ISSN 1644-0714
ISSN 2300-6145 (online)
www.asp.zut.edu.pl

Acta Sci. Pol. Zootechnica 14(1) 2015, 137–150

THE EFFECT OF DIFFERENT AGITATION MODES ON BACTERIAL CELLULOSE SYNTHESIS BY *GLUCONACETOBACTER XYLINUS* STRAINS

Anna Żywicka, Dorota Peitler, Rafał Rakoczy, Maciej Konopacki, Marian Kordas, Karol Fijałkowski

West Pomeranian University of Technology, Szczecin, Poland

Abstract. The study focuses on the effect of various agitation modes on bacterial cellulose (BC) synthesis by different strains of *Gluconacetobacter xylinus*. The BC biosynthesis process was performed for 7 days at 28°C in 50-mL plastic tubes, using a roller shaker, or in 50-mL plastic tubes equipped with disks made of metal ferrite or polycarbonate, using a shaker roller, or in 100-mL glass flasks on an orbital shaker. All the cultures were carried out in the agitated conditions with a variety of mixing speeds. After 7 days of incubation, the weight of the synthesized BC, density and pH of the culture medium were determined. It was found, that the stirring speed during the cultivation was one of the most important parameters affecting the process of bacterial cellulose biosynthesis. It has been shown that, depending on the stirring speed, BC formed spherical forms of different sizes or was produced as a mass with irregular shapes. Higher density of *G. xylinus* cells was found in the culture subjected to a higher stirring speed. It was also determined that the highest weight of BC was obtained in the culture agitated at the speed of 150 rpm using the orbital shaker.

Key words: bacterial cellulose, *Gluconacetobacter xylinus*, culture conditions, agitation speed, culture optimization

INTRODUCTION

Cellulose is the most abundant natural biopolymer on earth. It can be synthesized by plants, algae and microorganisms [Keshk 2014]. Bacterial cellulose

Corresponding author: Karol Fijałkowski, Department of Immunology, Microbiology and Physiological Chemistry, West Pomeranian University of Technology, Szczecin, al. Piastów 45, 70-310 Szczecin, Poland, e-mail: karol.fijalkowski@zut.edu.pl

(BC) has been widely recognized as a multifunctional biomaterial. BC performs better then plant cellulose in areas such as biomedicine, paper production, textile industry and environmental protection, for its distinct superior chemical purity, crystallinity, biocompatibility and ultrafine network architecture [Hornung et al. 2003]. Moreover, this material shows high water holding capacity, therefore it can be used as an excellent dressing for wound healing, without causing any side effects or allergic reactions [Bielecki et al. 2012]. BC pellicle may also be used in medical implantation [Kubiak et al. 2009]. By using the appropriate technique, it is possible to create e.g. implants blood vessels of different diameter and length or prosthesis of intervertebral discs, nose septum and breast [Hornung et al. 2003]. The BC nanofibryls form a regular and ordered structure, which makes this material extremely strong mechanically. Such unique properties make the BC pellicle a good carrier for immobilization of microbial cells, enzymes and natural dyes. The BC is also used for manufacturing of high quality paper, acoustic membranes and filter material with high strength and chemical stability [Keshk 2014]. The BC may also be used in human food and animal feed industries, especially as a thickening and gelling substance, stabilizer, water binding material and as packaging material. The BC has also been shown to be a good low-calorie food additive [Shi et al. 2014].

From the practical point of view, only *Gluconacetobacter xylinus* is a species of bacteria that is able to produce high amounts of cellulose and only this bacterium can produce cellulose at commercial levels [Keshk 2014]. The BC synthetized by *G. xylinus* is chemically pure as it does not contain any other substances, such as lignin and hemicellulose [Ruka et al. 2012]. The quantity and physical-chemical properties of BC mainly depend on the cultivation conditions, such as the culture medium composition, pH, temperature, dissolved oxygen content and the type of cultures used [Lee et al. 2014]. The current methods of BC production are based on static or agitated cultures.

