Bacterial Cellulose Production Enhancement in Repeated Static Batch Culture of *Acetobacter xylinum* in Bench-Scale

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ABSTRACT: *Bacterial Cellulose (BC) is commonly produced by a static batch culture, which is a time-consuming and low-yield process. Therefore, this study developed a new repeated static batch culture with optimal conditions to reduce production time, increase production value, and thus reduce BC production costs. First, by examining the volume of the surface ratio (depth) of the culture medium at 5 different levels and then the effect of cultivation time on the production efficiency of BC, at the desired depth of 1.6 cm, 5.6 g/L of BC per week was obtained. Then, for more production enhancement of BC, a new repeated static batch culture was developed at the obtained optimal conditions in the previous step. Then, by investigating the effect of the number of feed addition cycles in the repeated-batch culture, the maximum BC production of 13.06 g/L was obtained at the optimum cycle number 4 (7 days per cycle) with aeration. The highest amount of produced BC at the end of the 5th cycle was 41.15 g in a culture volume of 3.5 L at 6 cm depth. Aeration at the rate of 0.1vvm increased BC production in all cycles and decreased overall production time. The highest BC concentration was 13.27 g/L at the end of the third cycle, and the maximum production was 44.2 g at the end of the 4th cycle. A comparison of shear stress and Young's modulus of BC sheets produced in different cycles of the repeated-batch static culture with and without aeration showed that increasing the number of cycles as opposed to aeration makes a significant difference in the mechanical properties of the produced BC sheets.*

KEYWORDS: *Acetobacter xylinum; Bacteria cellulose; Production; Repeated static batch culture.*

INTRODUCTION

Cellulose is the most abundant renewable biopolymer on earth, produced by plants and some bacteria. Bacterial Cellulose (BC) produced by aerobic bacteria *Acetobacter xylinum* [1,2] has a neat three-dimensional structure [1,2]. Thus, BC has unique properties such as a high degree of polymerization, high purity, strong mechanical properties, and high water holding capacity and porosity, which distinguish it from plant cellulose. Unlike plant cellulose, BC is free from lignin and other plants' impurity and has an ultra-fine nanofibrous structure [3-5]. BC can be used in various industrial fields including, food, electronics, textiles, composites, and biomedical applications [6].

One of the most important challenges in BC production is high cost and low production yield, which is affected

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by various parameters such as the culture medium composition, the type of process, and culture conditions such as temperature and pH [7]. Therefore, the development of a new low-cost culture medium, optimal culture conditions, and high-efficiency fermentation processes are important factors in achieving commercial applications of BC.

BC is produced by static and agitated cultivation methods [8-10]. Under static cultivation, BC is produced as gelatinous floating pellicle on the culture surface and suspended irregular-shaped granule or fibril BC in agitated cultivation methods [10-12]. Furthermore, BC produced in agitated culture has a lower degree of polymerization, lower crystallinity, lower mechanical strength, lower content of $I\alpha$ cellulose, and higher water holding capacity than those for the static culture method [13]. Static culture is simple technique and does not need high technology but there is a problem with oxygen and nutrient mass transfer limitations. However, there are some problems with agitated culture including strain instability, high viscosity, and non-Newtonian behavior of the culture medium due to the production of exopolysaccharides [14]. Static culture is preferred because of cellulose deficient mutant strains appearance in agitated culture. However, BC production by static culture is a time-consuming, and low-yield process [10], and the production rate is limited when the BC pellicle traps all producing bacteria. Under these conditions, bacteria face oxygen restrictions. Therefore, we need a useful and practical approach to BC production.

Repeated batch culture is a cultivation method that fraction of the final cell culture medium is retained in a bioreactor for use as inoculation in subsequent batch culture. This method is often used to achieve higher productivity than batch cultivation. Repeated-batch culture has been used to substantial production enhancement of ethanol, acetic acid, and L-lactic acid. Repeated-batch culture has some advantages compared with batch culture such as inoculum with higher volume and cell concentration, minimizing cultivation time, less time, and cost wasting for washing and sterilization of bioreactor and inoculum preparation [15, 16].

The cycle number and amount of adding fresh medium to cultivation vessel and aeration are the most important factors that can influence BC production in repeated batch culture process, which is necessary optimized for any biological products.

Therefore, in this study, first, the optimal depth (volume to surface ratio) of culture medium in batch culture was determined and then a new repeated static batch culture was developed. Then, the effects of cycle numbers of feed adding and aeration were investigated on BC production enhancement. Finally, the amount of the produced BC in different processes and their mechanical properties were compared together.

