Preparation of *Komagataeibacter xylinus* inoculum for bacterial cellulose biosynthesis using magnetically assisted external-loop airlift bioreactor

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Abstract

The aim of this study was to demonstrate the applicability of a novel magnetically-assisted external-loop airlift bioreactor (EL-ALB), equipped with RMF generators for the preparation of *Komagataeibacter xylinus* inoculum during three-cycle repeated fed-batch cultures, further used for bacterial cellulose (BC) production. The fermentation carried out in the RMF-assisted EL-ALB allowed to obtain an inoculum of more than 200x higher cellular density compared to classical methods of inoculum preparation. The inoculum obtained in the RMF-assisted EL-ALB was characterized by a high and stable metabolic activity during repeated batch fermentation process. The application of the RMF-assisted EL-ALB for *K. xylinus* inoculum production did not induce the formation of cellulose-deficient mutants. It was also confirmed that the ability of *K. xylinus* to produce BC was at the same level (7.26 g/L of dry mass), regardless of inoculum age. Additionally, the BC obtained from the inoculum produced in the RMF-assisted EL-ALB was characterized by reproducible mechanical strength, nanostructure and total crystallinity index. The results obtained in this study may find multiple applications in any biotechnological processes requiring a high-quality bacterial inoculum.

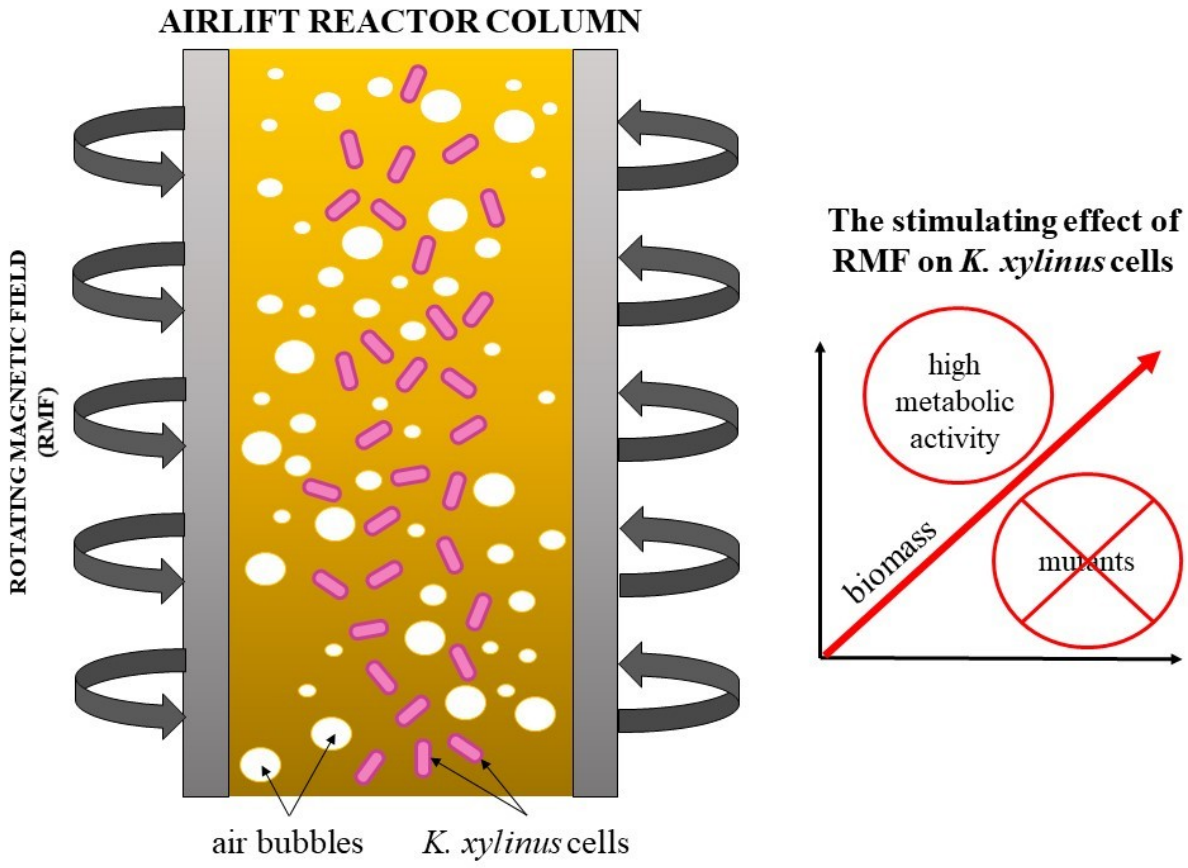
Keywords: rotating magnetic field, airlift bioreactor, inoculum, bacterial cellulose, fermentation.

Highlights

- A magnetically-assisted external-loop airlift bioreactor was used to produce *K. xylinus* inoculum.
- A higher cell density was obtained compared to conventional methods.
- The inoculum of *K. xylinus* cells was characterized by a high and stable metabolic activity.

- The number of cellulose-deficient mutants did not increase during fermentation.

Graphical Abstract



1. Introduction

Bacterial cellulose (BC) is a polysaccharide polymer synthesized by a variety of bacteria, including non-pathogenic *Komagataeibacter* genus (formerly known as *Gluconacetobacter*). BC has attracted significant interest thanks to its unique physical and chemical properties. Such characteristics as high tensile strength, extremely hydrophilic surface, unique nanostructure, excellent biodegradability and biological affinity make BC a promising material in a broad spectrum of applications, including medicinal dressings and

food production, to name just a few (Azeredo, Barud, Farinas, Vasconcellos & Claro, 2019; Ullah, Santos & Khan, 2016; Wang, Tavakoli & Tang, 2019). Nevertheless, a wide application of BC depends on such practical considerations as the scaling-up capability and process cost reduction.

The current methods of BC production are based mainly on static and agitated cultures, as well as various types of airlift bioreactors. Regardless of the type of culturing, the biosynthesis process is carried out as semi-continuous, continuous or fed-batch fermentation and it must always be preceded by inoculum preparation (Chao, Ishida, Sugano & Shoda, 2000; Gorgieva & Trček, 2019; Ullah et al., 2016). Inoculum preparation is a process in which dormant microbial cells are introduced from a stock culture to a favorable environment and grow to form a metabolically active microbial population. Such cells can be used for inoculation in the productive stage in a bioreactor (Sood, Singhal, Bhat & Kumar, 2011). An appropriate inoculum of microorganisms consists of a sufficient volume of a metabolically active culture retaining its product-forming ability (Nguyen, Ton & Le, 2009; Stanbury, Whitaker & Hall, 2017; Żywicka et al., 2020). Inoculum preparation is an extremely important stage in every biotechnological process using live cells and it directly affects the efficiency of fermentation. According to Webb and Kamat (1993), the variability in the yield and productivity of the fermentation process can be attributed to a poorly controlled process of initial inoculation. In other words, the quantity and quality of the inoculum have a significant impact on the quantity and quality of the final product (Blasco et al., 2020; Rajput & Sheikh, 2019). Although the above statement concerns also the production of BC, with the exception of a single scientific publication by Wang, Xiang, Wang and Li (2016), there are presently no reports on the optimization of *K. xylinus* inoculum preparation or comparisons of inoculum preparation methods.