Most of the studies are carried out on the BC synthetized in the static culture [Czaja et al. 2014]. In that type of culture, the microbiological medium is placed in shallow trays and inoculated with bacteria. This BC method production is expensive and characterized by low productivity. The efficiency of BC production in stationary cultures is strongly connected with the area of air–bioliquid surface. Agitating cultures require higher power supply when compared with the static BC method production. The main advantage of application of agitating culture for the BC biosynthesis are high cell concentration and productivity [Kralisch et al. 2010, Shezad et al. 2010]. In order to produce BC at industrial scale and achieve tangible profits from its synthesis, the production process must be adapted to stirred cultures in bioreactors, which requires optimization of the entire process [Czaja et al. 2004, Hornung et al. 2006]. The appropriate type of agitator and agitation speed

may be the key factor which defines the amount of obtained BC but also effect on its quality. Therefore, the aim of the present study was to assess and extend the current state of knowledge of the effects of various agitation modes on bacterial cellulose synthesis by different strains of *Gluconacetobacter xylinus*.

MATERIAL AND METHODS

Microorganisms and culture conditions

For the production of cellulose, we used three reference strains of *Gluconacetobacter xylinus* (Deutsche Sammlung von Mikroorganismen und Zellkulturen): DSM 46602, DSM 5602 and DSM 46604. Initially, bacteria were cultivated in stationary conditions using a Herstin–Schramm (H–S) medium composed of glucose, 2 wv%, yeast extract, 0.5 wv%, bacto-pepton, 0.5 wv%, citric acid, 0.115 wv%, Na_2HPO_4, 0.27 wv%, $MgSO_4 \cdot 7H_2O$, 0.05 wv%, and ethanol, 1 vv% added after sterilization [Ciechańska et al. 1999]. Prior the experiment, the 7-day cultures were vigorously shaken and the obtained bacterial suspension was used to inoculate H–S medium. The cultivation was performed in agitated conditions on a rocking roller (20 and 70 rpm) for 7 days at 28°C in 50-mL plastic tubes (3.8-cm in diameter, polypropylene conical centrifuge tube, Becton Dickinson and Company, USA) or in 50-mL plastic tubes combined with discs made of ferritic metal (ferritic steel, 1.4016) or polycarbonate plastic of different levels of roughness and wrinkles (Fig. 1). The BC biosynthesis process was also performed in 100-mL glass flasks on the orbital shaker (100–300 rpm, ES–20/60, Biosan, Latvia).

Determination of BC production

The BC was harvested from the medium, washed carefully with distilled water, drained of excess water and weighed on an analytical balance (WTB 2000 Radwag, Poland).

Quantification of BC producing bacteria

The density of BC producing bacteria were determined in liquid H-S medium, after cellulose was removed from the culture tube. All the samples were centrifuged for 20 min at 3.300 × g. The resulting pellets were washed in PBS (Phosphate Buffered Saline, Sigma-Aldrich), centrifuged at 3.300 × g for 20 min and restored to the original volume with PBS. Next, the optical density (OD) of bacterial cultures were measured at the wavelength of 600 nm in 96 well plates (Becton

