Zeszyty Problemowe Postępów Nauk Rolniczych
nr 599, 2019, 53–63
DOI 10.22630/ZPPNR.2019.599.22

THE IMPACT OF CHEMICAL AND CHEMICAL-PHYSICAL MUTAGENISATION ON PHENOTYPIC CHANGES IN THE BACTERIA OF THE SPECIES *KOMAGATAEIBACTER XYLINUS*

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Summary. Acetic acid bacteria *Komagataeibacter xylinus* are characterized by, i.a., the ability to produce bacterial cellulose, gluconic acid and dihydroxyacetone. The aim of this study was to use non-directional chemical and chemical-physical mutagenisation and to invastigating the impact of this process on the selected phenotypic features of *K. xylinus*. Chemical mutagenisation was carried out with the use of methyl methanesulfonate. Chemical-physical mutagenisation has been extended with the application of UV radiation (λ = 254 nm). The obtained mutant was characterized by higher of 56% ability to biosynthesize bacterial cellulose (compared to wild type). The study showed that chemical-physical mutagenisation may be a sufficient method for increasing bacterial cellulose synthesis, while it does not ensure an increased yield of gluconic acid and dihydroxyacetone by the tested strain.

Key words: chemical mutagenisation, chemical-physical mutagenisation, *Komagataeibacter xylinus*, bacterial cellulose, gluconic acid, dihydroxyacetone

INTRODUCTION

Acetic acid bacteria (AAB) are gram-negative, aerobic rods. Their characteristic feature is the ability to oxidize ethyl alcohol to acetic acid. These microorganisms are also used to produce, i.a. bacterial cellulose, gluconic acid and dihydroxyacetone [Gomes et al. 2018].

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Bacterial cellulose (BC) is a natural biopolymer composed of multiple chains of ß-1,4-glucan connected by glycosidic bonds [Picheth et al. 2017]. BC has good mechanical properties, a high level of crystallinity and a high water retention capacity [An et al. 2017]. BC is used, a.i. in the food production (as a filler or stabiliser), cosmetics (a component of face masks, peels, contact lenses) and electronic industry (material for the production of flexible supercapacitors, biosensors, acoustic membranes), as well as in medicine and dentistry (building material of artificial skin, blood vessels, dressings) [Torres et al. 2019, Gullo et al. 2018, Chen et al. 2016, Ullah et al. 2016].

Gluconic acid (GA) is an interesting metabolite of AAB, commonly used in the food industry as an acidity regulator and preservative compound [La China et al. 2018]. Dihydroxyacetone (DHA) is another metabolite of AAB, widely used in industry. This substance, after contact with human skin, causes a brown color. This feature was used in the production of self-tanning preparations in which DHA is the main active compound [Braunberger et al. 2018]. In order to improve the biochemical potential of microorganisms to produce specific compounds, chemical, physical or combined mutagenesis may be employed. [Yu et al. 2020, Goodarzi 2016]. The aim of this study was to assess the impact of chemical and chemical-physical mutagenisation as potential factors enabling the improvement of selected biochemical features of acetic acid bacteria of the species *K. xylinus*.

MATERIALS AND METHODS

A biological material in this study was the strain *Komagataeibacter xylinus*, derived from the Pure Cultures Collection of the Department of Biotechnology and Food Microbiology, Institute of Food Science, Warsaw University of Life Sciences (WULS--SGGW). The strain was cultured on a (controlled) medium [$g \cdot L^{-1}$]: yeast extract 30, ethyl alcohol 20, agar 15 (if necessary) pH 5.5 for 48 hours at 28°C on a reciprocating shaker (180 rpm).

CHEMICAL AND CHEMICAL-PHYSICAL MUTAGENISATION

Chemical mutagenisation (Ch-m) of the strain *K. xylinus* was carried out on a medium composed of [$g \cdot L^{-1}$]: yeast extract 30, ethyl alcohol 20, pH 5.5, the mutagenic agent was methyl methanesulfonate (MMS, Fluka, USA). Two concentrations of MMS 0.3% and 0.6% were used for chemical mutagenisation.