An inoculum of *K. xylinus* is most often prepared in a static or shaken culture (Atwa, El-Diwany, El-Saied & Basta, 2015; Wang et al., 2016; Żywicka et al., 2018). Both methods have significant limitations, especially when the inoculation concerns several thousand liters of the fermentation medium (the volume applied in industrial-scale BC production). The static culture method results in the accumulation of a gelatinous membrane produced at the air-liquid interface. After incubation, in order to remove the cells embedded in the BC, the membrane is vigorously shaken or homogenized, or enzymatically digested (Atwa et al., 2015; Hornung, Ludwig, Gerrard & Schmauder, 2006; Ruka, Simon & Dean, 2012). The obtained cell suspension can then be used as an inoculum in another fermentation process. However, such methods provide insufficient inoculum volume of cells with a relatively low metabolic activity. As reported by Hornung et al. (2006), only some of the total number of cells which are immobilized within the aerobic zone (of approximately 1 mm in thickness), are able to produce BC. A previous study performed by our research group confirmed that due to the limited availability of oxygen in the deeper layers of the BC membrane, the number of living cells was relatively low as compared to its top layer (Żywicka et al., 2018). During static culturing, the floating cellulose pellicle becomes a barrier for the glucose and oxygen transfer in the later stages of its synthesis (Hornung et al., 2006). Therefore, bacteria in deeper zones are inactive and cannot synthetize BC. Equally important is the time needed to prepare a sufficient amount of inoculum, which in the case of static culture is relatively long and lasts at least a few (usually 7-10) days (Atwa et al., 2015; Wang et al., 2016; Żywicka et al., 2018). In contrast to static cultures, the main advantage of an agitated culture for inoculum preparation is a high bacterial cell concentration thanks to the increased rate of transfer of substrates and oxygen. Therefore, shaken culture requires less time to obtain a high number of bacterial cells (Ha, Shah, Ul-Islam, Khan & Park, 2011). However, intensive agitation and aeration of a *K. xylinus* culture can significantly affect the induction of a spontaneous

transformation of cellulose-producing bacteria into non-cellulose-producing (Cel^{-}) mutants, thereby significantly reducing the efficiency of the production process. Despite these disadvantages, the agitated method is the only one that can provide a sufficient volume and density of *K. xylinus* biomass, needed for an industrial-scale BC production (Ha et al., 2011; Wang et al., 2016).

The use of various types of force fields (e.g. magnetic, electrical, ultrasound) is a promising alternative to traditional methods of intensification of biomass production. It was already shown that static magnetic fields (SMF) display a high potential for stimulating the growth of certain microbial species and for increasing the level of enzyme production (Kthiri et al., 2019; Muniz, Marcelino, Motta, Schuler & Motta, 2007; Tomska & Wolny, 2008). In turn, recent reports of our team and other researchers indicate the applicability of the rotating MF (RMF) for various biotechnological processes, including BC biogenesis (Drozd, Rakoczy, Wasak, Junka & Fijałkowski, 2018; Drozd et al. 2021; Fijałkowski et al., 2016; Fijałkowski, Żywicka et al., 2017; Fijałkowski, Żywicka et al., 2015).

In our previous studies, we have proved that exposure to the RMF causes a significant increase in growth and metabolic activity of such versatile microorganisms as *Staphylococcus aureus, Streptococcus mutans*, *Staphylococcus xylosus*, *Escherichia coli, Serratia marcescens, Cronobacter sakazakii,* and *Klebsiella oxytoca* (Fijałkowski, Drozd et al., 2017; Fijałkowski, Nawrotek, Struk, Kordas & Rakoczy, 2013; Fijałkowski, Nawrotek, Struk, Kordas & Rakoczy, 2015). Similar results were obtained for different strains of *K. xylinus* capable of producing BC (Drozd et al., 2021; Fijałkowski et al., 2016; Fijałkowski, Żywicka et al., 2017; Fijałkowski, Żywicka et al., 2015). Additionally, the application of RMF-assisted bioreactors (described in our previous studies) allowed to significantly increase the BC yield in comparison with a conventional static process (Drozd et al., 2021; Fijałkowski et al., 2016; Fijałkowski, Nawrotek et al., 2015; Fijałkowski et al., 2013; Fijałkowski, Żywicka et al.,

2017; Fijałkowski, Żywicka et al., 2015). Noteworthy, the application of the RMF did not increase the number of mutants unable to produce cellulose within the exposure time of 72 h (Fijałkowski et al., 2016). It was also found that the observed effect was correlated with the time of magnetic exposure during *K. xylinus* cultivation (Fijałkowski, Drozd et al., 2017).

The hypothesis behind the current study was that the application of an RMF-assisted bioreactor enhances the quality and quantity of *K. xylinus* inoculum, which translates into high yield of the obtained BC polymer. The experiments of our and other research teams performed so far concerned the application of RMF-assisted bioreactors only in laboratory scale due to unresolved technological and operational constraints, related directly to the RMF generator (Fijałkowski, Drozd et al., 2017; Fijałkowski et al., 2013; Fijałkowski, Nawrotek et al., 2015; Fijałkowski et al., 2016; Konopacka, Rakoczy & Konopacki, 2019). Because of these constraints, the RMF-assisted bioreactors presented in previous studies allowed to conduct the process of BC production in tanks of no more than 3 L volume. In the current study we present the newly developed magnetically-assisted external-loop airlift bioreactor (EL-ALB), equipped with the RMF generators, enabling BC production in a significantly increased (up to 100 L) volume of the process chamber.

Performed prior to this study, the analyses of fluid hydrodynamics showed the enhancement of such hydrodynamic parameters as downcomer liquid velocity, mean liquid circulation velocity, mean circulation time and mixing time in RMF-assisted EL-ALB in comparison with an EL-ALB without the application of the RMF (Lechowska et al., 2019). These results suggest that the application of the RMF-assisted EL-ALB could improve the mass transfer rate during the fermentation process and thus increase the rate of *K. xylinus* cell multiplication. Therefore, the aim of this study was to demonstrate the applicability of the novel magnetically-assisted external-loop airlift bioreactor for the preparation of *K. xylinus* inoculum in repeated batch fermentation process, subsequently used for BC production.

2. Material and methods

2.1. Experimental apparatus

For the production of inoculum, a magnetically assisted external-loop airlift bioreactor (EL-ALB) equipped with RMF generators was used. The experimental apparatus consists of a cooling liquid chamber and upper chamber (separation zone), rising and falling columns, magnetic field generator and the necessary measurement equipment and installations **(Fig. 1)** (Lechowska et al., 2019). The concept and design of RMF-assisted EL-ALB was based on the results of our previous research (Drozd et al., 2021; Fijałkowski et al., 2016; Fijałkowski, Żywicka, et al., 2017; Fijałkowski, Żywicka et al., 2015).