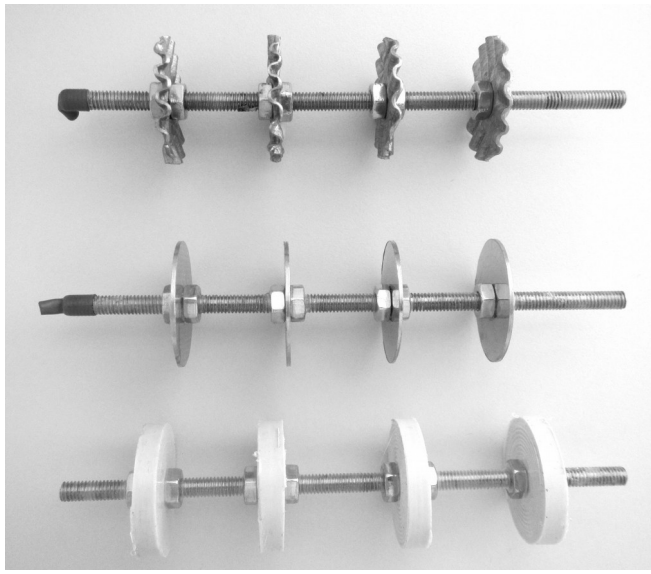

Fig. 1. Discs made of ferritic metal and polycarbonate plastic

Rys. 1. Dyski wykonane z metalu ferrytycznego i poliwęglanu

Dickinson and Co.) with 200 μL of each sample of bacterial suspension using microplate reader (Infinite 200 PRO NanoQuant, Tecan, Switzerland).

pH measurements

pH determination was performed using the pH meter (PH Lon Meter, CIP-501, Elentron, Poland) after cellulose was removed from the culture tubes and the medium was centrifuged for 20 min at 3.300 × g to remove *G. xylinus* cells.

RESULTS

Determination of BC production

The cultivation was performed in agitated conditions on a rocking roller at stirring speed of 20 and 70 rpm. In the case of biosynthesis performed at 20 rpm in the 50 mL tubes it was observed, that BC formed regular spherical shapes (Fig. 2).

With a rotation of 70 rpm, BC adhered on the walls of the tube in the form of biofilm. Analogous results were observed for all three strains used in the experi-

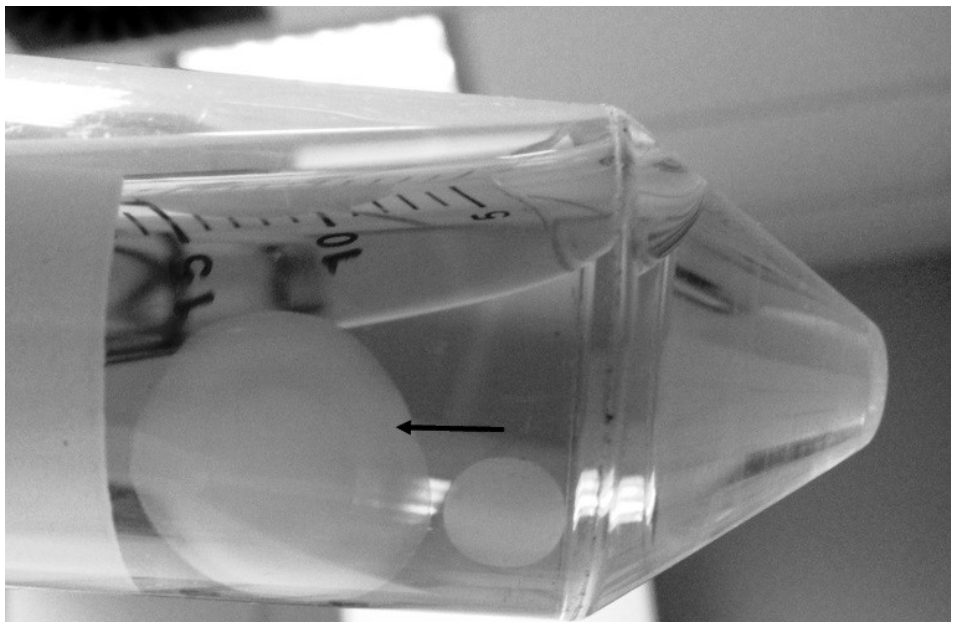

Fig. 2. The BC obtained using rocking roller at the stirring speed of 20 rpm (*G. xylinus* DSM 46602)

Rys. 2. Celuloza bakteryjna otrzymana przy użyciu wytrząsarki rolkowej z szybkością mieszania 20 rpm (*G. xylinus* DSM 46602)

ment. The results of the weight of obtained cellulose are summarized in the Table 1.

Table 1. Weight of wet BC (g) obtained in agitated cultures carried out on the rocking roller

Tabela 1. Waga mokrej celulozy bakteryjnej (g) otrzymanej w hodowlach prowadzonych na wytrząsarce rolkowej

Speed, rpm Prędkość, obr./min	DSM 46602	DSM 5602	DSM 46604
20	1.52	0.81	0.49
70	ND	ND	ND

ND – not determined – nie ustalono.

