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Bacterial cellulose yield increased over 500% by supplementation of medium with vegetable oil

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ABSTRACT

Bacterial cellulose (BC), produced by *Komagataeibacter xylinus*, has numerous applications to medicine and industry. A major limitation of BC use is relatively low production rates and high culturing media costs. By supplementing culture media with 1% vegetable oil, we achieved BC yield exceeding 500% over the yield obtained in standard media. BC properties were similar to cellulose cultured in standard methods with regard to cytotoxicity but displayed significantly higher water swelling capacity and mechanical strength. As we demonstrated herein, this significantly increased BC yield is the result of microscopic and macroscopic physiochemical processes reflecting a complex interaction between *K. xylinus* biophysiology, chemical processes of BC synthesis, and physiochemical forces between BC membranes, oil and culturing vessel walls. Our findings have significant translational implications to biomedical and clinical settings and can be transformative for the cellulose biopolymer industry.

1. Introduction

Cellulose is the most abundant organic polymer on Earth and is a major constituent of most plants and algae (Thongsomboon et al., 2018). Chemically, cellulose is a linear polysaccharide comprising β-1,4–linked glucosyl residues. Bacterial cellulose (BC) is a biopolymer produced by variety of bacteria, including non-pathogenic *Komagataeibacter xylinus*. This bacterium has been used as the model microorganism for basic and applied studies of BC (Hornung, Ludwig, Gerrard, & Schmauder, 2006a; Ruka, Simon, & Dean, 2012). The number of scientific reports on BC is expanding rapidly every year [https://www.ncbi.nlm.nih.gov/pubmed/?term=bacterial+cellulose], because compared to plant cellulose, BC demonstrates superior mechanical properties including high tensile strength, water-swelling capacity, crystallinity and biocompatibility (Bielecki et al., 2012; El-Saied, El-Diwany, Basta, Atwa, & El-Ghwas, 2008; Hornung et al., 2006a; Ruka et al., 2012).

The unique properties of BC have enabled broad applications of this material to medicine and industry. In the biomedical field, this multifaceted nanomaterial has already found a wide range of applications as a dressing for patients with extensive burns and for wound healing and management (Bielecki et al., 2012), as carriers for drug delivery and pharmacotherapy, as implants for bone, cartilage, dental, neural and dura mater, and as contact lenses, tympanic membranes, and artificial corneas (Picheth et al., 2017). In industry, BC is used for high quality paper production (Surma-Ślusarska, Presler, & Danielewicz, 2008), as a carrier for immobilization (Drozd, Rakoczy, Wasak, Junka, & Fijałkowski, 2018; Wei, Yang, & Hong, 2011), as membranes and acoustical filter materials, for the production of biocomposites (Keshk, 2014). BC is also used in the food industry as a source of dietary fiber for production of desserts as Nata de coco, fruit cocktails and fruit jellies (Jagannath, Raju, & Bawa, 2010), as a low-caloric food additive,

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stabilizing substance, and water binding or packaging material (Shi, Zhang, Phillips, & Yang, 2014).

However, low production rates and high culture media costs limit commercial uses of BC. Therefore, the prerequisite for the broad use of BC in various industries is the development of efficient and novel, yet cost-effective, methods for production (Chao, Ishida, Sugano, & Shoda, 2000). Modification of culture medium is one of the approaches used to improve BC yield and to reduce production costs. In order to increase the production of BC, the culture medium can be modified by enriching it with substances such as caffeine and related xanthenes, or by adding different concentrations of ions such as Mg, Cu, Fe, Zn, Ag, and Cr (Fontana, Franco, de Souza, Lyra, & de Souza, 1991). The synthesis of BC can also be increased by addition of insoluble microparticles: diatomaceous earth, silica, or small glass beads to agitated/aerated *K. xylinus* culture (Vandamme, de Beets, Vanbaelen, Joris, & de Wulf, 1998). In order to decrease cost of BC production, there has been a growing need to develop culture media using industrial and food stuff wastes, for example fruit and vegetable waste, glycerol, molasses, or whey (Keshk, 2014; Kurosumi, Sasaki, Yamashita, & Nakamura, 2009). Production of BC from agricultural products and residues, which include konjak glucomannan (Hong & Qiu, 2008), wheat straw (Chen, Hong, Yang, & Han, 2013; Hong, Zhu, Yang, & Yang, 2011), and cotton-based waste textiles (Hong et al., 2012), has also been previously demonstrated. Another way to increase BC yield is a genetic rearrangement of chromosomes in BC-producing bacteria in order to significantly intensify the process of BC fibril synthesis. Florea et al. (2016) created a set of genetic tools that enables biosynthesis of patterned cellulose, functionalization of the cellulose surface with proteins, and tunable control over cellulose production, thus greatly expanding the ability to control and to engineer new cellulose-based biomaterials. Other research teams have been investigating potential bacteria able to perform cellulose synthesis processes in conditions of reduced oxygen concentration, but this approach along with other ones have resulted in limited efficacy. Ji et al. (2016) have identified an *Enterobacter* sp. FY-07 which can efficiently produce BC under aerobic and anaerobic conditions.

It is important to note that methods seeking to produce a significant increase in the yield of BC in a single and timely culture process has been the Holy Grail for research teams all over the world for decades. These efforts intensified in recent years due to the many aforementioned reports of possible BC applications in the fields of medicine, cosmetics, pharmacy, food industry, and many others. Many elaborated methods were developed to reach this aim; however, no significant successes were achieved (Cacicedo et al., 2016). In this paper, we present a method to obtain significantly increased BC yield (up to 550%) in a single culturing process by cheap and simple modification of media composition with vegetable oil, specifically rapeseed oil. Although simple to perform this has been elusive for decades, and these modifications initiate complex processes and interactions between media, bacteria, and oxygen availability, as well as between the synthesized biopolymer and culture vessel walls, which we present herein. Our findings have significant translational implications to biomedical and clinical settings and can be transformative for the cellulose biopolymer industry.

2. Material and methods

2.1. Microorganisms and culture conditions

Komagataeibacter xylinus (Deutsche Sammlung von Mikroorganismen und Zellkulturen - DSM 46602) strain was cultivated in stationary conditions for 7 days at 28 °C, using a Herstin-Schramm (H-S) medium. Prior the experiment, the 7 day-old cultures were shaken and obtained bacterial suspension (2×10^5 CFU/mL) was used to inoculate 1 L of H-S medium in 3 L glass flasks with diameters at the upper level of the culture medium equivalent to 200 mm. The BC synthesis was conducted for 7 days at 28 °C.