2.2. Inoculum preparation in RMF-assisted EL-ALB

The inoculum preparation was carried out during three-cycle repeated fed-batch cultures using *Komagataeibacter xylinus* ATCC 53524 (American Type Culture Collection) strain. A standard culture medium for BC production Hestrin–Schramm (H–S) was used for fermentation (glucose —2 w/v%, yeast extract — 0.5 w/v%, bacto-pepton — 0.5 w/v%, citric acid — 0.115 w/v%, Na_2HPO_4 — 0.27 w/v%, $MgSO_4 \cdot 7H_2O$ — 0.05 w/v% and ethanol — 1 v/v).

The scheme of the experiment is presented in **Fig. 2.** Initially, several colonies of *K. xylinus* were transferred from an agar plate to the H–S medium in order to obtain cell concentration of 2×10^5 Colony Forming Units (CFU/mL). In the first cycle, 1 v/v% of *K. xylinus* suspension was added to 40 L of a sterile fermentation medium. The inoculum preparation process was carried out in RMF-assisted EL-ALB at 28 °C with constant aeration at the level of 0.05 vvm (the vol. of air/bioreactor working vol. per min). *K. xylinus* cells were

exposed to RMF for 12 h in each subsequent 24 h of fermentation. The magnetic induction was equal to 16 mT. The entire process was carried out for 168 h. The bacterial culture was exposed to the RMF as it passed through the rising and falling columns of the bioreactor system. Exposure parameters were selected on the basis of our previous research, considering the effect of the exposure to the rate of cell proliferation as well as the cost consumption associated with the generation of the magnetic field (Fijałkowski, Drozd, et al., 2017; Fijałkowski et al., 2013; Fijałkowski, Nawrotek et al., 2015; Konopacka et al., 2019; Lechowska et al., 2019).

After the first batch lasting 96 h, 35 L of the medium was removed from the RMF-assisted EL-ALB. Next, the bioreactor was supplemented with a fresh medium to the initial volume (40 L) for the second batch. The second and third batch was carried out for 36 h under the same conditions as described above **(Fig. 2).** The duration of the first fermentation cycle was chosen on the basis of our preliminary study which showed that the number of *K. xylinus* cells reached a maximum value after 96 h of fermentation (after 96 h no further increase was noticed within the next 48 h of incubation) **(Fig. S1).** Subsequent fermentation cycles were carried out until the number of *K. xylinus* cells reached the values from the first cycle.

2.3. Assessment of fermentation parameters

During fermentation, the pH of the medium was measured using CX701 Multifunction meter (Elmetron, Poland). Dissolved oxygen (DO%) was also controlled using a ProODO Optical Dissolved Oxygen Instrument (YSI Inc., China). Measurements were performed every 12 h.

2.4. Evaluation of inoculum quality produced in the RMF-assisted EL-ALB

2.4.1. Determination of the number of living cells

The number of living cells was determined by quantitative plating. For the test, serial dilutions of the bacterial suspension taken from the bioreactor were spread on Petri dishes containing the H–S agar medium. After 48 h of incubation at 28 °C (Galaxy R PLUS CO2 Incubators, RS Biotech, UK), the number of CFU per 1 mL was determined.

2.4.2. Determination of the cells' metabolic activity

In order to determine the metabolic activity of *K. xylinus* cells, the AlamarBlue (Life Technologies, USA) assay was performed. For the test, 50 mL of the sample taken from the bioreactor was centrifuged for 20 min at 3.300 x *g* (Centrifuge 5804 R, Eppendorf, Germany). The resulting pellets were washed in PBS (Phosphate Buffered Saline, Sigma–Aldrich), centrifuged again at 3.300 × *g* for 20 min and restored to 1 mL of PBS. Next, 100 µL of bacterial suspension was transferred to a 96 well plate (Becton Dickinson and Co., USA) and 10 µL of AlamarBlue reagent was added to the wells. The plate was incubated for 45 min at 28 °C. The fluorescence signal was measured using microplate fluorescence reader at wavelengths of 530 nm (excitation) and 590 nm (emission).

2.4.3. Determination of cellulose-deficient (Cel^{-}) mutants

The number of Cel^{-} mutants was determined in the culture medium taken from the bioreactor during the fermentation process by performing quantitative plating as describe in the section *Determination of the number of living cells*. Next, the grown colonies were flooded with 20 mL of PBS with 0.01% of Tinopal LPW dye (Calcofluor White M2R, Tinopal UNPA-GX, Sigma-Aldrich, Germany) and incubated for 24 h in darkness. The colonies were examined using an UV Cabinet (CAMAG, Switzerland) at a wavelength of 366 nm. BC fibrils stained with a fluorescent dye were observed only around the cellulose-producing colonies (in contrast to the Cel^{-} mutants).

2.4.4. Assessment of the cells' ability to produce BC

Every 24 h of the fermentation process, 100 mL of the bacterial suspension was taken from the bioreactor and transferred into the Petri dishes (dimensions: 150 x 20 mm) with vents. The BC production was carried out under static conditions for 5 days at 28 °C without any interruptions. After incubation, the BC was harvested from the media and weighed on an analytical balance (WTB 2000**,** Radwag, Poland). Next, the BC pellicles were purified by treatment with 0.1 M NaOH (Chempur, Poland) at 90 °C for 30 min to remove bacterial cells and media components and rinsed with sterile water until the pH become neutral.

2.5. Evaluation of the classical methods of inoculum preparation

In order to compare the effectiveness of inoculum production using the RMF-assisted EL-ALB, the standard stationary and agitated conditions were evaluated. For this purpose, 1 v/v% of *K. xylinus* suspension at a concentration of 2×10^5 CFU/mL was added to 100 mL of the H–S medium. The culture was carried out in stationary conditions for 7 days or in agitated conditions for 4 days at 28 °C. Then, the obtained BC was digested with the enzyme cellulase (100 µL/1 mL 0.05 M citrate buffer, pH 4.8, Sigma-Aldrich, Germany). The number of living cells in the bacterial suspension was determined as described in the section *Determination of the number of living cells*. Additionally, the cells' ability to produce BC was also analyzed. For this purpose, the bacterial suspension obtained after the digestion of BC with the cellulase enzyme (Sigma-Aldrich, Germany) was washed twice in a fresh H–S medium in order to remove the enzyme. Next, the inoculum was restored to 100 mL of the H–S medium and used as described above in the section *Assessment of the cells' ability to produce BC.*

2.6. Analysis of physicochemical properties of BC depending on the age of the inoculum produced in RMF-assisted EL-ALB

2.6.1. Mechanical strength

Purified and wet BC samples were cut into bars of 30 mm length and 10 mm width using a pneumatic press machine (CEAST Instron, Massachusetts, USA). The tensile strength test was performed using the MTS Synergie 100® machine (MTS System Corp, Minnesota, USA). The measurements were carried out at a speed of 10 mm/min at room temperature. Based on the recorded values of force (F) and displacement (Δl), stress-strain graphs were prepared based on which tensile strength was determined.

