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EVALUATION OF TOXICITY OF BIOLOGICALLY SYNTHESIZED SILVER NANOPARTICLES (Ag-NPs) USING LEMNA TEST AND ALGALTOXKIT F

OCENA TOKSYCZNOŚCI NANOCZĄSTEK SREBRA (Ag-NPs) SYNTETYZOWANYCH BIOLOGICZNIE Z ZASTOSOWANIEM TESTÓW LEMNA I ALGALTOXKIT F

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Streszczenie. Srebro jest jednym z częściej używanych nanomateriałów w wielu gałęziach przemysłu. Nanocząstki srebra (Ag-NPs) okazały się dobrym środkiem antybakteryjnym i dezynfekującym, mogą być także nośnikami różnych substancji. Celem badań była ocena toksycznego działania nanocząstek srebra syntetyzowanych metodą biologiczną na *Selenastrum capricomutum* (Algaltoxkit FTM) oraz *Lemna minor*. Do syntezy użyto płynów pohodowlanych szczepu *Bacillus subtilis*, do których dodawany był azotan srebra o stężeniu końcowym 1 mM. Obecność nanocząstek srebra monitorowana była za pomocą specktrofotometru UV-Vis. Testy toksyczności zostały wykonane zgodnie z normami OECD Guideline 201 (2006) i ISO standard 20079 (2006). Test Algaltoxkit oceniał dwa parametry – hamowanie wzrostu komórek glonu oraz zawartość w nich chlorofilu a. Badanymi parametrami w teście *Lemna minor* były: liczba i powierzchnia frondów, biomasa oraz zawartość barwników (chlorofilu a i b oraz karotenoidów). W badaniach stwierdzono, że jony Ag+ były bardziej toksyczne dla organizmów *Selenastrum capricomutum* i *Lemna minor* niż biologicznie syntetyzowane nanocząstki srebra. Toksyczność AgNPs względem badanych organizmów była niższa, w porównaniu z jonami Ag+, w przypadku wszystkich badanych parametrów. Wyniki wskazują, że nanocząstki syntetyzowane na podłożu, którym były ścieki browarnicze, są bardziej toksyczne niż nanocząstki powstałe na podłożach LB i melasie.

Key words: silver nanoparticles (Ag-NPs), toxicity, *Lemna minor*, *Algaltoxkit*.
Słowa kluczowe: nanocząstki srebra (Ag-NPs), toksyczność, *Lemna minor*, *Algaltoxkit*.

INTRODUCTION

Engineered silver nanoparticles (Ag-NPs) have received a lot of attention due to their rapidly increasing applications. Rapid developments in the manufacture and use of engineered nanoparticles have also led to an urgent need for assessing their possible risk to humans and the environment. As presented by Kadukova et al. (2015) there were more than 1300 nanotechnological consumer products on the market by March 2011 and 313 of them contained nanosilver. Currently, nanosilver is perhaps the most preferred antimicrobial

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nanomaterial (Ivaska et al. 2014). However, the release of nanoparticles from consumer and household products into the waste streams and further into the environments is widely now observed (Wang et al. 2014).

Processes used for nanoparticles synthesis are chemical, physical, and a recently developed biological method (Prabhu and Poulose 2012; Firhouse and Lalithe 2015; Swany et al. 2015). Chemical methods have various drawbacks including the use of toxic solvents, generation of hazardous by-products, and high energy consumption, which pose potential risks to human health and to the environment. Therefore, the biological method has an advantage over chemical and physical methods of nanoparticle synthesis, as it is cost-effective and environmentally friendly (Nabhikha et al. 2009). However, these methods also have the drawback of being rather slow (Balaji et al. 2009). In the biological synthesis are involved: bacteria, fungi, and plant extracts (Prabhu and Poulose 2012; Quester et al. 2013).

The potential effects and impacts of Ag-NPs to organisms and ecosystems have been studied (Handy 2008). Biological properties of silver nanoparticles are mostly studied using common tests with bacteria, rarely by tests involving algae or higher plants. It is well-known fact that Ag-NPs are highly toxic to microorganisms which include 16 major bacterial species (Kim et al. 2007). However, most of the papers describe the toxicity effects of chemically synthetized Ag-NPs against the procarytoic and eucaryotic organizmes (Juganson et al. 2015). Among them, only few papers present the results of chemically synthetized Ag-NPs toxicity on algae (Navarro et al. 2008; Renault et al. 2008; Van Hoecke et al. 2008; Park and Craggs 2010; Sadiqet et al. 2011). However, the knowledge on the toxicity of biologically synthesized Ag-NPs is limited.

