and combination of pH control at 5.0 could further promote BNC production. The twin pitched blade (large) impeller configuration performed better than the helical ribbon and frame impellers as well as the five combined impellers. This investigation found that the optimal performance of the twin pitched blade (large) impeller could be related to the high oxygen K_{La} value it afforded. Under the optimized cultivation conditions with the twin pitched blade (large) impeller, the BNC volumetric productivity and yield from the consumed glucose was enhanced 2.25 and 2.37 times, respectively, from that obtained using the single pitched blade (large) impeller. Moreover, it was shown that the high DP_v and crystallinity of BNC could be retained during STR cultivation with a high BNC productivity and yield. Additional studies are needed to elucidate how the agitator configuration in an STR affects BNC productivity and quality, K_{La} change, by-product formation, and the BNC DP_v value during cultivation. Other factors influencing BNC productivity and quality, such as strain growth sensitivity to shear force, the effect of shear force on the polymerization chain length of cellulose, and strain degeneration, should also be assayed in future studies.

Experimental procedures

Bioreactor and agitators

The bioreactor used in this study was a commonly used 3-l STR equipped with two baffles (10.0-mm width) at opposite sides (3BG, Shanghai BaoXing Bio-Engineering Equipment Co., Ltd., Shanghai, China). Scheme 1 shows all of the agitator configurations used in this study. The parameters of the agitators are listed in Table 5. Five kinds of agitators, including agitators A, B, C, D and E, were used in the comparison of single impellers (group I). An additional five types of agitators, including agitators F, G, H, I and J, were used in the comparison of the helical ribbon and frame impellers with the combined impellers under pH control (group II). Seven kinds of agitators, including agitators F, G, H, K, L, I and M, were used in the comparison of the helical ribbon and frame impellers with the twin impellers at the optimized agitation speed of 100 rpm (group III).

Microorganism and culture media

Komagataeibacter xylinus ATCC 23770 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained on agar slants. The agar slant medium consisted of 25 g l^{-1} glucose, 5 g l^{-1} peptone, 3 g l^{-1} yeast extract and 20 g l^{-1} agar, with the pH 5.0 adjusted using a 4 M aqueous solution of H_2SO_4. The content of the seed culture medium used for preparing inocula was the same as that of agar slant but without the agar. The volume of the seed culture was 160 ml in a 500-ml shake flask. The fermentation medium

contained 40 g l^{-1} glucose, 10 g l^{-1} peptone, and 6 g l^{-1} yeast extract, with the initial pH adjusted to 5.0 using a 4 M aqueous solution of H$_2$SO$_4$. The working volume of the fermentation culture was 2 l in the 3-l STR.

Production of BNC in STRs

Effect of single impellers on BNC production. The group I agitators in Scheme 1 were used to study the effect of single impellers on BNC production. The inocula used for each STR were prepared by transferring one loop of the bacteria from an agar slant to the seed culture medium, with cultivation performed in a shaker at 30°C and 160 rpm for 24 h. After inoculation, fermentation was performed with an agitation speed of 300 rpm and at 30°C and with an airflow of 1.5 vvm in the STRs equipped with the impellers. Similar to the study by Kouda, no pH adjustment was conducted (Kouda *et al.*, 1997). During cultivation, broth samples were collected every day to determine the concentration of residual glucose, and the DO content and pH were also recorded online every day. After 6 days, the BNC was harvested by centrifugation. Each cultivation was only repeated once, which is a common practice in STR studies because of the heavy manual work required.

An agitation speed of 300 rpm was selected based on the following considerations. A low agitation speed is favourable to ensure high BNC quality and production. However, the speed should not be too low to achieve a high BNC productivity. Speeds of 100–300 rpm have previously been reported to provide good results for BNC production during agitated cultivation with glucose as a carbon source (Tantratian *et al.*, 2005; Chen *et al.*, 2017; Singhsa *et al.*, 2018). In our previous study, a speed of 150 rpm with double impellers gave a higher DP of BNC than 50 rpm, while the use of 250 rpm with double impellers gave a lower DP of BNC than that obtained using a speed of 150 rpm (Chen *et al.*, 2017). In the current work, for the comparisons of single impellers, an agitation speed of 300 rpm was selected to allow for the number of impellers being halved compared to that used in the previous work, where the optimum speed was 150 rpm with double impellers (Chen *et al.*, 2017). Subsequently, a comparison of double impellers at a speed of 150 rpm was carried out.