2.6.2. Scanning Electron Microscopy analysis

Purified and wet BC samples were fixed in glutaraldehyde (POCH, Poland) for 7 days and dried in a critical point dryer. Next, the BC samples were sputtered with Au/Pd (60:40) and examined using a scanning electron microscope (SEM, Auriga 60, Zeiss, Oberkochen, Germany). Fibril diameter was analyzed by means of the ImageJ software (NIH, Bethesda, USA).

2.6.3. ATR-FTIR analysis of BC

The BC sheet samples purified and dried at 60 °C for 12 h were analyzed using Attenuated Total Reflectance-FTIR spectroscopy with ALPHA FT-IR spectrometer (Bruker, Germany). After normalization of the obtained spectra at 1160 cm^{-1} wavenumber, the total crystallinity index (TCI) of the dry BC samples was determined as the ratio of absorbance values for bands 1370/2900 cm^{-1} (Drozd, Rakoczy, Konopacki, Frąckowiak & Fijałkowski, 2017).

2.7. Statistical analysis

Data was shown as the means ± standard errors of the means (SEM) obtained from three different measurements. Statistical differences between the samples were determined by one-way analysis of variance (ANOVA). All analyses were considered statistically significant

when the P value was less than 0.05. The statistical analyses were conducted using Statistica 9.0 (StatSoft, Poland).

3. Results and discussion

3.1. Measurements of cellular parameters

The applicability of BC in versatile areas of industry is limited mainly by the relatively low efficiency of its production. Therefore, a number of studies have been performed to improve this parameter. The main approaches included the search for highly effective cellulose producers, modification of their genome, optimization of medium content, fermentation parameters, and construction of customized bioreactors (Florea et al., 2016; Hong et al., 2012; Shezad, Khan, Khan & Park, 2009). Surprisingly, the process of inoculum preparation which is a starting point and one of the main factors determining the outcome of any BC production process, was barely investigated (Wang et al., 2016). Moreover, in a number of articles concerning BC production, the description of this specific stage of fermentation happens to be sketchy or even missing. To overcome this oversight, the current study focuses on a novel way of inoculum preparation.

Our previous studies showed that exposure to the RMF caused a significant increase in the growth rate and metabolic activity of various species of microorganisms, including different strains of *K. xylinus* (Drozd et al., 2021; Fijałkowski et al., 2016; Fijałkowski, Żywicka, et al., 2017; Fijałkowski, Żywicka et al., 2015). Nevertheless, these studies were conducted in static conditions, and with a volume of the culture not exceeding 25 mL. It should be noted that a successful optimization of the process in a laboratory scale does not guarantee an analogical success (with regard to yield, enzymatic activity, etc.) of a pilot-scale or industrial process (Ha, Kim, Seo, Oh & Lee, 2007; Jung, Kim, Moon, Oh & Lee, 2007). Therefore, in the current study, inoculum production was carried out using a new multiphase

apparatus - magnetically assisted external-loop airlift bioreactor (EL-ALB) equipped with RMF generators in the riser and downcomer columns. This RMF-assisted EL-ALB is a fully innovative design which has not been previously used for any biological processes.

The procedure of inoculum preparation must ensure high density of cells, especially if the process is conducted on an industrial scale (Sood et al., 2011). Moreover, the cells should be able to produce a satisfactory and reproducible yield of product (Nguyen et al., 2009; Żywicka et al., 2020). The time of inoculum preparation is also an important parameter due to operational costs of the process. In the current study, the maximum density of cells was $11x10^9$ CFU/mL after 96 h in the first cycle, which was preceded by a 48 h adaptation (lag) phase. The subsequent fermentation cycles were carried out until the number of *K. xylinus* cells reached the values from the first cycle, which was achieved after 36 hours (**Fig. 3A)**. There were no statistically significant differences in the cell density obtained in subsequent fermentation cycles.

Additionally, the AlamarBlue assay was performed to investigate the effect of RMF on the *K. xylinus* cell metabolic activity. The AlamarBlue assay incorporates an oxidation-reduction (REDOX) indicator which changes color and fluorinates in response to cell metabolic activity (Rampersad, 2012). The results showed that the metabolic activity of *K. xylinus* increased dynamically during the first 96 h. Importantly, there were no statistically significant differences in the metabolic activity of the cell suspension in subsequent cycles **(Fig. 3B),** which proves the stability of the process during all fermentation cycles.

In our previous reports it was confirmed that RMF has a stimulatory effect on the metabolic activity and growth of *K. xylinus* cells (Drozd et al., 2021; Fijałkowski et al., 2016; Fijałkowski, Żywicka, et al., 2017; Fijałkowski, Żywicka et al., 2015). It may be explained by the impact of RMF on microfluidic mixing of charged particles within the bioreactor, altering the transfer process between the cell surface/liquid phase and affecting the chemical

composition of the cells (Anton-Leberre et al., 2010; Gaafar, Hanafy, Tohamy & Ibrahim, 2008). Additionally, the use of RMF generators improves the downcomer liquid velocity and mean liquid circulation velocity (Lechowska et al., 2019). Moreover, as also proved by Lechowska et al. (2019), the mean circulation time and mixing time under the RMF action have significantly lower values in comparison with conventional EL-ALB.

The optimal process of *K. xylinus* inoculum preparation requires high multiplication of cells and lack or very low production of BC. It is because the freshly-formed BC fibers can deposit on the elements of the bioreactor, obstructing air supply, sampling or measurements of temperature, pH and DO% (Ha et al., 2011; Hornung et al., 2006; Ruka et al., 2012). The above-mentioned disadvantages can be reduced significantly by the addition to the fermentation medium of enzymes such as various types of microbial cellulases (Wang et al., 2016). However, this approach is also limited by the needs for further deactivation of the used enzymes. In the current study, a small amount of BC was produced during the fermentation process, despite the fact that the cell concentration obtained in the RMF-assisted EL-ALB was high **(Fig. 3A)**. Taking into account the results of our previous study (Lechowska et al., 2019), it may suggest that the application of rising and falling columns in the RMF-assisted EL-ALB improved the hydrodynamic parameters and prevented the formation and deposition of BC. It bestowed the cells with optimal conditions for multiplication without the addition of cellulose-digesting enzymes or inhibitors to the bioreactor.

The results presented in **Table 1** confirmed that fermentation carried out in the RMF-assisted EL-ALB allowed to obtain an inoculum with a significantly higher density of *K. xylinus* cells compared with the bacterial suspension obtained after enzymatic digestion. The application of the RMF-assisted EL-ALB allowed to obtain more than 200 x and 8000 x higher cell density as compared to the bacterial suspension obtained after enzymatic digestion of BC biosynthesized in an agitated and static culture, respectively. It should be noted that the

number of *K. xylinus* cells was determined in the same volume of the H–S medium (100 mL) for each of the inoculum production methods.