Discs made of metal and plastic with different levels of roughness and wrinkles were used to improve the stirring of medium during the experiment. However it was found, that at both stirring speeds (20 and 70 rpm) the BC biosynthesis did not occurred and cellulose did not appeared on any disks, regardless the *G. xylinus*

strain used in the experiment. It was only observed, that the thin mass of BC was formed on the threaded bar between disks (Fig. 3).

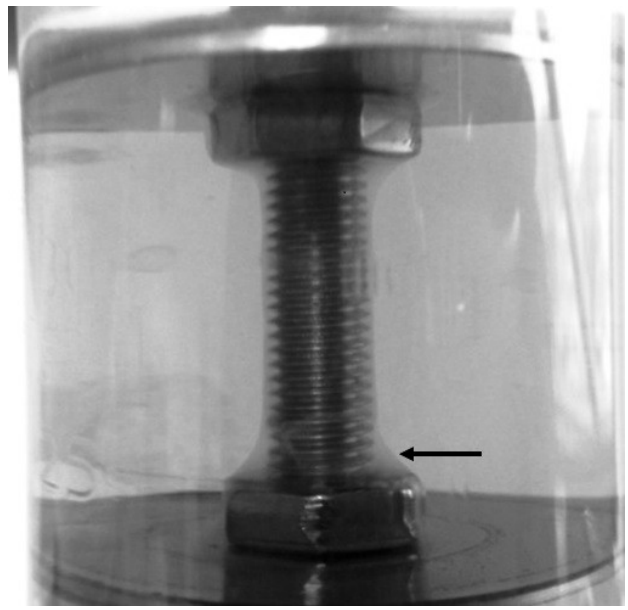

Fig. 3. The BC obtained in cultures carried out on the rocking roller with the use of discs, at the stirring speed of 20 rpm (*G. xylinus* DSM 46602)

Rys. 3. Celuloza bakteryjna otrzymana przy użyciu wytrząsarki rolkowej z wykorzystaniem dysków, z szybkością mieszania 20 rpm (*G. xylinus* DSM 46602)

In the case of cultures carried out on the orbital shaker, it was observed that at lower rotation speed (100 rpm) the BC was produced as uniform, large, spherical forms (0.5–1 cm, Fig. 4A), whereas the higher rotational speed (150 rpm to 250 rpm) caused the decrease in size of the formed BC spheres (Fig. 4B, 3C). At the highest rotation speed (300 rpm), the BC was produced as mass with irregular shapes (Fig. 4D). Similar results were observed for all three strains used in the experiment.

Based on the obtained results, it was also shown that the highest weight of BC was obtained in the case of cultured agitated at 150 rpm on the orbital shaker (Table 2).

Quantification of cellulose producing bacteria

In agitated cultures carried out on the rocking roller in 50-mL tubes, with and without discs, the densities of *G. xylinus* cells were below the detection limit.

However, in cultures agitated on the orbital shaker, it was shown that the density of *G. xylinus* bacterial cells increased with the stirring rate (Table 3).

pH measurements

It was recorded that in 7 days cultures agitated on a rocking roller pH values did not drop below 4.5 (Table 4).