In order to assess the possibility of using the proposed modification of the culture medium, several additional experiments using two additional *K. xylinus* strains (DSM 46604, DSM 5602), two other oils (sunflower or linseed), and vessels of different volumes, surface and shape were performed. To this end, static cultures of *K. xylinus* strains were grown in 50 mL plastic tubes (3.8 cm diameter; 25 mL of medium) (Polypropylene Conical Centrifuge Tube, Becton Dickinson and Company, USA), glass beakers (dimensions: 80 × 100 mm; 200 mL of medium and 180 × 320 mm; 1 L of medium), glass flasks (dimensions at the upper level of the culture medium: 50 mm; 150 mL of medium), and in Petri dishes (dimensions: 150 × 20 mm; 100 mL of medium). As a control, regardless of the type of culture vessel used, BC was produced in a static culture using H-S medium without addition of oil.

2.2. Determination of BC production

BC was harvested from the media and weighed on an analytical balance (WTB 2000, Radwag, Poland). Then, BC pellicles were purified by treatment with 0.1 M NaOH at 90 °C for 30 min to remove bacterial cells and media components prior to being rinsed with sterile water. The obtained cellulose was dried at 60 °C overnight, weighed again and investigated as described below. The thickness of wet BC (mm) was measured using a ruler, whereas the thickness of dry BC (μm) was determined using a micrometer caliper.

2.3. Determination of the oil content in BC

The Soxhlet extraction method was used to determine the percentage of oil in BC samples. The extraction using diethyl ether was performed according to the ISO 3596:2000 norm: Animal and vegetable fats and oils – Determination of unsaponifiable matter – Method using diethyl ether extraction. After extraction, the weight of BC was measured again.

2.4. Physicochemical properties of BC

2.4.1. Mechanical strength

BC specimens to be used in tensile strength tests were cut out using a pneumatic press machine (CEAST Instron, Massachusetts, USA). The tensile strength test was performed using MTS Synergie 100® machine (MTS System Corp, Minnesota, USA). The tests 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 mechanical parameters, such as tensile strength, were determined. The results were presented as average tensile strength values from multiple samples. All measurements were performed in six repeats.

2.4.2. Scanning electron microscopy

NaOH purified BC was fixed in glutaraldehyde (POCH, Poland) for 7 days and dried in a critical point dryer. Subsequently, the cellulose was subject to sputtering with Au/Pd (60:40) using EM ACE600, Leicasputter (Leica Microsystems, Wetzlar, Germany). The sputtered samples were examined using a scanning electron microscope (SEM, Auriga 60, Zeiss, Oberkochen, Germany). Fibril diameter was assessed using a built-in analyzer; the size of cellulose pores was analyzed by means of ImageJ software (NIH, Bethesda, USA).

2.4.3. Scanning-transmission electron microscopy analysis

BC samples were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH = 7.2) for 24 h. Materials were post-fixed in 2% osmium tetroxide in cacodylate buffer for 1 h at room temperature (RT), washed three times with buffer and one time in water, and dehydrated using a graded ethanol series. Samples were rinsed in pure acetone, followed by infiltration with epon resin (agar) 1:1 with acetone for 24 h at RT, and in

pure resin for 2 h, after which the samples were embedded in resin and polymerized at 60 °C for 24 h. Resin blocks were trimmed to obtain cross-sections at the superficial and basal level of hydrogel samples. Samples were cut with an ultramicrotome (Leica UC7, Leica Microsystems, Wetzlar, Germany) for 60 nm-thin sections collected on copper transmission electron microscopy grids. Specimens were analyzed by means of Auriga 60 (Zeiss, NY, USA) dual-beam electron-ion microscope at 20 kV using scanning-transmission electron microscopy (STEM) detector with bright field mode enabled. Micrographs were normalized using Fiji software (LA, USA) and a local contrast normalization plugin.

2.4.4. Determination of water swelling ratio

To assess water swelling in BC, the cellulose pellicles were cut into 1 cm^2 samples, dried at 60 °C for 6 h to remove any water content. Next, the dried BC samples were immersed in distilled water and weighed every 1 min until a constant weight of the wet sample was achieved. The results are shown as percentage of swelling ratio (SR) (Pandey, Mohamad, & Amin, 2014) calculated using the formula:

$$SR\ [\%] = \frac{(W_{wet} - W_{dry})}{W_{dry}}$$

Where, W_{wet} is the weight of the swollen BC and W_{dry} is the dry weight of the sample.

2.4.5. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Infrared spectra for BC was determined by the ATR-FTIR method, using ALPHA FT-IR Spectrometer (Burker Co., Germany) with a DTGS detector and the platinum-ATR-sampling module with a robust diamond crystal and variable angle incidence beam. For each sample 32 scans of the resolution of 2 cm^{-1} were recorded within the spectral range of 4000–400 cm^{-1}. The spectra were collected and processed initially using the Omnic software package.

The crystallinity index was calculated using the ratio of absorbance values for peaks 1430/900 (Cr.R1) and 1370/2900 (Cr.R2). The fraction of the cellulose *Iα* was calculated from ATR-FTIR spectra according to the method described by Kataoka and Kondo (1999). The area of the peaks at *A*710 (710 cm^{-1}) for *Iβ* and at *A*750 (750 cm^{-1}) for *Iα* was determined from the spectra deconvoluted in Peakfit software. The percentage of *Iα* was calculated according to the formula:

$$I\alpha\ [\%] = \frac{A710}{(A750 + A710)} \times 100$$

2.5. Cytotoxic effect of oil-modified BC

A Neutral Red (NR) cytotoxicity assay was performed in fibroblast (L929) cell cultures treated with extracts obtained from BC membrane-conditioned medium. The extracts were prepared according to the ISO 10993 norm: Biological evaluation of medical devices; Part 5: Tests for in vitro cytotoxicity; Part 12: Biological evaluation of medical devices, sample preparation and reference materials (ISO 10993-5:2009 and ISO/IEC 17025:2005). Briefly, BC membranes of approximately 1 g of wet mass were introduced to plate wells filled with MEM cell culture media without serum (Sigma-Aldrich, Germany) and incubated for 72 h in 5% CO_2 at 37 °C with shaking at 500 rpm (Schuttler Microplate Shaker, MTS-4, IKA, Germany). The BC discs incubated with 75% ethanol (POCH, Poland) for 72 h served as negative controls for this experiment. After incubation, membranes were extruded from the wells, and the supernatants were introduced to the cell cultures and incubated for 24 h, 48 h, and 72 h in 5% CO_2 at 37 °C. After incubation, media was removed and 100 µL of NR solution (40 µg/mL; Sigma-Aldrich) was introduced to wells of the plate. Cells were incubated with NR for 2 h at 37 °C. After incubation, the dye was removed, wells were rinsed with PBS (Sigma Aldrich, Germany) and left to dry at room temperature. Subsequently, 150 µL of de-stain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid; POCH, Poland) was introduced to each well. The plate was vigorously shaken in a microtiter plate shaker for 30 min until NR was extracted from the cells and formed a homogenous solution. Next, the value of NR absorbance was measured spectrometrically using microplate reader (Multi-scan GO, Thermo Fisher Scientific, USA) at 540 nm wavelength. The absorbance value of cells not treated with extracts was considered 100% of potential cellular growth (positive control).