BNC production under pH control. In the study on BNC production under pH control, arrowhead disc turbine and pitched blade (large) impellers (A and D, respectively, in Scheme 1 and Table 5) were used. The fermentation was conducted the same as described above, except that the pH was manually controlled at 5.0 with 4 M NaOH.

As BNC envelops pH electrodes to form a BNC jacket in STRs at low agitation speed, the monitored pH value heavily lags behind the true value in the culture broth, and there is currently not a good method to perform automatic pH control. Therefore, the fermentation results would be distorted if the pH control strategy was carried out at a lower speed, such as 50 rpm. Additionally, since the work of Kouda did not describe performing pH adjustments (Kouda *et al.*, 1997), we stopped performing pH control in the optimization of agitation speed. To control the pH after the pH probe was enveloped by the BNC jacket, a manual pH adjustment was performed, where sodium hydroxide was added, followed by waiting for some minutes until the pH stabilized and the addition of alkali was repeated. As the BNC concentration increased, the time lag became longer.

Comparison of helical ribbon and frame impellers with combined impellers under pH control. The agitators of group II in Scheme 1 were used. Fermentation was carried out using the same method, except the agitation speed was 150 rpm, and the pH was manually controlled at 5.0.

Optimization of agitation speed. For the optimization of agitation speed, the twin pitched blade (large) impeller was used (impeller I in Scheme 1 and Table 5). Fermentation was performed as described for the investigation of the effect of single impellers on BNC production, except that the agitation speed was 50, 100 or 150 rpm. Considering that the accurate adjustment of pH is not easy if the pH electrode becomes more heavily wrapped at lower agitation speeds, the optimization of agitation speed was performed without pH control.

Comparison of helical ribbon and frame impellers with twin impellers at the optimized agitation speed. The agitators in group III in Scheme 1 were used to compare the helical ribbon and frame impellers with the twin impellers for fermentation at the optimized agitation speed of 100 rpm. Fermentation was performed as described for the investigation of the effect of single impellers on BNC production. The oxygen mass transfer coefficient K_{La} was measured in each STR using the method reported by Hou *et al.* (2017), except that the measurement temperature was set at 30°C in the current study. The K_{La} measurement in culture medium was conducted only once before inoculation, as measuring the K_{La} without cells has been reported to be a good means of evaluating the difference in oxygen transfer in bioreactor (Chao *et al.*, 2001; Song *et al.*, 2009). It is believed that the difference in oxygen transfer in medium before inoculation would continuously

affect BNC production during all the cultivation phases. Furthermore, the final BNC productivity would be driven by the difference in oxygen transfer. A method for *in situ* measurement of K_{La} is not currently available. If the measurement of K_{La} is performed during cultivation using the current method, there is a high risk of contaminating the culture as well as affecting bacterial metabolism.

However, it should be noted that the metabolites excreted by the bacteria, mainly BNC, would change the physical properties of the culture broth (principally, viscosity and surface tension) along with incubation time, reducing the efficiency of oxygen transfer rate and the K_{La} value. To precisely determine how the agitator configuration affects BNC production via oxygen transfer, it is best to follow the time-course of K_{La} during the fermentation process.