In order to perform Ch-m, the *K. xylinus* (10% v/v) inoculum was transferred to a suitable MMS medium, mutagenisation was carried out at 28°C for 60 minutes on a reciprocating shaker (180 rpm).

Chemical-physical mutagenisation (Ch-ph-m) was carried out in the same way as Ch-m, except that after 60 minutes of incubating the cells in the MMS medium, 1 cm^3 of suspension was transferred to Petri dishes and exposed to UV light at 254 nm for 120 seconds, the distance of the suspension from the light source was 60 cm. After this time, the samples were incubated in the darkroom for 45 minutes to prevent photoreactivation.

From each sample of the suspension after mutagenisation (Ch-m or Ch-ph-m), 0.1 cm^3 of cell suspension was taken and spread on Petri dishes. The culture was carried out at 28°C for 96 hours. The survival of *K. xylinus* cells, measured in percentages, in the samples after mutagenisation were calculated relative to the control no exposed to mutagens. Calculations were made in three repetitions for each performed dilution (10^{-1}, 10^{-2}, 10^{-3}) [Hu et al. 2011].

MUTANTS SCREENING

Fifty mutants were randomly selected to next stages of research. Each mutant was tested for changes in the examined phenotypic traits, i.e. BC synthesis, GA and DHA production. Single pure colonies were taken from Petri dishes and suspended in 2 cm^3 of 0.75% NaCl, then 0.6 cm^3 of this suspension was taken and transferred to an appropriate liquid medium for selection aiming at: BC synthesis ($g \cdot L^{-1}$: glucose 30, yeast extract 20, ethyl alcohol 20, pH 5.0), DHA production ($g \cdot L^{-1}$: glycerol 30, yeast extract 20, pH 5,0) and GA production ($g \cdot L^{-1}$: glucose 30, yeast extract 20, pH 5.0). All cultures were carried out at 28°C for 72 hours.

MUTANTS SELECTION FOR INCREASED BACTERIAL CELLULOSE PRODUCTION

The ability of BC synthesis by obtained mutants was investigated, based on the presence or absence of BC on the surface of the liquid broth. During the initial selection, cultures were kept in test tubes for 72 h at 28°C. In the case of presence of BC, the mutant was subjected to the second selection stage, involving culturing in the media containing various sources of carbon ($g \cdot L^{-1}$: glucose or technical glycerol or waste glycerol 30, yeast extract 5, $(NH_4)_2SO_4$ 7.5, pH 5,0). To achieve this stage, 5 cm^3 of the cell suspension was transferred to 45 cm^3 of an appropriate medium. Cultures were grown at 28°C for 48 hours using the shake flask method and then with the stationary method for 120 h. The resulting CB was weighed and then dried in order to quantify the dry matter. In parallel with the cultivation and evaluation of mutants, wild-type (WT) cultures were carried out on the control medium.

MUTANTS SELECTION FOR INCREASED GLUCONIC ACID PRODUCTION

The first stage of selection was to measure the pH of the medium after 72 hours of culturing the mutants and WT. The mutants, showing a WT-like capability of lowering the pH of the medium, were subjected for a second selection step. It consisted in conducting the culture in a larger volume of medium (50 ml) with increased glucose concentration ($g \cdot L^{-1}$: glucose 80, yeast extract 1, $(NH_4)_2SO_4$ 1, $MgSO_4 \cdot 7H_2O$ 1, pH 5.5) and determining the content of GA by titration. Selected samples were titrated with 0.1 M NaOH in the presence of phenolphthalein, where 1 cm^3 of NaOH corresponded to 0.0196 g of GA. The

study was performed both on a sterile control medium and on the media used for mutant and WT cultures. The obtained results were converted into the GA content in 1 dm^3 of the medium and then subjected to statistical analysis.