EXPERIMENTAL SECTION

Strain and culture

Acetobacter xylinum PTCC 1734 was used for the production of cellulose. The culture medium contains 20 g glucose, 5 g yeast extract, 5 g peptone, 6.8 g $Na_2HPO_4.12H_2O$, 1.15 g citric acid, 1.25 g $MgSO_4.7H_2O$, and 10 ml ethanol per liter. The pH of the medium was adjusted with 1 N HCl and NaOH to 5. To achieve strains with the same characteristics, strains were stored in the culture medium comprising 20% glycerol at -70°C.

Cultivation of acetobacter xylinum

In all experiments, the inoculum was prepared by inoculating 200 ml of sterile culture medium with a stock culture vial and incubated at 30 °C and 50 rpm for three days. Then, BC production stage was started by adding 10% (v/v) inoculum to the culture medium. In all experiments, the same number of cells per unit volume was used for the reproducibility of the production process. Cell count was performed using a Neubauer Chamber. The heat-sterilizable plastic containers with a surface area of 30×21 cm^2 and a total volume of 5 liters were used to optimize production conditions.

First, by examining the effect of the volume-to-surface area ratio (depth) of the culture medium at five levels of 0.8, 1.6, 2.4, 3.2, and 4 cm, the height of the culture medium in static batch culture was optimized. Then, the effect of cultivation time on cellulose production was evaluated in three levels of one, two, and three weeks.

In repeated batch culture, a fraction of the final cell culture suspension is kept in the culture vessel for use in subsequent batch culture. BC sheets absorb moisture strongly due to their high hydrophilic properties. As a result, a significant part of the culture medium is placed in the cellulose layer and its value changes in each process cycle. Therefore, in this study, instead of using a constant amount of culture medium at the end of each cycle as the inoculum

of the next cycle, the total volume of culture medium (without cellulose) was the same at the beginning of all cycles. At the end of each repeated batch culture cycle, the BC-free culture medium was discharged (remaining BC) and mixed with freshly sterilized medium to reach the volume of the initial culture medium cycle. Then its pH and glucose concentration was regulated at pH=5 and 10 g/L, respectively, and brought back to the vessel by a peristaltic pump under sterile conditions. Repeated batch culture cycles can be continued until the efficiency of the process decreases. For this purpose, the effect of the number of repeated batch culture cycles at five levels (cycles 1, 2, 3, 4, and 5) was investigated on the production and mechanical properties of the produced BC. Then, the effect of aeration on BC production improvement and its mechanical properties was investigated in 0.1 vvm.

Treatment and drying bacterial cellulose

To purify BC, the final culture medium was first shaken vigorously to separate the bacterial cells attached to the cellulose sheets. Then, BC sheets were harvested and washed twice with distilled water to remove the residual culture medium. In the following, the BC sheets were treated with NaOH solution 0.1 M at 90 ℃ for 1 hour. After that, the BC sheets were rinsed again with distilled water until the pH of the water became neutral. Prepared BC sheets were placed on a glass plate and dried at 80 °C for 1-2 days.

Analytical method

The glucose concentration was enzymatically analyzed using kits (ChemEnzyme CO., I.R. Iran). The BC production by *Acetobacter xylinum* was confirmed by FT-IR spectroscopy. The morphology of the produced BC was determined using Scanning Electron Microscopy (SEM). The BC concentration was determined by the BC dry weight measurement. A universal instrument (2126 HIWA, Hiwa Company, Iran) was used to measure tensile strength. The speed of tension tests was adjusted at 2 mm/min with spam of 35 mm. The samples of each BC layer were cut in rectangular shape (10 cm × 2 cm) with a paper cutter.

RESULTS AND DISSCUSION

Effect of volume to surface area ratio and cultivation time

Table 1 shows that a maximum of 7.12 g/L BC was produced in vessel 2 with a volume to the surface area ratio (depth) of 1.6 cm. Then, as the depth of the culture medium increased, BC production decreased significantly, and in vessel number five with a deeper culture medium (4 cm depth), BC just formed like a circle around the vessel wall. By the way, by increasing the depth of the culture medium, the time of cellulose pellicle appearance on the surface medium was delayed (Table 1). Hence, the productivity of BC declined in deeper culture medium.