As there are some assumptions that biologically produced nanoparticles are less toxic against organisms than chemically produced nanoparticle, the aim of the study was to focus on the toxic effects of biologically produced Ag-NPs on microscopic algae and *L. minor*. The toxicity of biologically synthesized Ag-NPs was compared with Ag+ ions.

SILVER NANOPARTICLES CHARACTERIZATION

Synthesis of Ag-NPs by the *Bacillus subtilis* producer of biosurfactants was carried out according to the method described previously (Płaza et al. 2016). In the synthesis protocol, a silver nitrate solution (Sigma-Aldrich) was added to 50 ml of the *Bacillus subtilis* culture supernatant to final concentration of 1 mM, and kept for stirring at 200 rpm at room temperature for 48 h. The following media were used for culturing of *Bacillus subtilis*: brewery effluents, molasses and LB medium. The scheme of the biologically synthesis Ag-NPs is presented in Fig. 1. The bioreduction of silver ions was monitored at regular intervals (2 h, 24 h and 48 h) by the UV–Vis spectra from 300 to 600 nm in a UV-Vis spectrophotometer (Lange DR5000 with a resolution of 0.72 nm). Prepared nanoparticles were estimated by dynamic light scattering technique (DLS) and transmission electron microscopy (TEM) using the JEM--2010 (Jeol Ltd., Japan).

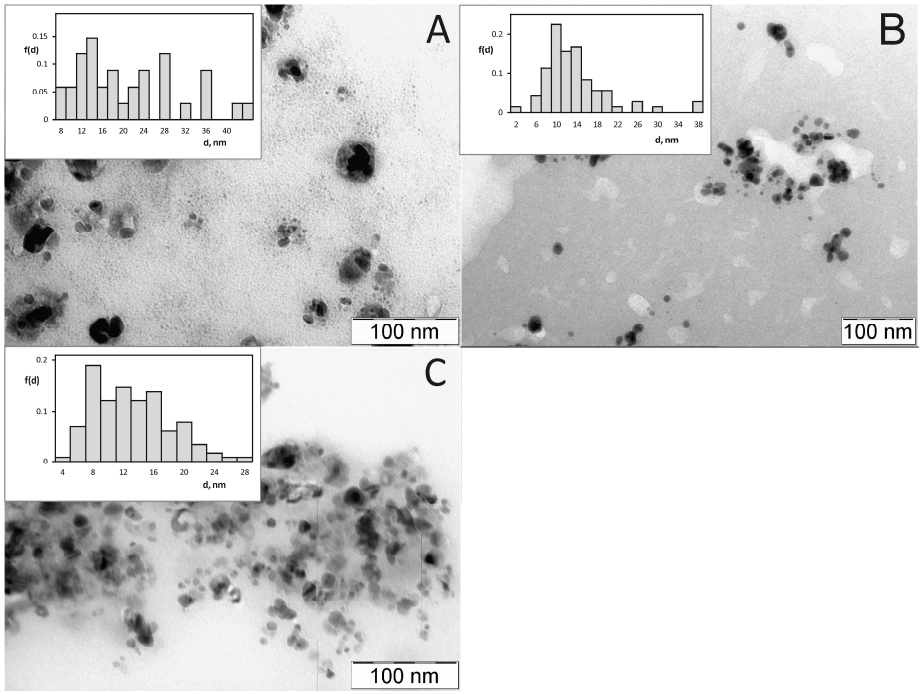

Fig. 1. TEM images of the biosynthesised Ag-NPs with size distribution in the case of *B. subtilis* cultivated on: A – LB medium, B – Molasses, C – Sterilised brewery effluents no. 6
Ryc. 1. Zdjęcia z transmisyjnego mikroskopu elektronowego (TEM) przedstawiające rozkład nanocząstek srebra syntetyzowanych przez *B. subtilis* hodowanych na: A – pożywce LB, B – melasie, C – sterylizowanym ścieku browarniczym nr 6

EVALUATION OF PHYTOTOXICITY

Algaltoxkit

The 72 h growth inhibition test with the green algae *Pseudokirchneriella subcapitata* (renamed *Raphidocelis subcapitata* or *Selenastrum capricornutum*) was performed according to the standard operational procedure of the Algaltoxkit F (Algaltoxkit F… 1996), which follows OECD Guideline 201 (OECD 1984).