MUTANTS SELECTION FOR INCREASED DIHYDROXYACETONE PRODUCTION

In the first stage of mutant selection, the method with Fehling's solution was used. The second selection stage involved culturing on a medium for activating glycerol dehydrogenase, composed of [$g \cdot L^{-1}$] yeast extract 5, glycerol 20, $(NH_4)_2SO_4$ 5, pH 5.0, at 28°C for 48 hours. Then, the shake flask culture was conducted in a medium composed of [$g \cdot L^{-1}$]: yeast extract 5, glycerol 30, $(NH_4)_2SO_4$ 7.5, pH 5.0, at 28°C for 96 hours [Stasiak-Różańska et al. 2018]. Samples for determining the concentrations of DHA were collected every 24 hours during a period of 96 hours. DHA concentration was determined with the spectrophotometric method, according to Chen et al. [2008].

ANALYSIS OF RESULTS

The Duncan test was used to analyse the study results statistically. The analysis was performed using the R-Commander program.

RESULTS AND DISCUSSION

Survival rate of bacteria after mutagenisation

The *K. xylinus* strain had their survival rate significantly reduced under the influence of Ch-m and Ch-ph-m.

Table 1. Average number of colony forming units ($ncf \cdot cm^{-3}$) *K. xylinus* after chemical and chemical-physical mutagenisation

Tabela 1. Średnia liczba jednostek tworzących kolonie ($jtk \cdot cm^{-3}$) *K. xylinus* po mutagenizacji chemicznej i chemiczno-fizycznej

	Dilutions – Rozcieńczenia		
	10^{-1}	10^{-2}	10^{-3}
Mutagenic factor type Czynnik mutagenny		$ncf \cdot cm^{-3}$	
Wild type Szczep dziki	>300	>300	174 ±16,82
0,3% MMS	>300	225 ±12,77	2,33 ±1,53
0,6% MMS	>300	98 ±8,19	3,0 ±1,0
0,3% MMS + UV	>300	181 ±9,17	0,0 ±0,0
0,6% MMS + UV	>300	60 ±4,58	0,0 ±0,0

As a consequence of using Ch-m, the assumed bacterial survival rate of 1.34% was reached and it fell within the suggested range from 1 to 5% [Błażejak et al. 2011] in relation to WT survival rate in the control medium (0.3% MMS R=10^{-3} survival rate 2.33 cfu·cm^{-3}, WT, R =10^{-3} survival rate 174 cfu·cm^{-3}, Table 1). No bacterial colonies were obtained from a 10^{-3} dilution after exposure to Ch-ph-m. In similar works [Błażejak et al. 2011, Hu & Zheng 2011], the survival rate in the range of 1-5% was achieved after applying UV radiation, however, the time of exposure to the mutagen was shorter – 45, 60 and 90 seconds, respectively, and in those cases physical mutagenisation was not preceded by chemical mutagenisation. The survival rates of mutants presented in Table 1 may suggest that the UV radiation time applied (120 sec) may have been too long, and yet it allowed some mutants to survive. 50 mutants were randomly selected for further analysis.

SCREENING FOR BACTERIAL CELLULOSE PRODUCING MUTANTS

The initial stage of selection of *K. xylinus* mutants with regard to an increased BC production demonstrated that only 1 out of 50 mutants tested showed an increased ability to produce BC (as compared to WT). It was the M15 mutant. In the subsequent selection stages, the M15 mutant produced more BC than the wild-type strain on each of the media tested (with glucose/technical glycerol/waste glycerol). Figure 1 shows the mass of BC obtained as a result of culturing the M15 mutant and the wild-type strain of *K. xylinus* on the media with varied carbon content.