The number of cells obtained from BC biosynthesized under agitated conditions was higher as compared to static conditions. The increased number of *K. xylinus* cells obtained in agitation or airlift bioreactors is associated with more oxygen in the medium, as previously confirmed by other authors (Hornung et al., 2006; Ruka et al., 2012; Wu & Li, 2015). However, the application of airlift bioreactors in a fermentation process provides lower shear stress and shutdowns of the medium, compared to agitated cultures. It should be also noted that the inoculum preparation time in the RMF-assisted EL-ALB was shorter as compared to the bacterial suspension obtained after enzymatic digestion.

3.2. Fermentation parameters - pH and DO%

K. xylinus is a strictly aerobic bacterium; thus, an appropriate oxygen supply is crucial for its cultivation (Hornung et al., 2006). An inadequate mass transfer rate of oxygen limits cell growth and product formation (Chao, Sugano & Shoda, 2001; Reiniati, Hrymak & Margaritis, 2017). **Fig. 3B** presents the changes in the amount of DO% in the medium during fermentation. The results show a substantial decrease in the DO% concentration during the first 48 h of fermentation. After 48 h of incubation, DO% remained at a relatively similar level (on average 3%) until the end of the first cycle. DO% increased to 35% and 30% respectively in the second and third cycle of fermentation after the addition of 35 L of a fresh medium. However, the level of DO% decreased rapidly to 3% during the next 24 h in the second and third cycle. Despite the decrease in DO%, the growth rate of *K. xylinus* cells was not affected **(Fig. 3A).** Similar observations were made by Cheng, Wang, Chen and Wu (2002) who revealed that despite DO% drop to 2%, no significant decrease in biomass production was observed. Moreover, the above authors also showed that the increased value of oxygen−transfer coefficient (k_La) during cultivation in an airlift bioreactor positively

influenced the growth rate of *K. xylinus*. Lechowska et al. (2019) showed that the k_La was significantly increased in an RMF-assisted EL-ALB in comparison with a conventional EL-ALB. It should also be mentioned that increasing the k_La is a more economical approach with regard to improving the performance of oxygen transfer than supplying a higher concentration of oxygen in the bioreactor (Cheng et al., 2002).

The pH of the culture medium is one the most limiting factors that can significantly influence *K. xylinus* cell growth and productivity of BC (Kongruang, 2007). Changes in the pH of the medium are associated mainly with the production by *K. xylinus* cells of gluconic and acetic acid during BC biosynthesis, when glucose and ethanol are used as a sole carbon source (Atwa et al., 2015; Hornung et al., 2006; Ruka et al., 2012). Optimal pH values range from 4.5 to 7.5, with the greatest efficiency with regard to BC production being around 6.5. If the pH drops below 3.5, cellulose synthesis is inhibited. It was demonstrated that the pH value of the fermentation medium decreased steadily, reaching approximately 4.5 after each cycle as shown in **Fig. 3B.** It should be noted that during 168 h of fermentation the pH did not drop below the level which could affect the product formation ability of *K. xylinus* cells; therefore there was no need to further stabilize the pH **(Fig. 3B).**

3.3. Determination of cellulose-deficient (Cel^-) mutants

As stated above, the microbial inoculum should be able to retain its ability to form a high yield of product. Several authors confirmed that the ability of eukaryotic and prokaryotic cells used in the fermentation processes to produce useful/valuable metabolites may be lost, especially in the case of repeated culture transfers. Therefore, strain stability is one of the major concerns (Sood et al., 2011; Stanbury et al., 2017).

It should also be mentioned that BC is a natural type of biofilm that protects the cells from adverse environmental conditions (Augimeri, Varley & Strap, 2015). Under optimal conditions provided in a bioreactor, a subpopulation of cells may lose its ability to produce

BC, with such subpopulations being referred to as Cel^- mutants. Several authors reported more frequent appearance of Cel^- mutants in agitated cultures with a high oxygen supply and high volumetric agitation power compared to static conditions (Hornung et al., 2006; Ruka et al., 2012). The frequency of mutant occurrence is one of the most important factors lowering the quality of inoculum produced in shaken cultures.

During three cycles of fermentation, the number of *K. xylinus* colonies which were unable to synthesize cellulose was on average 14.3 x 10^3 CFU/mL, which accounted for approximately 0.0012% of all *K. xylinus* cells present in the culture **(Tab. 2).** As can be seen in **Fig. 4**, luminescent zones were observed around the cellulose-producing colonies. The number of Cel^- mutants which appeared during fermentation in RMF-assisted EL-ALB was lower compared to the results presented by other authors. In the research presented by Park, Jung and Park (2003), the number of Cel^- mutants after 96 h of incubation in agitated conditions (200 rpm) was on average 2 x 10^5 CFU/mL. Wang et al. (2016) showed that after 96 h of incubation in a shaking culture (120 rpm) the number of Cel^- mutants was 4 x 10^6 CFU/mL. Aydin and Aksoy (Aydin & Aksoy, 2014) found that mutant ratio (number of Cel^- /number of total cells) increased along with the shaking rate and the number of batches, obtaining a maximum of 2% in the fifth batch at the speed of 200 rpm.

The results obtained in the current study can be explained by the relatively low DO% level during the process (35–3% of DO) **(Fig. 3B)** being a result of the low air-flow applied during fermentation. The fermentation process in RMF-assisted EL-ALB was carried out with constant aeration at an air-flow rate of 0.05 vvm, which is much lower than the one commonly used for *K. xylinus* cultivation (0.5 - 2 vvm) (Chawla, Bajaj, Survase & Singhal, 2009; Song, Li, Seo, Kim & Kim, 2009; Wang et al., 2019).

3.4. Assessment of cell ability to produce BC

The ability to produce BC was determined for the inoculum obtained in the RMF-assisted EL-ALB and after enzymatic digestion of the cellulose membrane obtained in a static or agitated culture. For BC biosynthesis, the same volume of the H–S medium (100 mL) was used. The results showed statistically significant differences in the amount of the obtained BC depending on the method of inoculum production **(Fig. S2).** The average weight of the BC obtained from an inoculum produced in the RMF-assisted EL-ALB was 940 g/L and 7.26 g/L of wet and dry BC respectively and was on average 60% higher as compared to the BC obtained after enzymatic digestion of the cellulose membrane. It should be mentioned that the enzymatic digestion procedure did not affect the metabolic activity of *K. xylinus* cells **(Fig. S3).** The results of the current study also confirmed that *K. xylinus* ability to produce BC was at the same level regardless of the age of inoculum **(Fig. 5).** There were no statistically significant differences in BC weight obtained in subsequent cycles of the fermentation process.

It should be mentioned that there are no statistically significant differences in the amount of biosynthesized BC, regardless of the number of cells in the inoculum obtained in the RMF-assisted EL-ALB **(Fig. 3A, Fig. 5).** This can be explained by the mechanism of cellulose membrane formation in a stationary culture. BC formation within a static surface culture starts with the formation of island-like cellulose fragments on the broth surface (Hornung et al., 2006). In the next step, the fragments stick together to form a thin cellulose film. Further incubation results in the accumulation of a gelatinous membrane produced at the air-liquid interface, which becomes a barrier for the glucose and oxygen transfer in subsequent stages of its synthesis. At this point of the process, the initial cell concentration in the medium is no longer relevant. Therefore, in line with the available literature data, it can be stated that a higher density of *K. xylinus* cells in the inoculum determines BC formation rate, however only at the beginning of the process (Hornung et al., 2006; Ruka et al., 2012).