2.6. Analysis of bacteria viability and visualization of oil with confocal microscopy (CM)

For CM purposes, BC samples were cut aseptically into 3 even stacks using a razor in a laminar cabinet. Bacteria in cellulose samples were stained with Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (ThermoFisher Scientific, USA containing Syto 9 and Propidium iodide (PI)). DMSO stocks of both compounds were diluted 1000× in PBS and directly used for labeling. Oil droplets were stained with NR (Sigma Aldrich, Germany) – 10 mg/mL stock was diluted 250x in PBS. The imaging was performed on an upright Leica SP8 resonant scanning confocal system (Leica Microsystems, Germany). Stacks of confocal 8-bit images with a pixel size of 0.3 µm and a 5 µm Z step were acquired using a 25× water immersion objective (NA 0.95). The pinhole was set to 1 AU. Cellulose surface was visualized in a reflection mode using 638 nm laser line. Syto 9 and NR fluorescence was collected using a 488 nm laser line and 496–530 nm emission range. PI was excited with a 552 nm laser line and 561–618 nm emission range was recorded. The acquisition was performed in a sequential mode. Fluorescence intensity profiles were created in GraphPad Prism 6 (GraphPad Software, USA) based on rectangles (336 × 675 pixels) drawn in ImageJ/Fiji software (National Institutes of Health, USA) on maximum intensity projection images of confocal Z stacks.

2.7. Determination of glucose concentration

The concentration of glucose in the culture medium was determined enzymatically using the Glucose Assay Kit (BioMaxima, Poland) according to the manufacturer's protocol. The results were expressed as a percentage of glucose consumed within the H-S medium during cultivation.

2.8. Statistical analysis

The data obtained in this study were presented as mean values ± standard error of the mean (SEM) and were analyzed using a one-way analysis of variance (ANOVA). The Tukey-Kramer test was used for multiple comparison of means (post-hoc analysis) obtained from different *K. xylinus* strains, in control and cultures with different oils. The cultures were conducted in triplicate and all experiments were repeated at least three times. Differences were considered significant at a level of $p < 0.05$. The statistical analyses were conducted using Statistica 12.5 (StatSoft, Inc. Tulsa, OK, USA).

3. Results

The weight of wet BC pellicles obtained in rapeseed oil-modified medium from cultures in 3 L flasks was higher in comparison to control BC by 604%, while corresponding differences in dry mass was 650% (Fig. 1A). At the macroscopic level, oil from the medium was observed to be incorporated onto the surface of the cellulose (Fig. S1). The average mass of oil trapped inside cellulose was estimated as ca. 10% of the wet cellulose mass. However, even after oil removal, the weight of wet cellulose pellicles obtained in oil-modified medium was still significantly higher – by 540%, as compared with cellulose produced in a

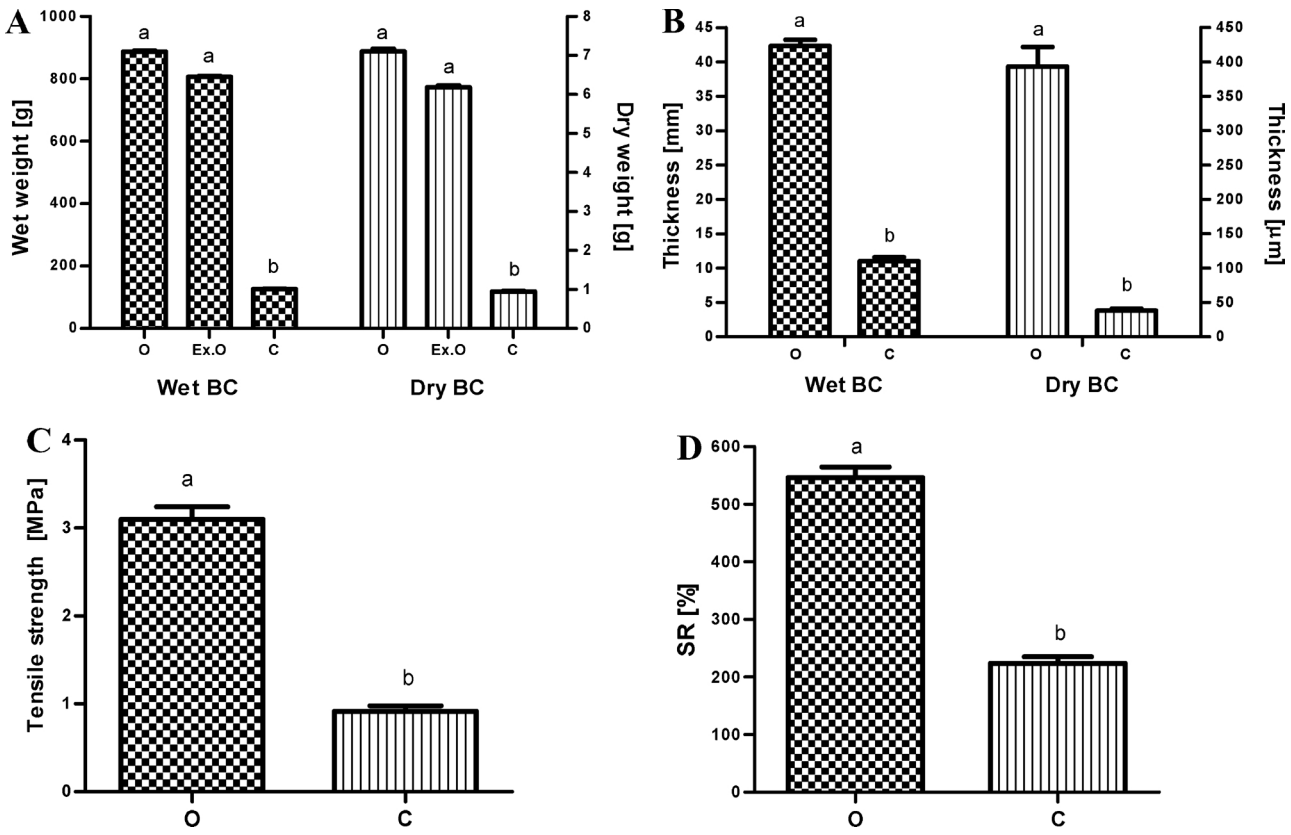

Fig. 1. Weight and properties of BC pellicles synthesized by *K. xylinus* DSM 46602 in control H-S medium and H-S medium with rapeseed oil. (**A**) Weight; (**B**) Thickness; (**C**) Tensile strength; (**D**) Swelling ratio.
O – weight of BC from medium with 1% of rapeseed oil; Ex. O – weight of BC after oil extraction; C – control (H-S medium without oil); Data are presented as a mean ± standard error of the mean (SEM); Values with different letters (a, b) are significantly different ($p < 0.05$).

standard culture medium. The weight recorded for dry oil-modified BC after oil extraction compared to the control was also higher – by 553% (Fig. 1A). The cellulose pellicles obtained from oil-supplemented media were approximately 285% and 192% thicker for wet and dry BC, respectively, as compared with cellulose produced in standard culture media (Fig. 1B).