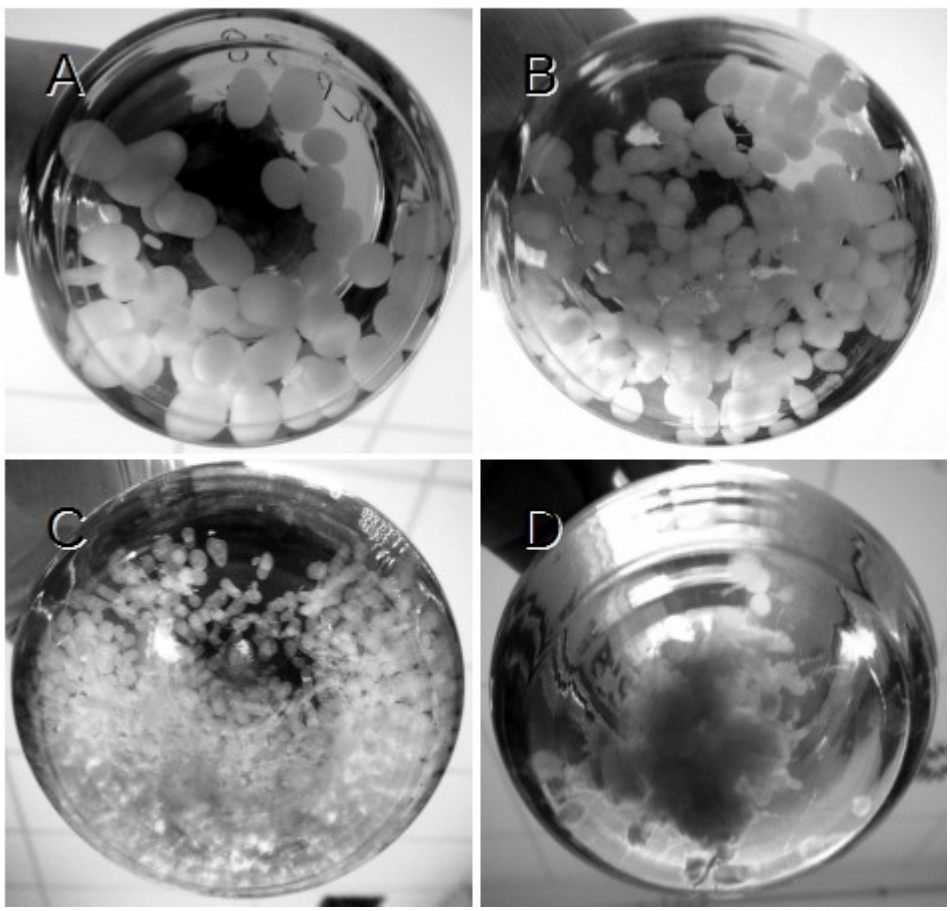

Fig. 4. The BC obtained in agitated cultures carried out on the orbital shaker at the different rotation speeds, A) 100 rpm, B) 150 rpm, C) 250 rpm, D) 300 rpm (*G. xylinus* DSM 46602)

Rys. 4. Celuloza bakteryjna otrzymana przy użyciu wytrząsarki orbitalnej z zastosowaniem różnej szybkości mieszania, A) 100 rpm, B) 150 rpm, C) 250 rpm, D) 300 rpm (*G. xylinus* DSM 46602)

Table 2. Weight of wet BC (g) obtained in agitated cultures carried out on the orbital shaker at different rotation speeds

Tabela 2. Waga mokrej celulozy (g) uzyskanej w hodowlach prowadzonych z wykorzystaniem wytrząsarki orbitalnej, przy różnych szybkościach mieszania

Speed, rpm Prędkość, obr./min	DSM 46602	DSM 5602	DSM 46604
100	3.10	1.95	2.96
150	11.46	3.72	5.13
250	7.73	1.53	3.56
300	3.91	0.96	2.72

Table 3. The density of *G. xylinus* cells in agitated cultures carried out on the orbital shaker at different rotation speeds

Tabela 3. Gęstość komórek *G. xylinus* w hodowlach prowadzonych z wykorzystaniem wytrząsarki orbitalnej, przy różnych szybkościach mieszania

Speed, rpm Prędkość, rpm	DSM 46602	DSM 5602	DSM 46604
100	0.759	1.005	0.658
150	0.872	1.027	0.674
250	1.613	1.434	1.181
300	1.634	1.493	1.199

Table 4. pH in H-S medium after 7 days of cultivation carried out on the rocking roller at different rotation speeds

Tabela 4. pH medium H-S po 7 dniach hodowli prowadzonych z wykorzystaniem wytrząsarki rolkowej, przy różnych szybkościach mieszania

	Speed, rpm Prędkość, obr./min	DSM 46602	DSM 5602	DSM 46604
Empty tube Pusta probówka	20	4.87	4.80	4.85
	70	4.53	4.64	4.53
With disc Z dyskiem	20	4.98	4.87	4.81
	70	4.67	4.53	4.46

However, in the case of cultures agitated on the orbital shaker, it was found that the pH dropped below critical for BC synthesis 4.0 (Table 5). Similar results were obtained for cultures with all included in that study *G. xylinus* strains.