Moreover, Rangaswamy, Vanitha and Hungund (2015) claimed that if the inoculum concentration is in excess, there would be competition between the cells in utilizing nutrients, which disrupts bacterial growth and thereby reduces the production of BC.

3.5. Analysis of physicochemical properties of BC

The parameters of the inoculum used in the biotechnological process determine the quantity and quality of the resulting product, as well as the duration of the process and the operating costs (Sood et al., 2011). It has been reported that inoculum age may affect the formation of BC and its properties (Cielecka, Ryngajłło & Bielecki, 2020; Yanti, Ahmad & Muhiddin, 2018). Therefore, in the current study, the physicochemical properties of BC obtained using the inoculum prepared in the RMF-assisted EL-ALB were analyzed.

The results of our study revealed that regardless of the age of the inoculum obtained, BC was characterized by similar mechanical strength, nanostructure and TCI. The results for all analyzed parameters are presented in **Table 3.** SEM analysis confirmed a coherent 3-D network formed by cellulose fibers **(Fig. 6, Fig. S4).** The average diameter of the fibrils determined on the basis of SEM was 53.84 ± 0.78 nm **(Table 3)**. In addition, the average tensile strength of BC was 2.41 ± 0.18 MPa. The analyses showed that BC displayed a TCI of 1.61 ± 0.26 **(Table 3).** There were no statistically significant differences in the analyzed parameters between samples obtained in subsequent fermentation cycles.

4. Conclusions

A successful implementation of a biotechnological process relies directly on the optimization of inoculum preparation, which ensures high efficiency and optimal operating costs. In the current study, a novel magnetically-assisted external-loop airlift bioreactor (EL-ALB), equipped with RMF generators was used for *K. xylinus* inoculum production. The proposed approach allows to significantly increase cell density as compared to the

conventional method. Additionally, *K. xylinus* inoculum was characterized by a high and stable metabolic activity. The application of RMF-assisted EL-ALB did not induce the formation of cellulose -deficient mutants during three-cycle repeated fed-batch cultures. The ability to produce BC was at the same level regardless of inoculum age. Furthermore, the BC obtained using the inoculum produced in the RMF-assisted EL-ALB was characterized by repeatable mechanical strength, nanostructure and total crystallinity index.

It should be mentioned that in the current study, inoculum preparation was carried out during three-cycle repeated fed-batch cultures in the working volume of 40 L. However, the design of the RMF-assisted EL-ALB can be modified relatively easily, which would allow further expansion of scale and the application of different RMF exposure modes during continuous or semi-continuous culturing of bacteria. The application of RMF-assisted EL-ALB in the production of inoculum could possibly eliminate the need for a lengthy, continuous biomass multiplication. The results obtained in this study may find multiple applications in all biotechnological processes requiring a high quality of inoculum.

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Conflict of Interest Statement

All authors declare no conflict of interest.

References

Anton−Leberre, V., Haanappel, E., Marsaud, N., Trouilh, L., Benbadis, L., Boucherie, H., ... & François, J. M. (2010). Exposure to high static or pulsed magnetic fields does not affect cellular processes in the yeast Saccharomyces cerevisiae. *Bioelectromagnetics: Journal of the Bioelectromagnetics Society, The Society for Physical Regulation in Biology and Medicine, The European Bioelectromagnetics Association*, *31*(1), 28-38.https://doi.org/10.1002/bem.20523 .

Atwa, N. A., El-Diwany, A. I., El-Saied, H., & Basta, A. H. (2015). Improvement in bacterial cellulose production using Gluconacetobacter xylinus ATCC 10245 and characterization of the cellulose pellicles produced. *Egyptian Pharmaceutical Journal*, *14*(2), 123. https://doi.org/10.4103/1687-4315.161286

Augimeri, R. V., Varley, A. J., & Strap, J. L. (2015). Establishing a role for bacterial cellulose in environmental interactions: lessons learned from diverse biofilm-producing Proteobacteria. *Frontiers in microbiology*, *6*, 1282. https://doi.org/10.3389/fmicb.2015.01282

Aydın, Y. A., & Aksoy, N. D. (2014). Isolation and characterization of an efficient bacterial cellulose producer strain in agitated culture: Gluconacetobacter hansenii P2A. *Applied microbiology and biotechnology*, *98*(3), 1065-1075. https://doi.org/10.1007/s00253-013-5296-9.

Azeredo, H., Barud, H., Farinas, C. S., Vasconcellos, V. M., & Claro, A. M. (2019). Bacterial cellulose as a raw material for food and food packaging applications. *Frontiers in Sustainable Food Systems*, *3*, 7. https://doi.org/10.3389/fsufs.2019.00007.

Blasco, L., Kahala, M., Tampio, E., Vainio, M., Ervasti, S., & Rasi, S. (2020). Effect of Inoculum pretreatment on the composition of microbial communities in anaerobic digesters producing volatile fatty acids. *Microorganisms*, *8*(4), 581. https://doi.org/10.3390/microorganisms8040581.

Chao, Y., Ishida, T., Sugano, Y., & Shoda, M. (2000). Bacterial cellulose production by Acetobacter xylinum in a 50−L internal−loop airlift reactor. *Biotechnology and bioengineering*, *68*(3), 345-352. https://doi.org/10.1002/(sici)1097-0290(20000505)68:3<345::aid-bit13>3.0.co;2-m.

Chao, Y., Sugano, Y., & Shoda, M. (2001). Bacterial cellulose production under oxygen-enriched air at different fructose concentrations in a 50-liter, internal-loop airlift reactor. *Applied microbiology and biotechnology*, *55*(6), 673-679. https://doi.org/10.1007/s002530000503

Chawla, P. R., Bajaj, I. B., Survase, S. A., & Singhal, R. S. (2009). Microbial cellulose: fermentative production and applications. *Food Technology and Biotechnology*, *47*(2), 107-124.

Cheng, H. P., Wang, P. M., Chen, J. W., & Wu, W. T. (2002). Cultivation of Acetobacter xylinum for bacterial cellulose production in a modified airlift reactor. *Biotechnology and Applied Biochemistry*, *35*(2), 125-132. https://doi.org/10.1111/j.1470-8744.2002.tb01180.x.

Cielecka, I., Ryngajłło, M., & Bielecki, S. (2020). BNC biosynthesis with increased productivity in a newly designed surface air-flow bioreactor. *Applied Sciences*, *10*(11), 3850. https://doi.org/10.3390/app10113850.