According to observations, in the first few days of culture, the thickness of cellulose produced increased rapidly but decreased with time. Therefore, following the study of BC production optimization, the effect of cultivation time on production was examined in three levels of one, two, and three weeks in optimized depth. Fig. 1 indicates that BC production has diminished over time. The highest amount of BC production productivity was achieved at one week with a culture depth of 1.6 cm.

On the other hand, the study of the presence of microorganisms in the BC sheets and the culture medium at the end of the cultivation process showed that a significant part of the bacteria along with the culture medium was located in the bacterial cellulose network. The number of bacteria in BC sheets and culture medium after 7 days was 1.39×10^6 and 0.21×10^6 cells per ml, respectively, which is comparable with a bacterial cell number in the inoculum of the initial batch culture. Also, the produced BC sheets cover the surface of the culture medium and limit the oxygen transfer from the air to the culture medium. As a result, the consumption of culture medium is limited, and the BC production rate reduces. Therefore, a repeated-batch process was developed to inoculum development time reduction and increase cellulose production efficiency using an unconsumed culture medium containing sufficient amounts of *A. xylinum* as the new inoculum.

Development of new repeated static batch culture

Due to the presence of microorganisms and nutrients in the BC network and the oxygen mass transfer limitation in the presence of cellulose layers, a new repeated-batch culture was developed for productivity enhancement. In the new strategy, BC free culture medium was discharged in a sterile vessel and mixed with fresh medium to obtain 1-liter medium (equal optimized depth 1.6 cm), and its pH and glucose concentration was adjusted to 5 and 10 g/L, respectively. A powerful factor in the performance

Table 1: The effect of the volume to surface area ratio (depth) of the culture medium at five levels on BC production in static batch culture of A. xylinum PTCC 1734 with cultivation time 2 weeks.

Experiment number	1	2	3	4	5
Volume to surface ratio (cm)	0.8	1.6	2.4	3.2	4
Volume (L)	0.5	1	1.5	2	2.5
BC production (g/L)	6.96±0.01	7.12±0.02	5.81±0.01	3.93±0.04	2.65±0.02
BC appearance time (day)	3	3	5	8	10

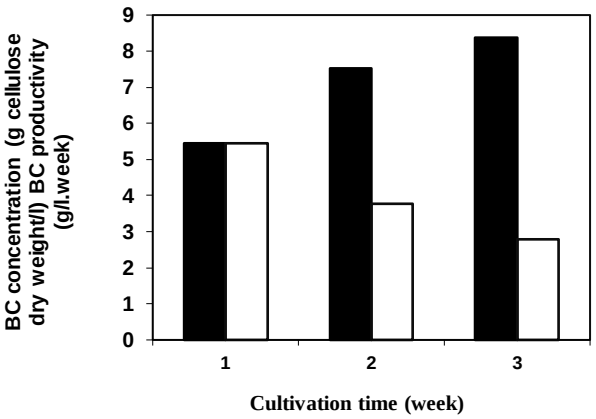

Fig. 1: Effect cultivation time of static batch culture of Acetobacter xylinum on concentration (black column) and productivity (white column) of BC.

of the repeated static batch fermentation system in BC production is the entry point of the fresh medium.

Due to the trapping of a significant proportion of microorganisms within the cellulose network, and the lower density of BC relative to the culture medium and its placement on the surface that prevents efficient transfer of oxygen to the culture, a mixture of fresh and unused culture medium was added on top of the cellulose layer. Also, the experimental results showed that when a fresh feed added at the top of cellulose layer, BC production increases significantly respect to the feed entrance from bottom. Produced BC in second cycle in two case of fresh medium addition from top and below of cellulose layer were 9.32 and 7.13 g/L, respectively.

Optimization of repeated static batch culture

The effects of the cycle number on amount of produced BC

The number of cycles is the most important parameter in the overall productivity of repeated batch cultivation. Therefore, repeated batch cultures were performed in 5 different cycles according to the dimensions of the test vessel. Table 2 displays the effects of number of cycles on amount of BC production and BC productivity. Harvesting the remaining culture medium at the end of each cycle and mixing it with fresh culture medium and then adding it to the culture vessel resulted in the deposition of the previous cellulose layers (Fig. 2).

By increasing the number of cycles, the volume of the remaining culture medium at the end of each cycle decreases, and the amount of cellulose produced in each cycle increases until the fourth cycle and then decreases. This has led to a more than 50% increase in BC productivity. Also, by increasing the number of cycles, and consequently increasing only about 3.5 times the volume of culture medium, BC production increased more than 7 times from 5.1 to 41 g/L.