DISCUSSION

The present study concerns the assessment of various agitation modes applied during the cultivation on bacterial cellulose biosynthesis by three reference stra-

Table 5. pH in H-S medium after 7 days of cultivation carried out on the orbital shaker at different rotation speeds

Tabela 5. pH medium H-S po 7 dniach hodowli prowadzonych z wykorzystaniem wytrząsarki orbitalnej, przy różnych szybkościach mieszania

Speed, rpm Prędkość, obr./min	DSM 46602	DSM 5602	DSM 46604
100	3.32	3.56	3.55
150	3.67	3.78	3.83
250	3.36	3.63	3.53
300	3.43	3.42	3.64

ins of *Gluconacetobacter xylinus*. As it is known, under agitated conditions the cellulose produced by *G. xylinus* strains is formed as fibrous suspensions, pellets, or irregular masses within the medium [Watanabe et al. 1998, Czaja et al. 2004, Ruka et al. 2012]. Based on the results obtained it the present study, it can be concluded, that the stirring speed used during the cultivation was one of the most important parameters measured within the experiment, which significantly influenced the quantity and quality of the synthetized BC.

In the analysis on agitation conditions using a rocking roller, it was found that regardless of the stirring speed and disks combination, the *G. xylinus* strains produced very slight amounts of cellulose. However, it had been previously shown that horizontal fermentors equipped with rotating discs or rollers were successfully applied to improve culture conditions and BC biosynthesis [Krystynowicz et al. 2002, Cheng et at. 2011, Lin et al. 2014]. On the other hand, disks used in our study were made from other materials and had other shapes compared to those used by other authors, which could affect the production of BC. Furthermore, agitation rate could also limit the BC production process. As it was determined by Chawla et al. [2009] the optimal rotation speed of the rotating discs in fermentor was 15 rpm, whereas according to Krystynowicz et al. [2002] 4 rpm.

In the case of cultures agitated on the orbital shaker at different rotation speeds, it was observed, that along increasing rate of stirring speed, the synthesized BC was characterized by more irregular shapes; from the shape of spherical particles synthetized at the speed of 100–150 rpm to the mass of irregular shapes obtained at 300 rpm. The present studies also showed, that the highest weight of BC was obtained at the agitation speed of 150 rpm on the orbital shaker. This agree with the results reported by Attariansah [2003], obtained in the study in which the author compared different stirring speeds (100, 140, 180, 220 rpm) on the production of BC by the strain *G. xylinus* (DSM 46602). According to this author, the rotation speed of 140 rpm was optimal for the production of BC by the investigated bacterium strain. However, the author suggested, that the optimal

stirring speed for the BC synthesis may vary among different strains. In our study, the rotation speed of 150 rpm was found to be optimal for all three *G. xylinus* strains used in the experiments.

Bacterial cellulose is synthesized by metabolically active *G. xylinus* cells, then it is secreted into the external environment allowing to access oxygen on the surface of the medium [Bielecki and Kalinowska 2008]. In the present study, it was recorded, that more *G. xylinus* cells in medium were found at higher stirring speeds. Therefore, it can be concluded, that cultivation of *G. xylinus* under agitated conditions resulted in increased cell growth, but decreased BC production. Such findings agree with previous suggestions by Czaja et al. [2004], who found, that the increase in cell growth can be caused by the high aeration, achieved in the agitated cultures, which substantially decreases the BC synthesis needed to anchor the cells at the top of the media to provide sufficient levels of oxygen. However, according to Hornung et al. [2006] the access to the oxygen is not the only factor, that may limit BC formation. The authors showed, that except oxygen, also agitation can inhibit BC synthesis as it may affect the binding of BC fibrils to the edge of the flask. When cellulose is formed in static culture, its formation begins as a biofilm around the edge of the flask and spreads across the surface toward the centre.