This trend was consistent for all tested *K. xylinus* strains (Table S1). The significant increase in BC production observed in media supplemented with oils was observed regardless of the volume of media used in culture vessels of all tested shapes (Tables S2 and S3; Fig. S2). It should be noted that when a culture vessel (tubes, beaker or flask) of lower volume (f.e. 50 mL plastic tubes) was used, the differences between weight for BC obtained in oil-modified and control medium were not as significant as in the case of larger volumes and did not exceed 55% (Table S2).

BC membranes formed in oil-supplemented media displayed higher tensile strength value in comparison to control cellulose samples (Fig. 1C). In the case of cellulose grown in the presence of rapeseed oil, tensile strength exceeded 3× that of control samples and this result was statistically significant ($p < 0.05$). Moreover, the swelling ability of BC formed in modified media was significantly higher (over 2×) in comparison to control samples (Fig. 1D). As this parameter is directly related with cellulose cross-linking, we performed SEM analysis of fibril diameters (Fig. S3) and pore sizes (Fig. S4) in oil-modified and control samples. There were no significant differences between the two aforementioned parameters analyzed. The fibril diameter was 168.85 ± 12.89 nm vs. 178.93 ± 9.87 nm for oil-modified and non-modified cellulose, respectively. The pore size measured in feret diameters was 55.74 ± 6.76 vs. 61.32 ± 11.22. Furthermore, SEM images revealed that after oil extraction, the surface of cellulose was randomly covered with voids (Fig. S5).

The chemical structure of BC samples was studied by ATR-FTIR. The analyzed BC samples displayed a relatively high content of Iα fraction (Table S4). No significant differences were found between percentage of Iα calculated for the controls and oil-modified samples. All samples displayed high values of I.C. I1430/900. Similarly, the second crystallinity index calculated as a ratio between absorbance of the bands at 1370 cm^{-1} (CH bending) and 2900 cm^{-1} (CH and CH_2 stretching) was at similar levels in all types of investigated samples.

Extracts from cellulose membranes grown in a medium modified with oil were also non-cytotoxic to fibroblast cell lines according to binding norms (Fig. S6).

A distinct line of investigation performed by means of CM and STEM revealed that oil droplets are surrounded by a zone free from cellulose (Fig. 2A–C).

The proximity of *K. xylinus* cells to oil droplets suggests a lack of adverse conditions in this area for bacterial growth. Further investigation performed by means of CM shows that supplementation of media with oil correlates with the distribution pattern of *K. xylinus* cells within cellulose membrane. In control samples, bacteria were distributed with higher density in the top parts of BC, where they migrated from the lower part of the membrane (Fig. 3A). In BC from oil-modified medium, bacteria were distributed more evenly (Fig. 3C). Overall viability of *K. xylinus* cells was higher in oil-modified BC in comparison to cells embedded within control cellulose (Fig. 3B vs. Fig. 3D, respectively).

Results show that in oil-modified cellulose, not only is the total number of living bacterial cells higher, but more importantly the number of living cells in the middle and the bottom layer of BC is higher (Fig. 4). This suggests that the concentration of oxygen needed for *K. xylinus* to produce BC was higher in these areas, resulting in significantly higher production of BC polymer.

Measurement of glucose concentration within the culture medium

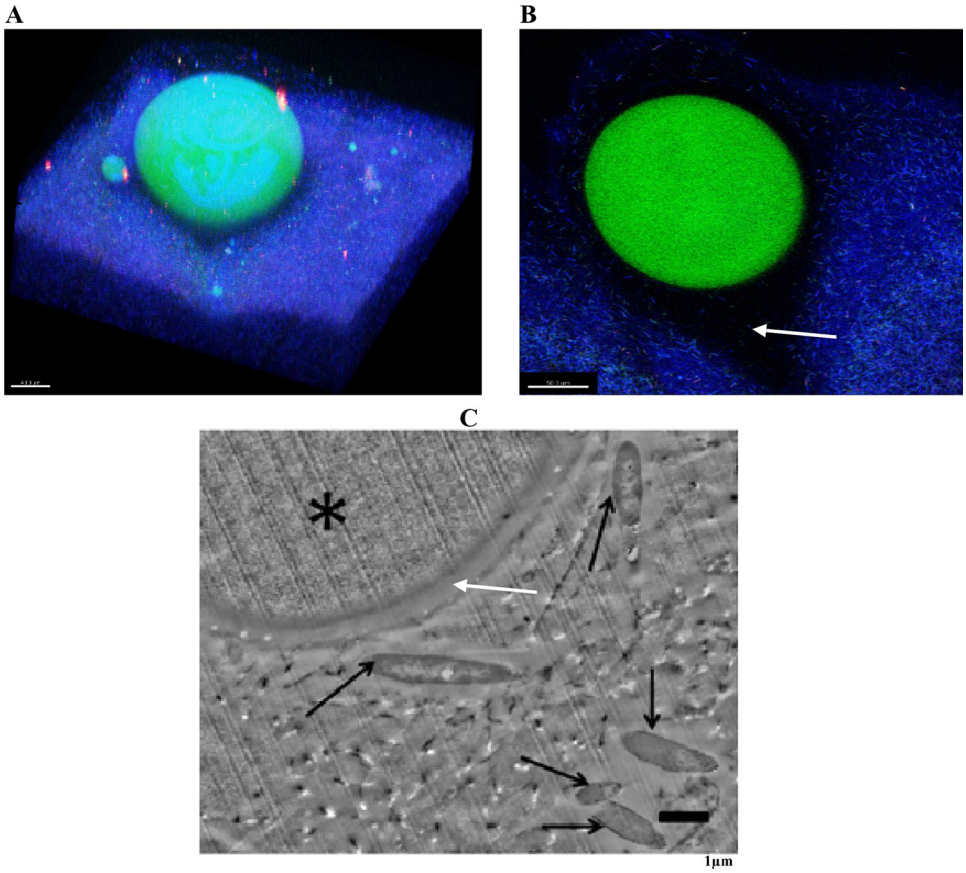

Fig. 2. Oil droplets visualized within BC membranes. (**A,B**) Oil droplet (green oval shape) in BC surrounded by *K. xylinus* cells visualized by CM; (**C**) Cross-section of cellulose performed by means of STEM.
Please note that in this CM setting, blue color stains both cellulose fibers and *K. xylinus* cells and provide no data concerning cell viability; White arrow in (**B**) indicate zone free from cellulose; In picture (**C**), oil droplet in is marked by asterisk, *K. xylinus* cells by black arrows, while clear zone between cellulose fibers and oil droplet by white arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

indicated almost complete consumption of this sugar and also lack of significant differences in value of this parameter after 7 days of between both, oil-supplemented and control cultures (Table S5).