Endpoinds

Two endpoints including: growth rate of biomass and chlorophylls *a* content were used to assess phytotoxicity. Plates were incubated in a light-temperature controlled chamber at 25°C for 72 h. Every 24 h the plates were manually shaken to re-suspend any settled cells and after 72 h, a sample from each well was read in a spectrophotometer at 670 nm.

Chlorophyll *a* concentration was determined spectrophotometrically after acetone extraction (Lawton and Robertson, 1999). The absorbance of the supernatant was measured at 664, 665 and 750 nm in Shimadzu 1260 spectrophotometer. Pigment content expressed in µg sample^{-1} were calculated with the following equations:

$$Chl\ a = 26{,}78(A_{664} - A_{750} - A_{665HCl} + A_{750HCl}) * Ve/Vs * L$$

where:

A_{664}, A_{750} – the absorbance of the extract at 664 and 750 nm, respectively;

A_{665HCL}, A_{750HCl} – the absorbance of the extract at 665 and 750 nm after acidification, respectively;

Ve, Vs – volumes of added acetone and volumes of samples, respectively;
L – length of cuvette.

Calculations

The average specific growth rate and EC_{50} value were calculated on the basis of spreadsheet uses the Macro "REGTOX" originally developed by Eric Vindimian and available at http://www.normalesup.org/~vindimian/en_index.html.

Effects on content of Ch a were determined on the basis of the two measurement variable at the end of the test. On the basis of all outcomes of parameters the percent inhibition was calculated for each concentration as follows:

$\% I = (rc - rT) / rc * 100$

where:

rc – a mean value for the control,
rT – a mean value for the treatment.

The EC_{50} values were estimated from the concentration-response relationship of the percent inhibition of the Chl *a* content.

The toxicity values EC_{50} express the concentration of Ag or Ag^+ in μg L^{-1} causing 50% toxic effect.

Lemna test

The 168 h growth inhibition assay with *L. minor*, was performed according to the ISO standard 20079 (ISO, 2006) with the modification according to Kaza et al. (2007) in disposable polystyrene microplates with covers. One *L. minor* plant with three to four fronds, was put to each well of the microplate containing 10 ml of the tested sample. The assay was conducted at 25 ± 1°C under constant cool white fluorescent light at 90–100 μE $m^{-2}s^{-1}$ for 7 days.

Endpoinds

Five endpoints including: number and total area of fronds, fresh weight (f.w.), chlorophylls *a* (Chl a) and *b* (Chl b) were used to assess phytotoxicity.

At days 0 and 7 images of the test plates were taken for analysis of the number and the total area of fronds with the use of image analysis program ImageTool (UTHSCSA, San Antonio, TX).

Pigments were analyzed after extraction of the plants with ethanol (POCH, Poland). *L. minor* from each well of the microplate were weighed, homogenized with 96% ethanol (pure P.A.) at 4°C, left in darkness at 4°C for 1h and centrifuged at 11.000 x g for 5 min. The absorbance of the supernatant was measured at 665, 649 and 470 nm in Shimadzu 1260 spectrophotometer. Pigment content expressed in μg $sample^{-1}$ were calculated with the following equations:

$Chl\ a = 13.7 * A_{665} - 5.76 * A_{649}$

$Chl\ b = 25.8 * A_{649} - 7.60 * A_{665}$

where:

A_{665}, A_{649} – the absorbance of the extract at 665 and 649 nm, respectively.

Calculations

The average specific growth rate was calculated as the logarithmic increase in the growth variables – frond number and their total area according to the following formula (OECD 2006):

$r = (\ln xt2 - \ln xt1) / (t2 - t1)$

where:

r – the specific growth rate per day,

xt1, xt2 – the values of parameter at t1 and t2,

t2 – t1 – the time period between xt1 and xt2 in days. The average specific growth rate was calculated for the entire test period (7–d).

Effects on yield were determined on the basis of the two measurement variable (fresh weight, Chl a and Chl b) at the start and at the end of the test.

On the basis of all outcomes of parameters, both growth rate and yield, the percent inhibition was calculated for each concentration as follows (OECD 2006):

$\% I = (rc - rT) / rc * 100$

where:

rc – a mean value for the control,

rT – a mean value for the treatment.