Determination of BNC weight and glucose concentration

Bacterial nanocellulose weight was determined using the following procedures. After 6 days of cultivation, the product was collected and centrifuged at 8000 *g* for 20 min. The precipitate obtained was washed with deionized water and then centrifuged again. The washed BNC was dried at 105°C and weighed. Washing BNC with NaOH and followed by deionized water several times is the best way to remove all bacterial cells and other impurities (Chen *et al.*, 2017). No further purification apart from water washing was performed because the BNC in the current study was in forms of small stellate particles or floc forms. During purification using NaOH, small pieces of the cellulose could be lost due to dissolution in the alkaline solution or due to losses during repeated washing steps, which would introduce large errors. In this study, any large errors would be assumed to be introduced with the purification step to account for the possible difference among the different agitators. The resulting purification errors would make the current comparison investigation futile. The degradation of cellulose with cellulase to retain the cells was also considered to give a corresponding result of BNC weight after deduction of the cell weight. However, because of the large volume of culture and the high weight of BNC, from a 3-l fermenter, it is not easy to completely degrade the BNC with cellulase efficiently, since BNC has a much higher crystallinity compared to plant cellulose. Cellulase hydrolysis is actually a method used for nanocrystal production from BNC. Therefore, it is better to dry BNC directly without further purification to obtain stable results for making reliable comparisons. We think that it is good that the current study at least provides a believable comparison result of crude BNC productivity among 13 agitator configurations. One of the purposes of this study was to assess the changing trends in the BNC yields obtained with various agitators and conditions. The weight of the bacterial cells would not be expected to influence the comparisons made in the study.

The residual glucose concentration was measured using the following method. The broth samples were centrifuged at 10 000 *g* for 20 min, and the supernatant was used to measure the concentration of residual glucose using the DNS method (Lindsay, 1973).

Preliminary study on computational fluid dynamics modelling

In the comparison of the helical ribbon and frame impellers with the twin impellers at the optimized stirring speed of 100 rpm, the agitators corresponding to the highest and lowest BNC productivity were further studied using computational fluid dynamics (CFD) simulation. First of all, the STR and fermentation system were simplified. Only the agitator and the tank wall were in the model. Properties of water rather than culture broth were used in the simulation in the tank and no airflow was adopted so as to make the system simple. A commercial grid generation tool of Gambit 2.4 was used to generate the 3D grids of the bioreactor model for running Fluent (version 15.0; Ansys, Inc.). The mathematical model was a Multiple Reference Frame model (MRF), in which impeller rotation region was set as the moving zone and the other regions were set as the tank zone. The upper surface of liquid was defined as free, and a no-slip wall condition was used for both the STR walls and the surface of the agitators. A non-uniformed grid with tetrahedral element was used for the agitator, while the other area was gridded with hexahedral element. The mesh for the STR was around 500 000 volume elements. The Fluent software was used to perform calculations. Convergence was achieved when residuals on continuity, velocities, kinetic energy and energy dissipation rate all became $< 10^{-5}$.

Verification of the optimum impeller in BNC production

The twin pitched blade (large) impeller was used (agitator I in Scheme 1 and Table 5) to perform fermentation at 30°C with an agitation speed of 100 rpm and an airflow of 1.5 vvm. The medium pH was manually regulated to 5.0 with 4 M NaOH. After 6 days, the BNC was harvested by centrifugation and weighed. A portion of the BNC was thoroughly washed five times at 80°C, each time for 4 h, using a 0.05 M aqueous solution of NaOH. Subsequently, the BNC was washed five times with deionized water at 80°C, each time for 4 h. The washing step with the NaOH solution was performed a greater number of times and for a longer period of time than in

most other studies, where washing was performed only once and for no more than 1 h (Watanabe *et al.*, 1998; Bae *et al.*, 2004). Extensive washing would yield a purer product but might negatively affect the BNC yield. The purified BNC was then freeze-dried for subsequent characterization. The obtained BNC was then characterized using SEM, viscometry and XRD analyses, as previously reported (Chen *et al.*, 2018). The DP_v value was measured based on the American National Standard of ASTM D 4243-16 (Institute, A.N.S, 2016). In the current study, the quality characterization included surface morphology analysis, crystallinity index and DP_v analyses of BNC, which are the basic and most commonly analysed qualities of BNC (Shibazaki *et al.*, 1997; Watanabe *et al.*, 1998). High quality is defined depending on the application. The presence of a nanostructure, high crystallinity and high DP_v are characteristics of high-quality BNC. Many applications of BNC are based on its unique properties (Bielecki, *et al.*, 2005). For these applications, such as cellulose nanocrystals and additives for mechanical enhancement, the nanostructure, high crystallinity and high DP_v are the desired qualities of high-quality BNC.

Acknowledgements

Conflict of interest

None declared.

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