4. Discussion

The aim of the current study was to report a novel, simple, and inexpensive method for increasing BC mass and production by addition of vegetable oil, and to explore and explain the biochemical and physical mechanisms leading to this phenomenon. The rationale behind the application of 1% oil was that this specific volume of oil did not significantly cover the entire culture medium surface and it allowed bacteria to produce cellulose at the first stage of this process. Our preliminary data showed (data not presented) that further increase in oil volume had a negative impact on final cellulose yield with a significant decline in BC production when 2.5% or higher oil concentrations were applied. Addition of 1% oil to culture medium had a positive effect on *K. xylinus* cell numbers and BC production. This modified BC did not display any significant cytotoxicity against a standard eukaryotic fibroblast cell line. The present study also demonstrates that BC membranes formed in oil-modified medium display higher tensile strength in comparison to control or traditional non-oil modified cellulose. The data from this study taken together with earlier reports in this field (Hornung, Ludwig, Gerrard, & Schmauder, 2006b) have allowed us to develop the following explanations for the phenomenon observed herein.

During culturing, BC takes the form of a membrane on the air-medium surface, because *K. xylinus* bacteria display strictly aerobic metabolism and need oxygen to synthetize BC (Chawla, Bajaj, Survase, & Singhal, 2009). Since BC membrane is of a slightly higher density than glucose medium (1030 kg/m^3 vs. 1008 kg/m^3, respectively), there is a downward weight or force contributing to a sinking movement of the material (Hornung et al., 2006b). However, the microscopic and macroscopic roughness of the vessel exerts an upwards frictional force, which hinders the sinking process (Hornung et al., 2006b). This interaction inhibits the formation of new cellulose layers due to a lack of fresh medium and a drop in oxygen availability. We observed that the addition of oil to the medium reduces the frictional force between BC and the vessel walls and intensify the process of downward sinking of the membrane (Fig. 6B). Moreover, BC takes the form of the vessel in which it is cultured. Therefore, as an example, in a conical beaker (we found this culture vessel the best in terms of the observed effect of oil-caused increasing the weight of BC), BC takes form of a cone. The majority of BC mass is therefore deposited in the lower part of this structure. This is another factor contributing to the sinking process of BC, resulting in increased availability of nutrients and in the formation of several new adherent cellulose layers on top of the original or primary layer. Our observations herein indicate that this process is escalating and self-fueling – e.g. after the first layer sinks and a new layer forms, the two-layer cellulose stack sinks again and a third BC layer is formed. In fact, we observed BC stacks formed by up to four coherent, but easy-to-discriminate, layers (Fig. S7).

In addition to the aforementioned mechanisms of increased BC production, there is a third mechanism related to the chemical properties of oil and its interaction with *K. xylinus* cells and cellulose fibrils. Oil has an impact on processes critical to BC formation, namely the availability of oxygen and media nutrients. Considering the hydrophobic nature of oil molecules, it is likely that elastic cellulose nanofibrils are stretched out by oil droplets. This should result in lower fibril density, lower mechanical strength, but also higher water-holding capacity of cellulose grown in oil-enriched medium. However, results presented in this study indicate that BC grown in media supplemented with 1% oil does display higher swelling ratio but has higher resistance to mechanical tension and has similar microfibril cross-linking as compared to control samples of BC without addition of oil. Moreover, the number of live *K. xylinus* cells in oil-modified cellulose was higher

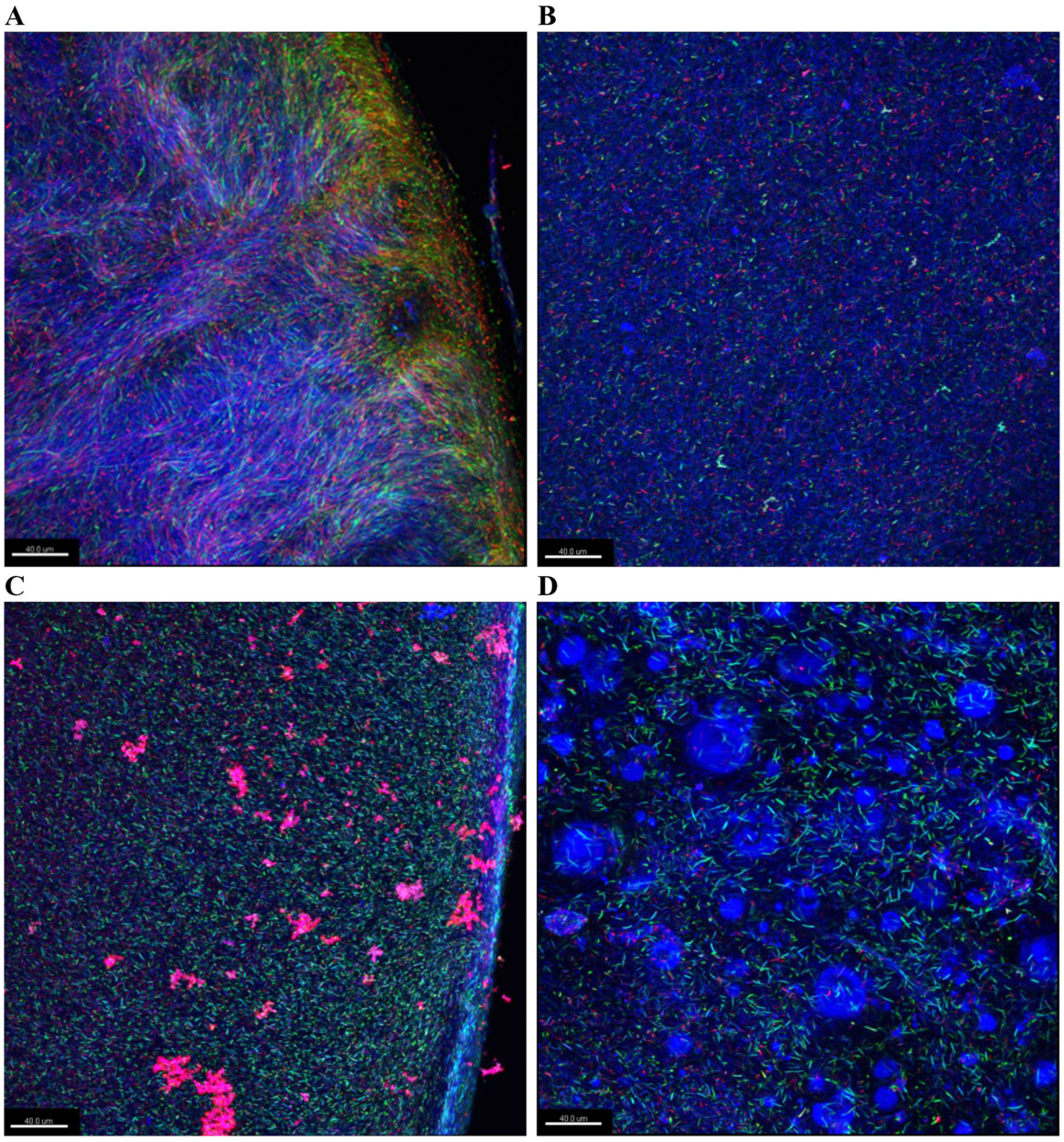

Fig. 3. Distribution (**A**,**C**) and overall viability (**B**,**D**) of *K. xylinus* DSM 46602 cells in BC pellicles synthesized in (**A**,**B**) control H-S medium and (**C**,**D**) H-S medium with rapeseed oil.
Green cells are live, while red ones are dead. All pictures were taken from the outer rim of cellulose discs, which edge (top part of BC) is seen in the right part of (**A**) and (**C**). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