The pH optimum for the production of cellulose ranges from 4.0 to 6.0 [Keshk 2014]. During long *G. xylinus* cultivation, gluconic acid or lactic acid accumulate, which decreases pH below the optimal range. In our study, it was recorded, that pH of cultures cultivated on the orbital shaker dropped from 6.5 (pH of H–S medium before inoculation) to the range between 3.32–3.83. As it was shown by Aramwit and Bang [2014], BC production intensity decreases at pH below 4 and can even be inhibited. Therefore, pH values during the BC biosynthesis should always be monitored and maintained on the optimal level [Keshk 2014].

CONCLUSIONS

In conclusion, our study demonstrates that the stirring mode during the cultivation was one of the most important parameters of BC production. The study shows that the highest weight of BC was obtained at the agitation speed of 150 rpm on the orbital shaker. It has also been shown that with an increasing stirring speed, the synthesized BC was characterized by more irregular shapes.

ACKNOWLEDGEMENT

This study was supported by the National Research and Development Centre (Grant No. LIDER/011/221/L-5/13/NCBR/2014).

REFERENCES

Aramwit, P., Bang, N. (2014). The characteristics of bacterial nanocellulose gel releasing silk sericin for facial treatment. BMC Biotechnol., 14(104), 1–11.

Attariansah, O. (2003). Cultivation medium selection and optimization agitation speed of bacterial cellulose production by *Acetobacter pasteurianum* in shaking culture. Scientific Repository – Bogor Agricultural University (IPB). Indonesia.

Bielecki, S., Kalinowska, H. (2008). Biotechnologiczne nanomateriały [Biotechnological nanomaterials]. Post. Mikrobiol., 47 (3), 163–169 [in Polish].

Chawla, P.R., Bajaj, I.B., Survase, S.A., Singhal, R.S. (2009). Microbial Cellulose: fermentative production and applications. Food Technol. Biotechnol., 47(2), 107–124.

Cheng, K.C., Catchmark, J.M., Demirci, A. (2011). Effects of CMC addition on bacterial cellulose production in a biofilm reactor and its paper sheets analysis. Biomacromolecules, 12, 730–736.

Czaja, W., Romanowicz, D., Brown, R.M. (2004). Structural investigations of microbial cellulose produced in stationary and agitated culture. Cellulose, 11(3–4), 403–411.

El–Saied, H., El–Diwany, A.I., Basta, A.H., Atwa, N.A., El-Ghwas, D.E. (2012). Production and characterization of economical bacterial cellulose. BioResources, 3(4), 1196––1217.

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. Eng. Life Sci., 6(6), 537–545.

Keshk, S. (2014). Bacterial cellulose production and its industrial applications. J. Bioproces. Biotechniq., 4(2), 2–10.

Kouda, T., Yano, H., Yoshinaga, F. (1997). Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. J. Ferment. Bioeng., 83(4), 371––376.

Kralisch, D., Hessler, N. (2012). Large-scale production of BNC: state and challenges. In: Bacterial NanoCellulose: A Sophisticated Multifunctional Material. CRC Press, USA.

Kralisch, D., Hessler, N., Klemm, D., Erdmann, R., Schmidt, W. (2010). White biotechnology for cellulose manufacturing – The HoLiR concept. Biotechnol. Bioeng., 105, 740–747.

Krystynowicz, A., Czaja, W., Wiktorowska-Jezierska, A., Gonçalves-Miśkiewicz, M., Turkiewicz, M., Bielecki, S. (2002). Factors affecting the yield and properties of bacterial cellulose. J. Ind. Microbiol. Biotechnol., 29(4), 189–195.