In addition, as the number of cycles increased to 4, BC production rate and consequently production efficiency increased but decreased further by 4 to 5.

In this process, the main reason for ending the process in the fifth cycle was the lack of residual culture medium to be used as the next cycle inoculum, as well as the limited space of culture vessels to add a new culture medium at the end of the fifth cycle.

The effect of aeration on increasing the production of bacterial cellulose

In the previous section, it was observed that after three cycles of adding the culture medium, the volume of free space of the culture vessel decreases due to increasing the volume of culture medium and cellulose production, and as a result, the surface oxygen transfers to the culture medium and consequently BC production decreases. Therefore, the effect of aeration on the BC production increase was investigated with an aeration rate of 0.1 vvm at the start of the process and at the beginning of the fifth cycle, which reduced the amount of production.

Table 2: Effect of cycle number in the repeated static batch culture without aeration on production amount and productivity of BC.

Cycle number	1	2	3	4	5
Added fresh medium (ml)	-	300	540	740	900
Residual culture medium at the end of each cycle (ml)	700	460	260	100	-
Final volume of culture medium (ml)	1000	1300	1840	2580	3480
final amount of BC production (g dry weight)	5.61±0.02	12.67±0.03	22.3±0.02	33.7±0.02	41.15±0.03
Final BC concentration (g dry weight/L)	5.61±0.02	9.74±0.03	12.11±0.02	13.06±0.02	11.82±0.03
BC productivity (g/L.week)	5.61	6.34	7.43	8.425	8.23

Table 3: Effect of cycle number in the repeated static batch culture with aeration on production amount and productivity of BC.

Cycle number	1	2	3	4
Added fresh medium (ml)	0	700	810	900
Final volume of culture medium (ml)	1000	1700	2510	3410
final amount of BC production (g dry weight)	8.56±0.02	19.74±0.03	33.3±0.02	44.2±0.02
Final BC concentration(g dry weight /L)	8.56±0.02	11.61±0.03	13.27±0.02	12.96±0.02
BC productivity (g/ L.week)	8.56	9.87	11.11	11.05

Fig. 2. BC production from Acetobacter xylinum in repeated static batch culture after 3 cycles.

Table 3 shows that aeration at the beginning of the cultivation process, the amount of BC production in all cycles increases, and production time reduces. On the other hand, due to the significant increase in production and consequently restriction of the volume of the cultivation vessel, the fermentation process continued until the fourth cycle. In the case of aeration from the beginning of the fifth cycle, the BC production in the fifth cycle increased by 15% and reached about 13.5 g/L. This result suggests the use of containers with more depth, volume, and aeration can significantly increase by reducing the time of inoculum preparation.

Effect of cycle number and aeration of repeated static batch process on shear stress and Young's modulus of the BC sheets

Table 4 shows shear stress and Young's modulus of the produced BC sheets in different cycles of the repeated static batch process with and without aeration. It can be found that increasing the number of cycles causes a greater difference in the mechanical properties of the produced cellulose sheets. Probably because the first sheets are more interconnected due to their more contact with the microorganisms of the nanofibers they form. Also, as the fermentation time increases and consequently the BC production amount increases, the thickness of the produced cellulose sheets enhances, which may affect the mechanical properties. Furthermore, aeration increases shear stress and Young's modulus of the produced BC sheets in all of the cycles. Increased rate and amount of BC production may lead to more compression and hardening of the BC sheets. Measurement and comparison of the wet and dry weight of the BC produced in the repeated batch cultivation with and without aeration also showed that despite the same wet weight, the dry weight is higher in the aerated conditions.

FT-IR spectrum of the BC film produced in this study has been presented in Fig. 3. The band that appeared at around 3480 cm^{-1} is ascribed to the –OH stretching vibration in the sugar residues of the cellulose [13].