than in cellulose grown in standard medium. Also, the apparent inconsistency between the observed high cross-linking and high water swelling parameters should be elucidated here, as the relationship between these two should be – to some extent – negative (Fijałkowski, Drozd et al., 2017; Fijałkowski, Żywicka et al., 2017). It should be noted that in our experimental settings we did not use an "oil in water emulsion" type of medium, but we simply added oil onto the medium surface (in the case of emulsion, oil droplets are relatively equally distributed throughout the medium volume). During the process of BC formation, we observed that oil droplets decreased in size and changed shape over time – from large and circular, to small and circular or irregular. We hypothesize that during the process of BC development, cellulose compresses oil droplets and has an impact on the overall morphology of this environment. Taking into consideration the laws of physics, oil droplets should adapt a funnel-type form within cellulose, with the base of the funnel directed at the surface of medium. At the same time, hydrophobic oil should push back on the cellulose matrix which consists of > 98% of water. These two opposing processes create a space, which is accessible to oxygen in the air. Indeed, we commonly observed such spaces when we performed STEM and CM analysis. These observed spaces possibly provide for oxygen exchange within deeper layers of cellulose membranes, allowing inhabiting *K. xylinus* cells to survive and continuously produce BC. Moreover, in the case of BC produced in control conditions, a higher density of viable cells was seen distributed at the margin where the availability of oxygen is high, while in oil-modified cellulose cells were distributed more evenly throughout the sample which confirms greater oxygen penetration due to addition of oil to the medium. Increased accessibility of oxygen is one of the key factors to successful cellulose production, and in the oil-supplemented setting *K. xylinus* is able to not only continue strictly aerobic processes of BC synthesis for a longer time than in control samples but is also able to produce this polymer at a greater distance from the oxygen-rich

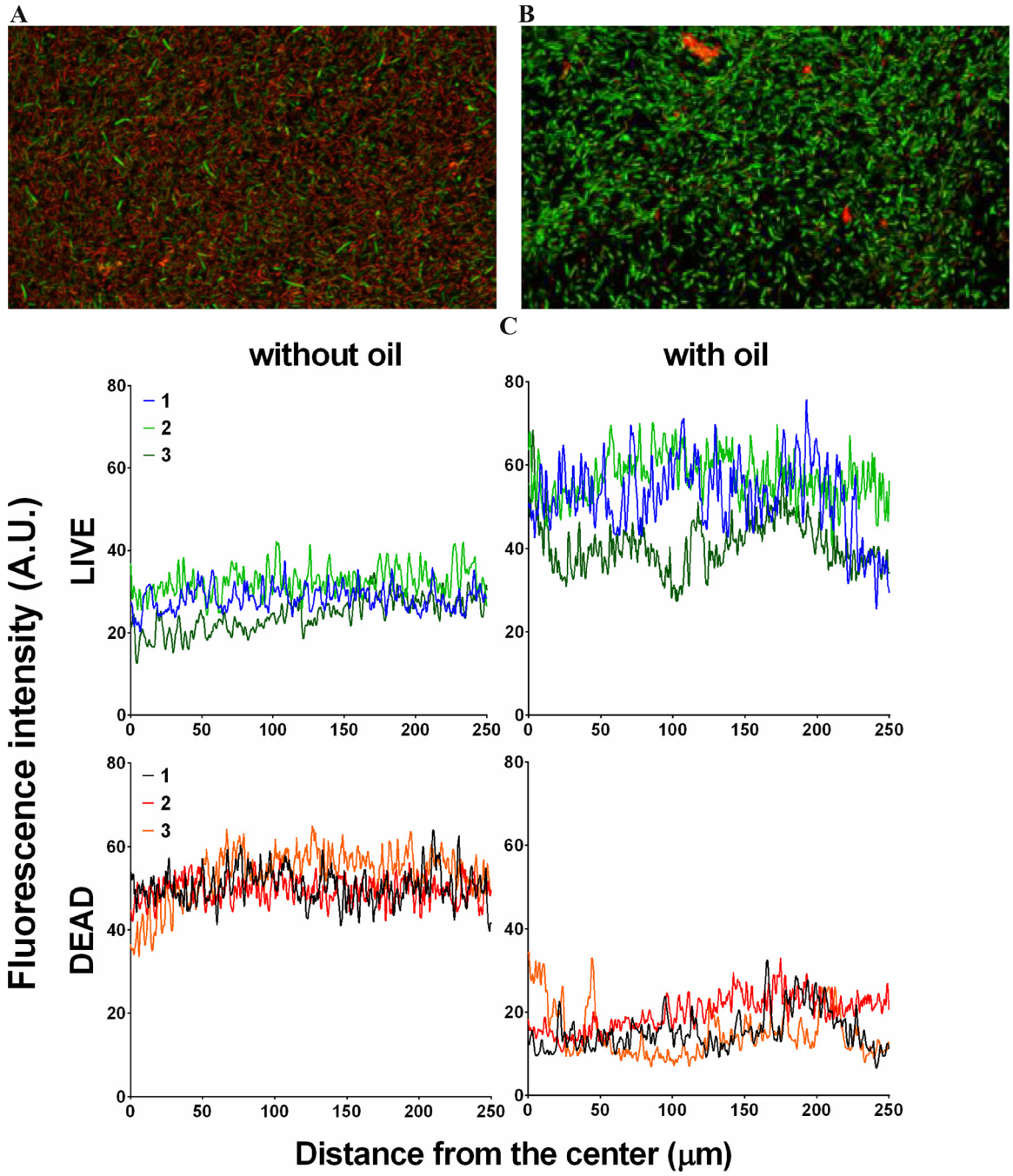

Fig. 4. Differences between number of live (green) and dead (red) *K. xylinus* DSM 46602 cells in BC pellicles synthesized in control H-S medium and H-S medium with rapeseed oil. (**A**) high number of dead cells (red color) in a bottom layer of cellulose grown in control medium; (**B**) high number of live cells in a bottom layer of cellulose grown in the oil-modified medium; (**C**) differences between live/dead cell number ratio within top, middle and bottom part of cellulose.
1 – the top part of BC; 2 – the middle part of BC; 3 – bottom part of BC with regards to the distance from air-liquid interface; AU – arbitrary units; Distance from the center – distance from the point where the analysis started. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

surface interface than normally observed or possible.