Drozd, R., Rakoczy, R., Konopacki, M., Frąckowiak, A., & Fijałkowski, K. (2017). Evaluation of usefulness of 2DCorr technique in assessing physicochemical properties of bacterial cellulose. *Carbohydrate polymers*, *161*, 208-218.https://doi.org/10.1016/j.carbpol.2016.12.063

Drozd, R., Rakoczy, R., Wasak, A., Junka, A., & Fijałkowski, K. (2018). The application of magnetically modified bacterial cellulose for immobilization of laccase. *International journal of biological macromolecules*, *108*, 462-470. https://doi.org/10.1016/j.ijbiomac.2017.12.031.

Drozd, R., Szymańska, M., Żywicka, A., Kowalska, U., Rakoczy, R., Kordas, M., ... & Fijałkowski, K. (2021). Exposure to non-continuous rotating magnetic field induces metabolic strain-specific response of Komagataeibacter xylinus. *Biochemical Engineering Journal*, *166*, 107855. https://doi.org/10.1016/j.bej.2020.107855.

Fijałkowski, K., Drozd, R., Żywicka, A., Junka, A. F., Kordas, M., & Rakoczy, R. (2017). Biochemical and cellular properties of Gluconacetobacter xylinus cultures exposed to

different modes of rotating magnetic field. *Polish Journal of Chemical Technology*, *19*(2), 107-114. https://doi.org/10.1515/pjct-2017-0036.

Fijałkowski, K., Nawrotek, P., Struk, M., Kordas, M., & Rakoczy, R. (2013). The effects of rotating magnetic field on growth rate, cell metabolic activity and biofilm formation by Staphylococcus aureus and Escherichia coli. *Journal of Magnetics*, *18*(3), 289-296. https://doi.org/10.4283/JMAG.2013.18.3.289.

Fijałkowski, K., Nawrotek, P., Struk, M., Kordas, M., & Rakoczy, R. (2015). Effects of rotating magnetic field exposure on the functional parameters of different species of bacteria. *Electromagnetic biology and medicine*, *34*(1), 48-55. https://doi.org/10.3109/15368378.2013.869754.

Fijałkowski, K., Rakoczy, R., Żywicka, A., Drozd, R., Zielińska, B., Wenelska, K., ... & Mijowska, E. (2016). Time dependent influence of rotating magnetic field on bacterial cellulose. *International Journal of Polymer Science*, *2016*. https://doi.org/10.1155/2016/7536397.

Fijałkowski, K., Żywicka, A., Drozd, R., Junka, A. F., Peitler, D., Kordas, M., ... & Rakoczy, R. (2017). Increased water content in bacterial cellulose synthesized under rotating magnetic fields. *Electromagnetic biology and medicine*, *36*(2), 192-201. https://doi.org/10.1080/15368378.2016.1243554

Fijałkowski, K., Żywicka, A., Drozd, R., Niemczyk, A., Junka, A. F., Peitler, D., ... & Rakoczy, R. (2015). Modification of bacterial cellulose through exposure to the rotating

magnetic field. *Carbohydrate polymers*, *133*, 52-60. https://doi.org/10.1016/j.carbpol.2015.07.011

Florea, M., Hagemann, H., Santosa, G., Abbott, J., Micklem, C. N., Spencer-Milnes, X., ... & Ellis, T. (2016). Engineering control of bacterial cellulose production using a genetic toolkit and a new cellulose-producing strain. *Proceedings of the National Academy of Sciences*, *113*(24), E3431-E3440.https://doi.org/10.1073/pnas.1522985113.

Gaafar, E. S. A., Hanafy, M. S., Tohamy, E. Y., & Ibrahim, M. H. (2008). The effect of electromagnetic field on protein molecular structure of E. coli and its pathogenesis. *Rom. J. Biophys*, *18*(2), 145-169.

Gorgieva, S., & Trček, J. (2019). Bacterial cellulose: production, modification and perspectives in biomedical applications. Nanomaterials 9: 1–20. https://doi.org/10.3390/nano9101352.

Ha, S. J., Kim, S. Y., Seo, J. H., Oh, D. K., & Lee, J. K. (2007). Optimization of culture conditions and scale-up to pilot and plant scales for coenzyme Q 10 production by Agrobacterium tumefaciens. *Applied microbiology and biotechnology*, *74*(5), 974-980. https:// doi.org/10.1007/s00253-006-0744-4.

Ha, J. H., Shah, N., Ul-Islam, M., Khan, T., & Park, J. K. (2011). Bacterial cellulose production from a single sugar α-linked glucuronic acid-based oligosaccharide. *Process Biochemistry*, *46*(9), 1717-1723. https://doi.org/10.1016/j.procbio.2011.05.024

Hong, F., Guo, X., Zhang, S., Han, S. F., Yang, G., & Jönsson, L. J. (2012). Bacterial cellulose production from cotton-based waste textiles: enzymatic saccharification enhanced by ionic liquid pretreatment. *Bioresource technology*, *104*, 503-508. https://doi.org/10.1016/j.biortech.2011.11.028

Hornung, M., Ludwig, M., Gerrard, A. M., & Schmauder, H. P. (2006). Optimizing the production of bacterial cellulose in surface culture: Evaluation of substrate mass transfer influences on the bioreaction (Part 1). *Engineering in Life Sciences*, *6*(6), 537-545. https://doi.org/10.1002/elsc.200620162.

Jung, H. M., Kim, S. Y., Moon, H. J., Oh, D. K., & Lee, J. K. (2007). Optimization of culture conditions and scale-up to pilot and plant scales for vancomycin production by Amycolatopsis orientalis. *Applied microbiology and biotechnology*, *77*(4), 789-795. https://doi.org/10.1007/s00253-007-1221-4.

Kongruang, S. (2007). Bacterial cellulose production by Acetobacter xylinum strains from agricultural waste products. In *Biotechnology for Fuels and Chemicals* (pp. 763-774). Humana Press. https://doi.org/10.1007/s12010-007-8119-6.

Konopacka, A., Rakoczy, R., & Konopacki, M. (2019). The effect of rotating magnetic field on bioethanol production by yeast strain modified by ferrimagnetic nanoparticles. *Journal of Magnetism and Magnetic Materials*, *473*, 176-183. https://doi.org/10.1016/j.jmmm.2018.10.053.

Kthiri, A., Hidouri, S., Wiem, T., Jeridi, R., Sheehan, D., & Landouls, A. (2019). Biochemical and biomolecular effects induced by a static magnetic field in Saccharomyces cerevisiae: Evidence for oxidative stress. *Plos one*, *14*(1), e0209843. https://doi.org/10.1371/journal.pone.0209843.

Lechowska, J., Kordas, M., Konopacki, M., Fijałkowski, K., Drozd, R., & Rakoczy, R. (2019). Hydrodynamic studies in magnetically assisted external-loop airlift reactor. *Chemical Engineering Journal*, *362*, 298-309. https://doi.org/10.1016/j.cej.2019.01.037.

Muniz, J. B., Marcelino, M., Motta, M. D., Schuler, A., & Motta, M. A. D. (2007). Influence of static magnetic fields on S. cerevisae biomass growth. *Brazilian Archives of Biology and Technology*, *50*(3), 515-520. https://doi.org/10.1590/S1516-89132007000300017

Nguyen, D. N., Ton, N. M. N., & Le, V. V. M. (2009). Optimization of Saccharomyces cerevisiae immobilization in bacterial cellulose by 'adsorption-incubation' method. *International Food Research Journal*, 16, 59-64.