Table 4: Effect of cycle number of repeated static batch process with and without aeration on shear stress and Young's modulus of the produced BC sheets in different feeding cycles.

order of BC sheet production	shear stress (MPa)		Young's modulus (GPa)	
	Without aeration	With aeration	Without aeration	With aeration
first	320	355	2.65	2.75
second	293	315	2.54	2.62
third	275	290	2.45	2.53
forth	252	254	2.37	2.36
fifth	235	_	2.18	_

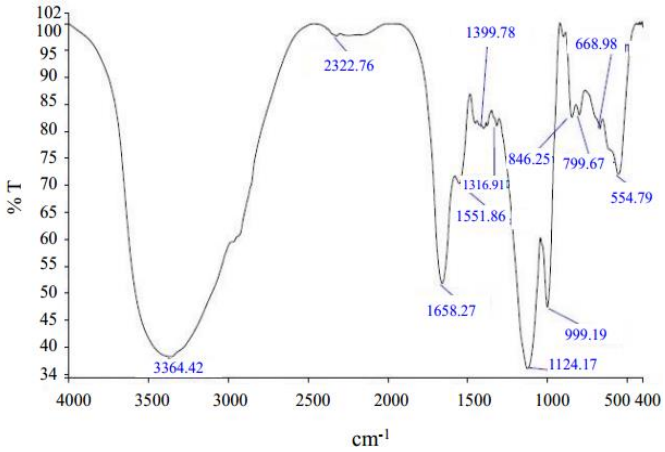

Fig. 3: FT-IR spectra of produced BC from repeated static batch culture of Acetobacter xylinum.

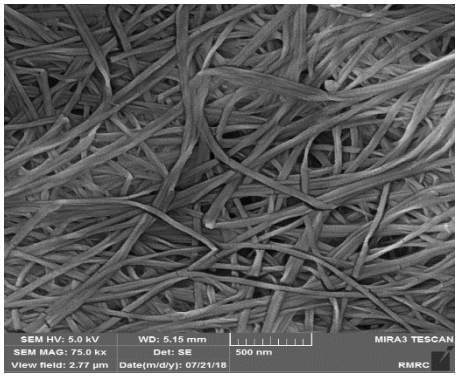

Fig. 4: SEM image of the produced BC from the third cycle of repeated static batch culture without aeration Acetobacter xylinum.

The band at 2890 cm^{-1} is related to the C─H stretching vibration of alkanes [15]. Two absorption bands at 1430 cm^{-1} and 1364 cm^{-1} originate from CH_3 bending [13]. The band at 1160 cm^{-1} attributes to C-O-C skeletal vibration [18]. These absorption bands confirmed the successful production of BC in our research. SEM image in Fig. 4 gives a picture of nano fibril structure of BC production in static batch culture. This fibrillar network provides desired porosity which enhance the water uptake capacity of the produced BC.

Discussion

As mentioned in the previous section, BC production from Acetobacter xylinum culture was performed statically. Due to medium homogeneity and mass transfer via diffusion phenomena in static culture [17, 18], the volume to surface area ratio (depth) of culture medium is a critical factor that influences production yield significantly. In this study, it was found that the maximum production of BC in a container containing 1 liter of culture medium obtains with a volume to surface ratio of 1.6 cm.

Results presented in Table 2 indicate that the BC productivity increases to fourth cycle and then decreases. The initial increase in yield is probably because with the increase in the number of cycles and the subsequent presence of cellulose layers containing significant amounts of bacteria in previous cycles, the presence of microorganisms in the culture medium increases at the beginning of the initial cycles. Also, the cellulose layers of previous cycles become thicker with increasing time due to the significant presence of bacteria and the availability of a new culture medium. Decreasing BC productivity from the fourth cycle onwards may be due to limited free space above the culture medium with increasing the number of BC sheets, limited oxygen transfer, reduced amount of culture medium remaining at the end of the cycles, and increased viscosity of the environment [19-21].

On the other hand, over time, the pH of the culture medium decreases to 3.5, which is less than the optimal

value for the growth of *Acetobacter xylinum* (pH = 5) [21]. Decreasing pH is related to acetate production by *Acetobacter xylinum* which its growth is inhibited by the residual concentration of acetate above 2 g/L [15]. Also, along with an increase in cultivation time, the growth rate of bacteria and its ability to the production of BC decrease due to harsh conditions and dissolved oxygen limitation.

As mentioned earlier, the static culture method has limited mass transfer, which is intensified by the emergence and increase of BC gelatin molecules on the surface of the culture medium. The produced pellicle BC covers the culture medium thoroughly and consequently restricts oxygen permeation into the medium culture. On the other hand, there are problems with the use of agitated culture medium, including the production of mutants with cellulose deficiency, which reduces the productivity of BC. Therefore, for overcoming the discrepancy in the increasing of BC layer thickness, optimum culture time was found in developed static batch culture. However, static culture is still the preferred method for high cellulose production due to the production of higher quality cellulose. Of course, to solve these problems and increase BC production, various solutions have been used, including increasing the surface to volume ratio of the culture medium, using a repeated batch process, and optimizations cultivation conditions.