However, although the total number of living bacterial cells in oil-modified BC was higher than in control cellulose and there was more BC synthetized in oil-supplemented medium in comparison to the medium without addition of oil, the level of glucose consumption by *K. xylinus* cells after 7 days of incubation did not differ significantly between cultures. Moreover, it was found that in both types of cultures, glucose was consumed almost completely. According to Ross, Mayer, and Benziman (1991) and Oikawa, Nakai, Tsukagawa, and Soda (1997), glucose is easily transported through the cell membrane and incorporated into the cellulose biosynthetic pathway and therefore, as reported by several authors it is consumed rapidly in the first days of fermentation. In our earlier study, we showed that glucose was almost entirely metabolized within the first 11 days of the cultivation (Fijałkowski, Drozd et al., 2017; Fijałkowski, Żywicka et al., 2017), i.e. in the period of cellulose biosynthesis. Moreover, our earlier study showed that even as much as 50% of glucose was metabolized within the time of formation of initial BC layer (3 days). Similarly, Keshk and Sameshima (2005) reported that glucose was consumed rapidly during the early stage of incubation and almost completely (97%) after 7 days

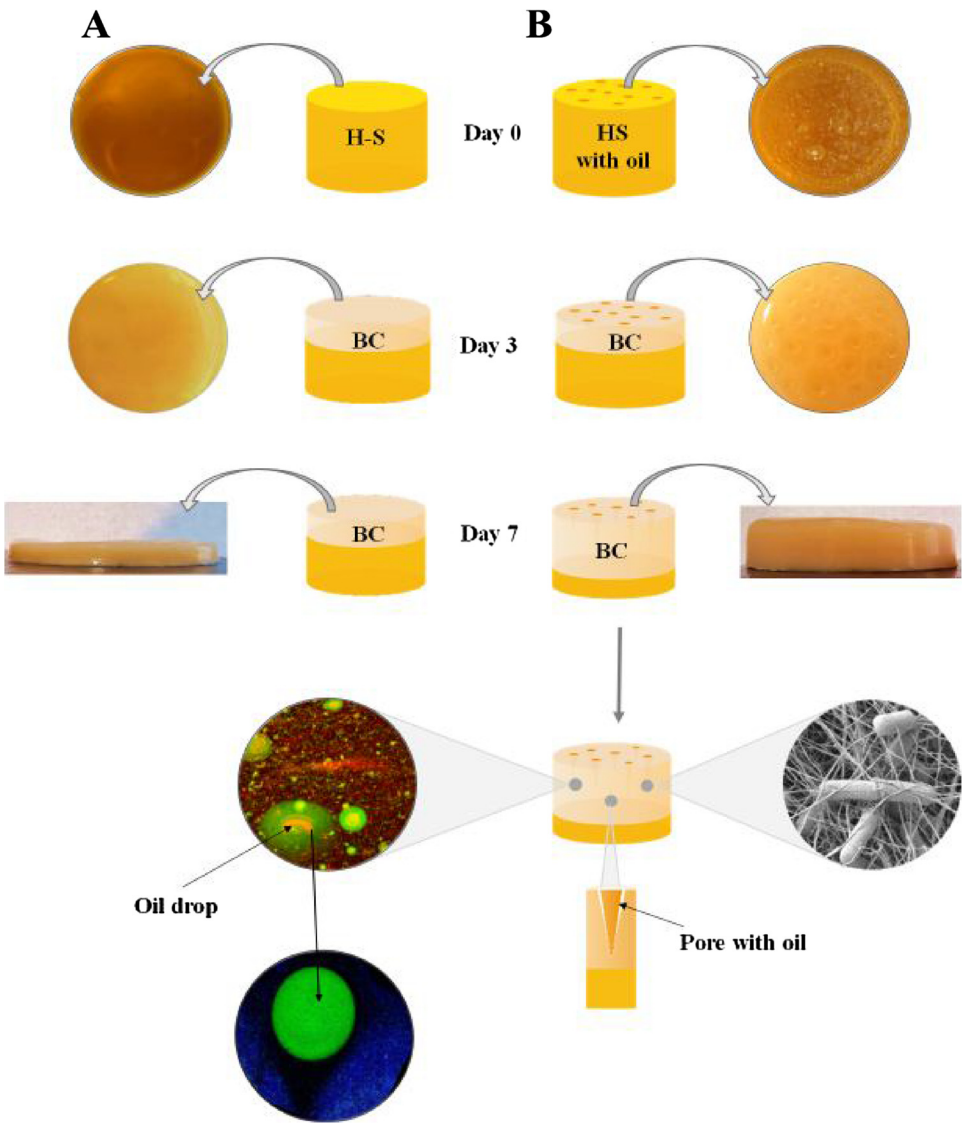

Fig. 5. Stages of BC synthesis in (**A**) control and (**B**) oil-supplemented medium.

of incubation, whereas Zhong et al. (2013) showed that glucose was almost completely depleted after 4 days of fermentation. On the other hand, although the capacity to form UDP-glucose is a prerequisite for cellulose synthesis, cellulose-producing bacteria may show high variations in the patterns of UDP- glucose metabolism. As an example, Ross et al. (1991) indicated that only 19% of glucose was incorporated into BC, and 40% of glucose was fluxed into the gluconic acid byproduct. This explains why, the differences in glucose concentration were not detected in the current study, despites obvious differences in cell number and BC weight between the different types of cultures.

BC in oil-modified media also demonstrated higher swelling ratios. To explain this phenomenon, it is important to once again note that we only used oil on the surface of media. Thus the aforementioned funnel-like oil droplets formed spaces, which after cellulose cleansing processes could be filled with water during swelling ratio tests. Indeed, spaces which were evidently remnants of oil containing regions could be easily seen in cellulose grown in supplemented medium even at the microscopic level (Fig. S5). A consolidated explanation of the mechanisms and phenomena leading to the yield of > 550% BC in oil-modified media as compared to standard media is presented in Fig. 5.

Day 0, 3 and 7 refers to the following culturing days.

In Fig. 6, which is divided with a timeline (thick green arrow), the left side represents BC production in standard medium, while the right one in oil-supplemented medium (oil droplets are shown with the thin green arrow). During the second day of incubation (Flask 1a), the top part of the cellulose covers the air-liquid surface of the medium, decreasing levels of gas exchange, which impedes and finally stops BC synthesis. However, with the increased weight of cellulose as grown in oil-supplemented medium (please refer to Fig. 1A) and owing to decreased friction between the cellulose and the slanted wall of the beaker (please refer to Fig. 6C), the BC membrane sinks (incubation day #3 and #5). Subsequently, media covers the top part of cellulose (red arrows) which allows bacteria located in this BC portion to produce greater yield of the polymer. Please refer to our Fig. S7 to see at least four layers of BC being formed as a result of this process. As it was shown in Figs. 5 and 6, the increased production of BC is related with strain-independent factors, i.e. increased oxygen and glucose availability within medium and shape of the culture vessel. Therefore, our findings may be directly extrapolated and applied not only to *K. xylinus,* but also to other cellulose-producing bacteria. Five-times increased BC yield during one culturing process can be of pivotal meaning in such industries as for example Nata de coco food branch (a cellulosic dessert originally from the Philippines, produced by *Acetobacter xylinum* during fermentation of coconut water).