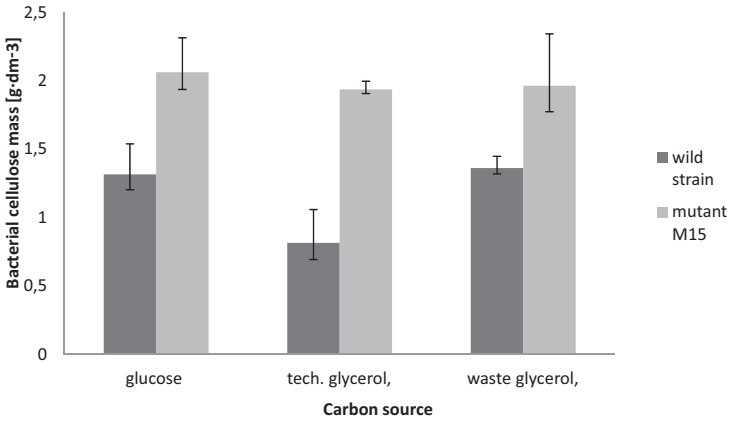


Fig. 1. Mass of bacterial cellulose [g·dm^{-3}] depending on the strain and carbon source

Rys. 1. Masa celulozy bakteryjnej [g·dm^{-3}] w zależności od szczepu i źródła węgla

On the medium containing glucose, the M15 mutant (obtained as a result of Ch-ph-m 0.3% MMS + UV) produced 56% (2.06 g) more BC than the WT (1.32 g). A similar result was achieved by Hungund & Gupta [2010]. The scientists obtained the GHUV4 mutant as a result of UV mutagenisation of the *G. xylinus* NCIM 2526. It was characterized by nearly 30% (3.92 g) higher efficiency of BC production when compared to that of the

wild type (3.04 g). This mutant was then exposed to the EMS chemical mutagen. Following this stage, the best of mutants, i.e. GHEM4, produced 5.96 g of cellulose, which was 50% more than the parental strain (GHUV4) and 98% more than the wild-type strain.

Similar results were also obtained by De Wulf et al. [1996]. In that study, a mutant of *Acetobacter xylinum*, obtained as a result of mutagenisation with UV radiation exposure, produced ca. 83% more cellulose than the parental strain. In addition, this mutant was characterized by a decreased ability to convert glucose into gluconic acid, which could also be noticed in the case of the M15 mutant obtained in this study. These studies indicate that the application of EMS together with UV radiation constituted an effective mutagenic agent for improving the phenotypic characteristics of AAB.

On the media with technical and waste glycerol (obtained from biodiesel production), the cellulose mass produced by the M15 mutant was almost the same and amounted to 1.94 g and 1.96 g, respectively (Fig. 1). On the medium with technical glycerol, the wild-type strain synthesized 1.24 g of cellulose, and on the glycerol waste medium it was 1.36 g (Fig. 1). Thus, on both media, the M15 mutant exhibited an increased ability to synthesize BC in comparison with the wild-type strain. Cutzu et al. [2013] obtained the 400A15 mutant after mutagenisation of *Rhodotorula glutinis* cells due to UV radiation exposure. The mutant exhibited a 100% increase (in relation to the wild-type) in the production of β-carotene on an optimized medium with waste glycerol [Cutzu et al. 2013].

Based on these results, it may be supposed that waste glycerol may be a very good source of carbon for the biosynthesis of various metabolites, including bacterial cellulose, and the development of effective and useful ways of managing this waste may contribute to environmental protection.

The decreased ability to biosynthesise BC by the wild-type on the medium with waste glycerol may suggest that the strain *K. xylinus* is sensitive to chemical pollutants present in the culture medium enriched with this waste. Waste glycerol contains, i.a., fatty acids, lipids and methanol, which could have had a negative impact on the production of bacterial cellulose [Kose et al. 2013]. According with these facts, it may be supposed that the changes leading to an increased tolerance to this type of pollutants may have occurred in the genome of the M15 mutant. It was confirmed by the research of Sandoval et al. [2015], in which the bacterium *Clostridium pasteurianum* was chemically mutated with the use of N-methyl-N-nitro-N-nitrosoguanidine, leading to the emergence of the M150B mutant, which exhibited a higher efficiency in the synthesis of butanol and an increased tolerance to waste glycerol. According to the scientists, the M150B mutant had a mutation in the key transcription regulator, Spo0A. Spo0A conditions the regulation of the expression of the genes responsible for sporulation and the production of butanol. Therefore, its inactivation resulted in a more tolerant and improved phenotype producing butanol. In addition, the M150B mutant produced more butanol (as compared to the wild-type) on the medium with pure glycerol, which could also be observed in the case of the M15 mutant and its biosynthesis of bacterial cellulose on the medium with pure technical glycerol [Sandoval et al. 2015]. The M15 mutant's increased ability to produce bacterial cellulose on the medium with waste glycerol suggests a significant research potential for broadening the knowledge about the possibility of using waste glycerol as a source of carbon for the production of BC.