The BC produced absorbs a significant portion of the culture medium and the bacteria that multiply during the culture process, and therefore a small amount of them are deposited under the cellulose sheets. On the other hand, agitating and mixing the residual culture medium of each batch culture with fresh medium increases dissolved oxygen content of culture medium for starting the subsequent cycle. Also, measurement of bacterial cell number in BC sheets and remaining medium in batch culture showed that there are enough bacteria for inoculum.

Therefore, if at the end of each batch cycle the BC layer is removed from the culture medium, a significant portion of the bacteria is removed, and the remaining culture medium alone is not sufficient to inoculate the next cycle. For this reason, instead of harvesting the constant percent of the culture medium, the total residual culture medium at the end of each batch cycle was used as inoculation of the new next batch cycle. However, significant amounts of bacteria in the cellulose layers formed in previous cycles, which are placed at the bottom of the culture dish with the addition of a new culture medium, also help to increase the efficiency of cellulose production in subsequent cycles. The increase in BC productivity in subsequent cycles was a confirmation of the efficiency of this method to increase the production of discontinuous cultures.

The results (Tables 2 and 3) show that the developed repeated batch cultivation process not only increases the amount and yield of BC production, but also can improve the BC production in a deeper culture medium or more volume culture vessel. The volume to the surface ratio (depth) of the culture medium is one of the limitations in the scale-up of BC production in the static batch culture of *Acetobacter xylinum*. In the new repeated static batch culture developed in this study, BC production in the case without aeration increased from 5.61 to 41.11 g with only a 3.6-fold increase in culture medium volume and the same surface area. Producing this amount of BC by the same static batch culture requires a container with 7.3 times the surface area. Also, this strategy allows sufficient inoculation to be provided for the next cycle, resulting in higher productivity BC production.

In this method, both cellulose-free residual culture medium and the produced BC sheets in the previous cycles were used in the next batch cycle. Therefore, all bacterial cells produced in the previous cycles were used in subsequent batch culture cycles. This increase in the number of cells in each subsequent culture cycle led to the possibility of increasing BC production even in a deeper culture medium. On the other hand, adjusting only pH and glucose content on initial values provide suitable conditions for BC production which shows that other components of the culture medium were in suitable concentration in every cycle of the process. So, we achieved a similar pattern for initial glucose concentration in all cycles and prevented the reduction of BC production upon glucose concentration reduction in each cycle to the previous one. On the other hand, reusing the fermented culture medium reduced the needed culture medium.

Increasing BC production in a static batch culture requires more surface area [21] while using this method without enlarging the surface of the culture medium, it was possible to cultivate at higher volumes. If we want to produce this amount of BC by static batch culture, a surface 7.3 times bigger than this is needed. Using repeated static batch culture can reduce cleaning, sterilization,

and inoculum preparation steps. On the other hand, by using more and more culture medium, the cost in consumption and preparation of cultivation environment was saved.

Compared to recent research reported, BC productivity obtained in this study is one of the highest values. By increasing the number of cycles in the repeated batch culture, the volume of free space above the culture medium and subsequently the mass transfer of its oxygen into the culture medium decreases. In this study, it was shown that forced surface aeration can increase production efficiency to a greater extent. In fact, in repeated batch cultures with aeration, BC can be produced with more productivity in containers with higher volumes without the need to change the cross-section.

Comparison of mechanical properties of cellulose sheets produced in repeated batch culture with and without aeration showed that with increasing the retention time of cellulose sheets in the culture vessel, Young's modulus and their shear stress increase so that the highest Young modulus and shear stress is related to cellulose sheets produced in the first cycles, and then the reduction must be until the last cycle. This may be because by adding a fresh culture medium at the beginning of the cycles, the bacteria trapped in the cellulose network gain access to the new culture medium, and as a result, the cellulose fibers become more entangled.

CONCLUSIONS

BC production in repeated static culture was compared with common static batch culture. Using new repeated static batch culture resulted in more yields and running the process in a higher volume to surface area ratio (deeper culture medium). On the other hand, this method eliminates the need for large surface area in BC production in a static manner. Repeated static batch culture not only increases the production yield but also eliminates the time and cost required for inoculum preparation and vessel cleaning, increasing the efficiency of the process. According to the results obtained from the high production of cellulose in this repeated batch process developed in this research, it can also be used to increase the microbial production of other solid exopolysaccharides.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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