Park, J. K., Jung, J. Y., & Park, Y. H. (2003). Cellulose production by Gluconacetobacter hansenii in a medium containing ethanol. *Biotechnology letters*, *25*(24), 2055-2059. https://doi.org/10.1023/B:BILE.0000007065.63682.18.

Rajput, A. A., & Sheikh, Z. (2019). Effect of inoculum type and organic loading on biogas production of sunflower meal and wheat straw. *Sustainable Environment Research*, *29*(1), 1-10. https://doi.org/10.1186/s42834-019-0003-x.

Rampersad, S. N. (2012). Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors*, *12*(9), 12347-12360. https://doi.org/10.3390/s120912347.

Rangaswamy, B. E., Vanitha, K. P., & Hungund, B. S. (2015). Microbial cellulose production from bacteria isolated from rotten fruit. *International journal of polymer science*, *2015*. https://doi.org/10.1155/2015/280784.

Reiniati, I., Hrymak, A. N., & Margaritis, A. (2017). Kinetics of cell growth and crystalline nanocellulose production by Komagataeibacter xylinus. *Biochemical Engineering Journal*, *127*, 21-31. https://doi.org/10.1016/j.bej.2017.07.007.

Ruka, D. R., Simon, G. P., & Dean, K. M. (2012). Altering the growth conditions of Gluconacetobacter xylinus to maximize the yield of bacterial cellulose. *Carbohydrate polymers*, *89*(2), 613-622. https://doi.org/10.1016/j.carbpol.2012.03.059.

Shezad, O., Khan, S., Khan, T., & Park, J. K. (2009). Production of bacterial cellulose in static conditions by a simple fed-batch cultivation strategy. *Korean Journal of Chemical Engineering*, *26*(6), 1689-1692.https://doi.org/10.1007/s11814-009-0232-5.

Song, H. J., Li, H., Seo, J. H., Kim, M. J., & Kim, S. J. (2009). Pilot-scale production of bacterial cellulose by a spherical type bubble column bioreactor using saccharified food wastes. *Korean Journal of Chemical Engineering*, *26*(1), 141-146. https://doi.org/10.1007/s11814-009-0022-0.

Sood S, Singhal R, Bhat S, Kumar A. Inoculum Preparation. In: Murray M-Y, editor. Comprehensive Biotechnology (3th ed.), Oxford: Pergamon; 2011, p.230-43. https://doi.org/10.1016/B978-0-444-64046-8.00076-8.

Stanbury, P. F., Whitaker, A., & Hall, S. J. (2017). *Principles of fermentation technology* (3rd ed.). Elsevier. https://doi.org/10.1016/B978-0-08-099953-1.00006-5.

Tomska, A., & Wolny, L. (2008). Enhancement of biological wastewater treatment by magnetic field exposure. *Desalination*, *222*(1-3), 368-373. https://doi.org/10.1016/j.biortech.2010.05.094.

Ullah, H., Santos, H. A., & Khan, T. (2016). Applications of bacterial cellulose in food, cosmetics and drug delivery. *Cellulose*, *23*(4), 2291-2314. https://doi.org/10.1007/s10570-016-0986-y.

Wang, J., Tavakoli, J., & Tang, Y. (2019). Bacterial cellulose production, properties and applications with different culture methods–A review. *Carbohydrate polymers*, *219*, 63-76. https://doi.org/10.1016/j.carbpol.2019.05.008

Wang, Z. G., Xiang, D., Wang, X. B., & Li, C. F. (2016). Preparation of an inoculum of Gluconacetobacter xylinus without mutants in shaken culture. *Journal of applied microbiology*, *121*(3), 713-720. https://doi.org/10.1111/jam.13193.

Webb, C., & Kamat, S. P. (1993). Improving fermentation consistency through better inoculum preparation. *World Journal of Microbiology and Biotechnology*, *9*(3), 308-312. https://doi.org/10.1007/BF00383069.

Wu, S. C., & Li, M. H. (2015). Production of bacterial cellulose membranes in a modified airlift bioreactor by Gluconacetobacter xylinus. *Journal of bioscience and bioengineering*, *120*(4), 444-449. https://doi.org/10.1016/j.jbiosc.2015.02.018.

Yanti, N. A., Ahmad, S. W., & Muhiddin, N. H. (2018, December). Evaluation of inoculum size and fermentation period for bacterial cellulose production from sago liquid waste. In *Journal of Physics: Conference Series* (Vol. 1116, No. 5, p. 052076). IOP Publishing. https://doi.org/10.1088/1742-6596/1116/5/052076.

Żywicka, A., Junka, A., Ciecholewska-Juśko, D., Migdał, P., Czajkowska, J., & Fijałkowski, K. (2020). Significant enhancement of citric acid production by Yarrowia lipolytica immobilized in bacterial cellulose-based carrier. *Journal of Biotechnology*, *321*, 13-22. https://doi.org/10.1016/j.jbiotec.2020.06.014.

Żywicka, A., Junka, A. F., Szymczyk, P., Chodaczek, G., Grzesiak, J., Sedghizadeh, P. P., & Fijałkowski, K. (2018). Bacterial cellulose yield increased over 500% by supplementation of medium with vegetable oil. *Carbohydrate polymers*, *199*, 294-303. https://doi.org/10.1016/j.carbpol.2018.06.126

Figure legends

Fig. 1. Experimental set-up: 1 – filters; 2 – fermentation medium dosing system; 3 – temperature probes; 4 – pH probes; 5 – DO% probes; 6 – measuring and control equipment; 7 – upper chamber (separation zone); 8 – inverters; 9 – electrical boxes; 10 – computers; 11 – rising column; 12 – falling column; 13 – cooling liquid chamber; 14 – heat exchanger; 15 – thermostat; 16 – magnetic field generators; 17 – coils; 18 – sparger; 19 – rotameter; 20 – inoculum removal system; 21 – circulation pump.

Fig. 2. Scheme of the experiment.

Fig. 3. A) The number of living cells and metabolic activity of *K. xylinus* based on fluorescence of AlamarBlue reagent; **B)** pH and DO% of the culture medium during the process of inoculum preparation. Data are presented as a mean ± standard error of the mean (SEM).

Fig. 4. Visualization of cellulose producing bacteria and non-producing mutants. A red arrow marks the cellulose around the *K. xylinus* colony. A black arrow marks the colony of non-producing mutants.

Fig. 5. Weight of BC obtained using inoculum produced in RMF-assisted EL-ALB during three-cycle repeated fed-batch cultures. Data are presented as a mean ± standard error of the

mean (SEM). There were no statistically significant differences between the samples ($p<0.05$).

Fig. 6. Nanostructure of BC obtained from the inoculum produced in RMF-assisted EL-ALB **A)** *K. xylinus* cells anchored in BC structure, **B)** BC structure after NaOH treatment.