SCREENING FOR GLUCONIC ACID PRODUCING MUTANTS

The initial selection of mutants with regard to their ability to produce gluconic acid demonstrated that only 11 out of 50 mutants tested exhibited a similar (as compared to the wild-type) level of GA production. The pH of the medium after culturing ranged from 3.82 for the wild type to 5.45 for the M23 mutant (Fig.2.). Such pH values of the medium may indicate that the DNA of the mutants underwent changes that prevented the production of acids. Thus, the negligible ability to produce gluconic acid may have been caused by the occurrence of mutations in the genes encoding proteins which are responsible for the oxidation of glucose to gluconic acid. Which is consistent with the results published by Raksha et al. [2012] in which the enzymes: glucose dehydrogenase and glucose oxidase, showed lower activity in a negative mutant (producing less GA than a wild type) compared to a positive mutant or wild type, which would indicate damage to the mechanisms associated with the bioconversion of glucose to GA.

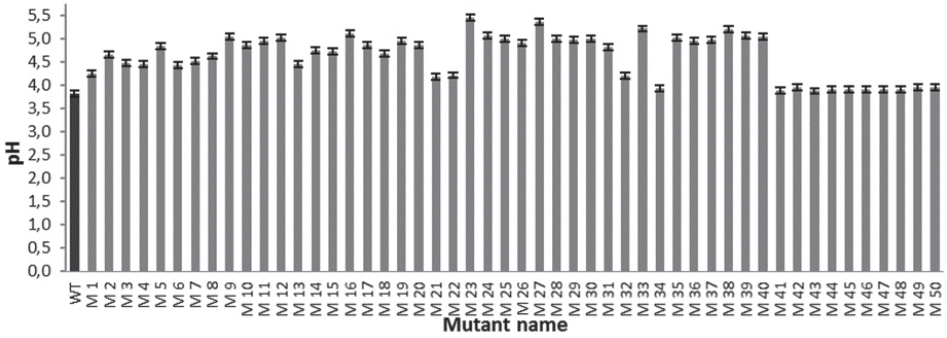


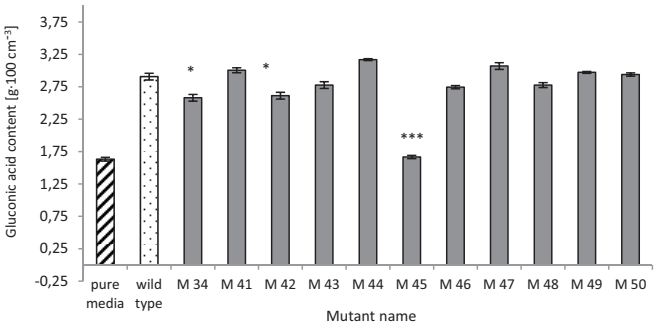
Fig. 2. The pH of the media after the first screening towards gluconic acid production

Rys. 2. Współczynnik pH podłoża po pierwszej selekcji w kierunku wytwarzania kwasu glukonowego

The correlation observed in the case of the M15 mutant, with the pH of the medium after its culture being 4.74, was equally interesting (compared to the pH of the wild type culture-3.8), Figure 2. It was demonstrated that M15 was characterized by an increased ability to biosynthesize bacterial cellulose. On this basis, it may be suspected that the increase in BC productivity caused a decrease in glucose bioconversion to gluconic acid, which consequently increased the pH of the medium.