The degree of crystallinity of cellulose microfibrils is affected by many factors relating directly to the strain used for BC production,

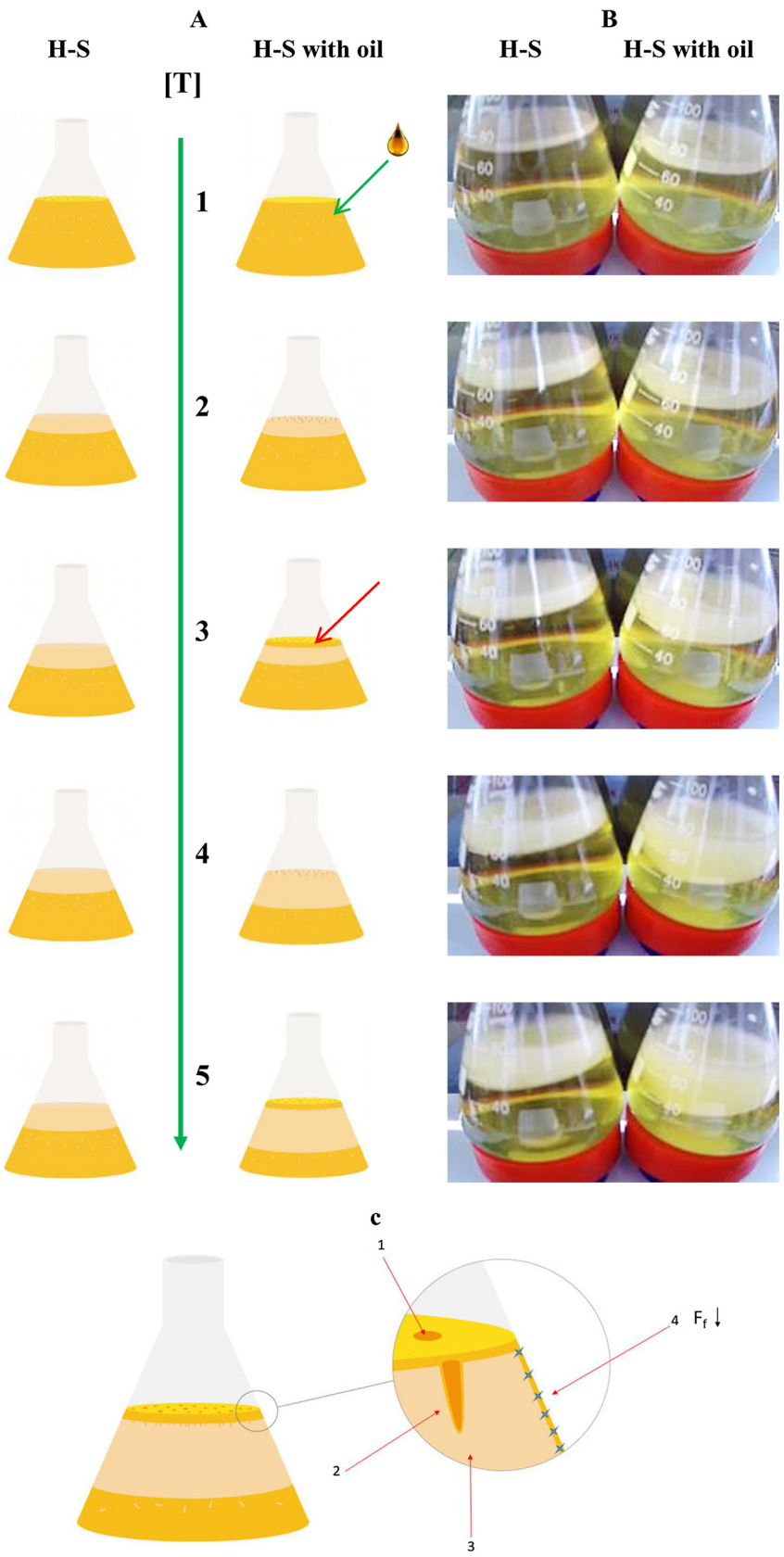

Fig. 6. Stages leading to significantly increased yield of BC produced in oil-supplemented medium and in a vessel of conical shape. (**A**) schematic illustration of increased BC production in oil-modified medium: numbers 1–5 refers to the following culturing days; (**B**) stages of cellulose formation in oil-supplemented medium, recorded by digital camera; (**c**) interaction between bacterial cellulose and vessel's wall: 1 – oil droplet on medium's surface; 2 – oil funnel in BC layer; 3 – BC; 4 – schematically presented lower friction and lower surface tension provided by oil presence in medium, respectively; F_f – force of friction. (For interpretation of the references to color in the text, the reader is referred to the web version of this article).

duration of the biosynthesis process, and pH or air pressure (Hornung et al., 2006a). In turn, the values of this parameter reflect important BC material properties such as tensile strength or elongation at break (Mihranyan, Llagostera, Karmhag, Strømme, & Ek, 2004). It should be noted that historically, modifications aimed at increasing BC yield frequently cause the depreciation of useful properties (Cheng, Catchmark, & Demirci, 2009; Czaja, Romanovicz, & Brown, 2004; Krystynowicz et al., 2002; Watanabe, Tabuchi, Morinaga, & Yoshinaga,

1998). Therefore, the lack of any negative impact on BC properties after adding oil to culture media, which is confirmed by characteristic absorption bands of BC functional groups observed in ATR-FTIR spectra, is of paramount importance in the context of future applications of oil-modified BC. This indicates that the application of oil-modified media does not negatively influence the internal architecture of this unique BC polymer. Moreover, oil-modified cellulose displays mechanical strength exceeding three times the value of this parameter recorded for cellulose cultured in standard media.

5. Conclusion

Herein, we present a significant increase of BC yield by simple, but effective and repeatable, technique of culturing medium supplementation with 1% vegetable oil. As we demonstrated, this significantly increased BC yield is the result of microscopic and macroscopic physiochemical processes reflecting a complex interaction between *K. xylinus* biophysiology, chemical processes of BC synthesis, and physiochemical forces between BC membranes, oil and culturing vessel walls. Our work and findings indicate that the use of oil added to culture medium provides an important novel technique for increasing and enhancing cellulose biogenesis by *K. xylinus*, which could have significant impact in multiple biotechnological applications of this material.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.06.126.

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