Kubiak, K., Kalinowska, H., Peplińska, M., Bielecki, S., (2009). Celuloza bakteryjna jako nanobiomateriał [Bacterial cellulose as a nanobiomaterial]. Post. Biol. Kom., 36, 85––98 [in Polish].

Lee, K.J., Buldum, G., Mantalaris, A., Bismarck, A. (2014). More than meets the eye in bacterial cellulose: biosynthesis, bioprocessing, and applications in advanced fiber composites. Macromol. Biosci., 14, 10–32.

Lin, P.S., Hsieh, S.C., Chen, K.I., Demirci, A., Cheng, K.C. (2014). Semi-continuous bacterial cellulose production in a rotating disk bioreactor and its materials properties analysis. Cellulose, 21(1), 835–844.

Ruka, D.R., Simon, G.P., Dean, K.M. (2012). Altering the growth conditions of *Gluconacetobacter xylinus* to maximize the yield of bacterial cellulose. Carbohydr. Polym., 89, 613–622.

Shezad, O., Khan, S., Khan, T., Park, J.K. (2010). Physicochemical and mechanical characterization of bacterial cellulose produced with an excellent productivity in static conditionsusing a simple fed-batch cultivation strategy. Carbohydr. Polym., 82, 173––180.

Shi, Z., Zhang, Y., Phillips, G.O., Yang, G. (2014). Utilization of bacterial cellulose in food. Food Hydrocolloids., 35, 539–545.

Watanabe, K., Tabuchi, M., Morinaga, Y., Yoshinaga, F. 1998. Structural features and properties of bacterial cellulose produced in agitated culture. Cellulose, 5, 187–200.

WPŁYW RÓŻNYCH SPOSOBÓW MIESZANIA NA BIOSYNTEZĘ CELULOZY BAKTERYJNEJ PRZEZ SZCZEPY *GLUCONACETOBACTER XYLINUS*

Streszczenie. Celem badań była ocena wpływu różnych sposobów mieszania hodowli na biosyntezę celulozy bakteryjnej przez różne szczepy *Gluconacetobacter xylinus*. Proces biosyntezy celulozy prowadzono przez 7 dni w temperaturze 28°C z zastosowaniem różnych szybkości mieszania, w 50 mL plastikowych probówkach z wykorzystaniem wytrząsarki rolkowej, w 50 mL plastikowych probówkach z dyskami wykonanymi z metalu ferrytycznego lub poliwęglanu z wykorzystaniem wytrząsarki rolkowej oraz w 100 mL szklanych kolbach na wytrząsarce orbitalnej. Po 7 dniach inkubacji oznaczano wagę zsyntetyzowanej celulozy, gęstość komórek bakteryjnych oraz pH medium hodowlanego. Na podstawie uzyskanych wyników stwierdzono, że szybkość prowadzonych hodowli była jednym z najbardziej istotnych parametrów wpływających na proces biosyntezy celulozy bakteryjnej. Wykazano, że w zależności od szybkości mieszania prowadzonych hodowli, syntetyzowana celuloza przybierała formy kuliste o różnych rozmiarach lub produkowana była jako masa o nieregularnych kształtach. Większą gęstość komórek *G. xylinus* odnotowano w hodowlach mieszanych z wyższymi prędkościami. Ponadto, ustalono, że największą wagą charakteryzowała się celuloza uzyskana w hodowlach prowadzonych na wytrząsarce orbitalnej z szybkością mieszania wynoszącą 150 rpm.

Słowa kluczowe: celuloza bakteryjna, *Gluconacetobacter xylinus*, warunki hodowli, szybkość mieszania, optymalizacja hodowli

Accepted for print: 27.03.2015

For citation: Żywicka, A., Peitler, D., Rakoczy, R., Konopacki, M., Kordas, M., Fijałkowski, K. (2015). The effect of different agitation modes on bacterial cellulose synthesis by *Gluconacetobacter xylinus* strains. Acta Sci. Pol. Zootechnica, 14(1), 137–150.