For the second selection stage, the mutants were selected from cultures where the pH did not exceed 4.0. These were M34, and from M41to M50 mutants. In Figure 3 gluconic acid content in media after mutant and wild type culture was shown. Based on the results of the second selection stage, it was found that no mutant with increased (compared to the wild type) GA biosynthesis capacity had been obtained.

Statistical analysis of the results demonstrated that the content of gluconic acid in the media after growing most mutants did not differ significantly ($\alpha = 0.05$) from the content



*Means the difference at the significance level α = 0.1; ***Means the difference at the significance level α = 0.05. *Oznacza różnicę na poziomie istotności α = 0,1; ***Oznacza różnicę na poziomie istotności α = 0,05.

Fig. 3. Content of gluconic acid depending on strain

Rys. 3. Zawartość kwasu glukonowego w zależności od szczepu

of this compound in the medium with the wild strain. The only significant difference was observed in the case of the M45 mutant, which was characterized by very low gluconic acid production (1.67 g·dm^{-3} medium) compared to the wild strain (2.91 g·dm^{-3}) (Fig. 3.). The 42.41% reduction in gluconic acid content obtained by the M45 mutant compared to the wild strain corresponds to the result obtained by Raksha et al. [2012]. In these studies, after chemical mutagenesis using N-methyl-N-nitro-N-nitrosoguanidine (NTG) applied to the strain *Aspergillus oryzae* RP-21, i.a., negative mutants were obtained, which showed a decrease in the content of produced GA by 66% compared to the wild strain (from 30 g·dm^{-3} to 10g·dm^{-3}). Probably, the changes in the biochemical pathway for GA production were caused by a wide spectrum of mutations caused by the chemical mutagen, which in consequence could damage the genome and thus inactivate gluconic acid synthesis enzymes at the level of transcription and / or translation [Raksha et al. 2012].

It is worth to mention that also the M34 mutant, which, unlike other mutants subjected to the second selection stage, was obtained as a result of combined mutagenisation (0.3% MMS + UV). Mutant M34 produced gluconic acid at a concentration of 2.5 g·dm^{-3} in the medium, at the same time the wild strain produced GA at a concentration of 2.91 g·dm^{-3}. A similar result after physical mutagenisation was obtained by Ahmed et al. [2015] in the case of the *Aspergillus terreus* strain, where the GA content in the mutant medium was 2.8 g·dm^{-3}. It was one of the lowest results obtained for a given radiation dose (0.2 kGy). For comparison, the maximum GA content obtained by the *A. terreus* wild strain not exposed to a mutagen was 4.21 g·dm^{-3} [Ahmed et al. 2015].

SCREENING FOR DIHYDROXYACETONE PRODUCING MUTANTS

After the first stage of selection of 50 mutants, 3 of them were selected for further inspection, being characterized by the colour of the medium similar to the wild type after the reaction using Fehling's solution. These were M1, M2 and M15 mutants. In the second selection stage, the mutants were moved onto the cultured medium, then onto the glycerol de-

hydrogenase activating medium and finnaly on the medium with glycerol. The culture continued for 96 hours, after which the spectrophotometer analysis of samples commenced.

Table 2 shows the change in DHA content during 96 hours of culture. The results obtained in the second selection stage did not confirm the presence of a mutant, which would be characterized by an increased DHA productivity compared to the wild type. All M1, M2 and M15 mutants tested showed very low levels of DHA produced, 0.11 mg·cm^{-3}, 0,18 mg·cm^{-3} and 0,12 mg·cm^{-3} respectively. At the same time, the highest DHA content obtained in the medium with the wild-type was about 8.77 mg·cm^{-3} (Table 2.). Probably, as a result of mutagenisation, the mechanisms involved in the biotransformation of glycerol to dihydroxyacetone might have been damaged.