The toxicity values EC_{50} were expressed the concentration of Ag or Ag^{+} in μg l^{-1} causing 50% toxic effect. The values were estimated from the concentration-response relationship of the percent inhibition of the average specific growth rate (frond number and their total area) and yield (fresh weight, Chl *a* and Chl *b*).

RESULTS AND DISCUSSION

The average diameters of the prepared Ag-NPs obtained from the TEM observations with drained nanoparticles were in the range of 1–100 nm, with average sizes of 13–19 nm (Płaza et al. 2016). The large nanoparticles visible in TEM images seemed to be formed as aggregates of those with smaller sizes (Fig. 1). The UV-Vis spectra of these dispersions showed sharp absorption maxima of the surface plasmon confirming smaller tendency of these Ag-NPs to form aggregates in dispersion (Fig. 2).

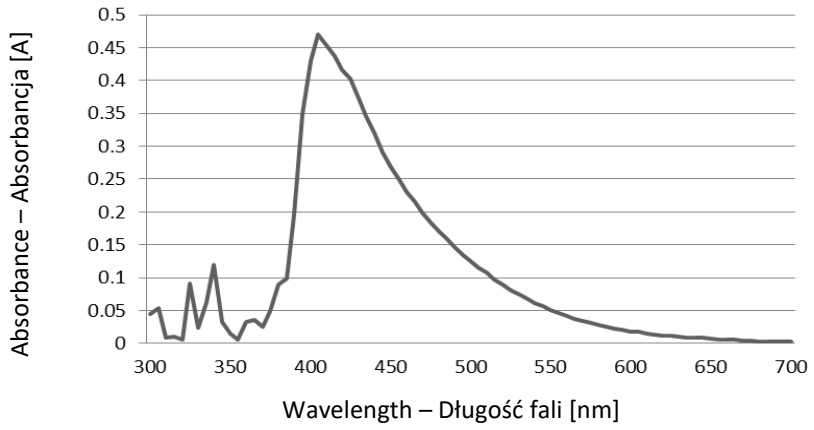

Fig. 2. Graph with absorption spectrum from UV-Vis spectrophotometer
Ryc. 2. Widmo UV-Vis mierzone spektrofotometrycznie

The results from the phytotoxicity test of the Ag-NPs and Ag+ are shown in Table 1 and Table 2. The toxicity of Ag-NPs to *S. capricornutum* and to *L. minor* was lower for all analyzed parameters than the toxicity of Ag+. The results showed that silver ions caused stronger inhibition effect towards the algae than silver nanoparticles. This is due to the fact that Ag+ can be easily taken up into living cells and thus it results in higher toxicity when compared with solid forms (Maneekarn et al. 2014). In the case of *S. capricornutum* the values of EC_{50} for Ag-NPs were 3.6–15.1 times higher than for Ag+. The similar differences were observed for *L. minor*. The EC_{50} values for Ag-NPs were lower and ranged from 1.7 to 7.9 times compared to Ag+.

Table 1. The EC_{50} values of silver nanoparticles (Ag-NPs) calculated from Algaltoxkit F
Tabela 1. Wartości EC_{50} nanocząstek srebra (Ag-NPs) liczone dla testu Algaltoxkit F

Name of sample Nazwa próby	Parameters Parametry	
	growth rate of biomass tempo wzrostu biomasy (EC_{50} [ug/L]$^{-1}$)	chlorophyll *a* chlorofil *a* (EC_{50}[ug/ L]$^{-1}$ ±SD)
I'1a/Ag-NPs (# 6)	3.59[a] 3.57–3.60	2.44 ± 0.04
I'1a/Ag-NPs (Mol)	5.79[a] 3.39–6.08	3.09 ± 0.23
I'1a/Ag-NPs (LB)	13.42[a] 13.04–13.94	7.72 ± 0.57
$AgNO_3$	0.89[a] 0.84–0.96	0.68 ± 0.15

[a] The median value – Mediana wartości.

Table 2. The EC_{50} values of silver nanoparticles (Ag-NPs) calculated from *Lemna* Test
Tabela 2. Wartości EC_{50} nanocząstek srebra (Ag-NPs) liczone dla testu Lemna

Name of sample Nazwa próby	Parameters Parametry (EC_{50} [ug/ L]$^{-1}$)				
	frond area powierzchnia frondów	frond numer liczba frondów	chlorophyll *a* chlorofil *a*	chlorophyll *b* chlorofil *b*	fresh weight świeża masa
I'1a/Ag-NPs (# 6)	200	>1000	214	189	161
I'1a/Ag-NPs (Mol)	328	627	439	225	213
I'1a/Ag-NPs (LB)	> 1000	> 1000	immeasurable niemierzalne[a]	immeasurable niemierzalne[a]	717
$AgNO_3$	101	136	98	91	91

[a] Immeasurable because due to high density of the solution – Niemierzalne ze względu na zbyt dużą gęstość badanego roztworu.