Table 2. Changes in dihydroxyacetone content during 96 hours of biotransformation

Tabela 2. Zmiany w zawartości dihydroksyacetonu podczas 96 godzin biotransformacji

	Time – Czas [h]				
	0	24	48	72	96
	DHA content [mg·ml^{-3}]				
Wild type Szczep dziki	0,00 ±0,0000	6,96 ±0,0110	8,77 ±0,0037	7,16 ±0,0068	6,63 ±0,0016
M1 mutant	0,00 ±0,0000	0,11 ±0,0001	0,09 ±0,0005	0,11 ±0,0006	0,10 ±0,0010
M2 mutant	0,00 ±0,0000	0,18 ±0,0015	0,17 ±0,0006	0,17 ±0,0018	0,19 ±0,0009
M15 mutant	0,00 ±0,0000	0,12 ±0,0026	0,12 ±0,0009	0,11 ±0,0007	0,12 ±0,0008

Examples of disabling the activity of glycerol dehydrogenase, an enzyme which converts glycerol to DHA, have been described in the literature. Habe et al. [2010] showed the possibility of using the damaged gene to change the metabolic pathway of the bacterium *Gluconobacter frateurii.* In this experiment, the *sldA* gene which is responsible for encoding the small glycerol dehydrogenase (GlyDH) subunit, was disrupted, resulting in the loss of GlyDH activity and thereby eliminating dihydroxyacetone production. This treatment was intended to modify the metabolism of this ketotriose towards bioconversion to glyceric acid. As a result, a strain with an increased capacity for glyceric acid biosynthesis was obtained with simultaneous lowering of DHA production.

Based on obtained results, it may be presumed that it is possible to use the obtained mutants that do not have the ability to produce DHA as precursors in the production of various compounds from glycerol, whose synthesis to date has been difficult due to the dominant effect of the activity of glycerol dehydrogenase.

SUMMARY AND CONCLUSIONS

The research carried out in the study showed that the Ch-m and Ch-ph-m methods can be effective in obtaining new strains of AAB of the species *K. xylinus* with modified phenotypic characteristics. Ch-ph-m has proved to be an effective tool for increasing the

biosynthesis of bacterial cellulose, also with alternative carbon sources, i.e. with technical and waste glycerol. Chemical and chemical-physical mutagenisation methods have resulted in the reduction of glucose to gluconic acid bioconversion as well as inhibition of glycerol to dihydroxyacetone biotransformation. In the example of the M15 mutant, it can be suspected that increasing BC productivity causes a decrease in gluconic acid biosynthesis.

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WPŁYW MUTAGENIZACJI CHEMICZNEJ I CHEMICZNO-FIZYCZNEJ NA ZMIANY FENOTYPOWE BAKTERII Z GATUNKU *KOMAGATAEIBACTER XYLINUS*

Streszczenie. Bakterie kwasu octowego *Komagataeibacter xylinus* charakteryzują się m.in. zdolnością do wytwarzania celulozy bakteryjnej, kwasu glukonowego oraz dihydroksyacetonu. Celem pracy było wykorzystanie nieukierunkowanej mutagenizacji chemicznej i chemiczno-fizycznej oraz zbadanie wpływu tych procesów na wybrane cechy fenotypowe *K. xylinus*. Mutagenizacja chemiczna została przeprowadzona przy użyciu metanosulfonianu metylu. Mutagenizacja chemiczno-fizyczna została rozszerzona o zastosowanie promieniowania UV (λ = 254 nm). Uzyskany mutant charakteryzował się wyższą o 56% zdolnością do biosyntezy celulozy bakteryjnej (w porównaniu do szczepu dzikiego). Badania wykazały, że mutagenizacja chemiczno-fizyczna może być wystarczającą metodą zwiększenia syntezy celulozy bakteryjnej, przy czym nie zapewnia zwiększonej wydajności wytwarzania kwasu glukonowego i dihydroksyacetonu przez badany szczep.

Słowa kluczowe: mutagenizacja chemiczna, mutagenizacja chemiczno-fizyczna, *Komagataeibacter xylinus*, celuloza bakteryjna, kwas glukonowy, dihydroksyaceton