The obtained value of EC_{50} for the growth inhibition of *S. capricornutum* for Ag+ (0.89 µg/l) was 24 times lower then EC_{50} obtained by Ribeiro et al. (2014) equal to 21.4 µg/l. In case of *L. minor* the EC_{50} calculated for frond number (136 µg/l) was only two times higher than obtained by Topp et al. (2011) equal to 78 µg/L.

It has been also demonstrated that Ag-NPs can be less toxic than Ag+ (Griffitt et al. 2009; Kvitek et al. 2009). The toxicity of Ag-NPs to the freshwater alga, *C. reinhardtii*, appeared to be much greater than silver ions when comparing an equal mass of Ag+, indicating a potential

NP-mediated effect due to the interaction of the NP with the algal cells (Navarro et al. 2008). As reported by Stokes (1981) the EC_{50} of Ag+ on general algae is between 24 and 190 nM. The minimum value of the NOEC (no observable effect concentration) for Ag+ in freshwater and marine algae was between 0.002 and 2 mg L^{-1}, depending on the type of alga (Ratte 1999). The Ag-NPs had a less toxic effect probably because solid forms of such nanoparticles showed less uptake than Ag+ in solution. In addition, aggregated forms of Ag-NPs could not get into cells of the green alga due to the larger size of these particles.

The Ag-NPs was synthetized in the three culture supernatants of *Bacillus subtilis* growing on molasses (Mol.), brewery waste (# 6) and LB medium. The toxicity of Ag-NPs to both plants was indicated with the following decreasing sensitivity:: Ag+ > Ag-NPs (# 6) > Ag-NPs (Mol.) > Ag-NPs (LB). This could be explained by the fact that some physic-chemical parameters of Ag-NPs like shape and size were different in the tested media. The results show that the increase of toxicity in case of parameter - growth rate of biomass was reflected in higher inhibition with Ch-a production. At the same time the Ch-a was 1.31–1.87 times more sensitive parameter then growth rate of biomass.

The toxicity of Ag-NPs to *L. minor* was lower compared to microalgae, but the decreasing sensitivity of Ag-NPs and Ag+ were near the same. Only the EC_{50} calculated for frond number indicated different order. The Ag-NPs (LB) in the tested concentrations (1000–62.5 ug/l) was no toxic for plants, but in higher concentration caused the turbidity of the sample caused a significant increase in saprophytic organisms (bacteria, fungi). Simultaneously the same samples caused increase of the Ch-a and Ch-b production in studied concentrations. Considering the sensitivity of analyzed parameters the results indicated that frond number was the lowest sensitive parameter while the fresh weight was the highest sensitive parameter to Ag-NPs biologically synthesized. Simultaneously the EC_{50} values calculated for frond area, Ch-a and Ch-b were the similar values.

Antimicrobial properties of silver nanoparticles are mostly studied using common tests with bacteria, rarely by tests involving algae or higher plants. In the paper of Kadukova et al. (2015) inhibitory effects of biologically prepared silver nanoparticles on the growth of bacteria *E. coli* CCM 3954 and *Staphylococcus aureus* CCM 3953, green microscopic alga *Parachlorella kessleri* LARG/1 and seed germination and root growth of plant *Sinapis alba* seeds were investigated. The inhibitory effect of silver ions was much higher compared to silver nanoparticles for all tested organisms. Miao et al. (2009) suggested the toxicity of Ag-NPs on various organisms may derive from the release of Ag+ from Ag-NPs that affects the cell growth, photosynthesis and the process of chlorophyll production.

The better knowledge of Ag-NPs toxicity mechanisms is required in order to evaluate the environmental risk of their toxicity (Oukarroum et al. 2012). The information on relevant characteristics such as particle size distribution, aggregation, surface properties, morphology, dissolution rate and solubility is necessary in ecotoxicological studies (Fabrega et al. 2011).

CONCLUSION

In conclusion, Ag+ ions were considered to be the most toxic for *Selenastrum capricornutum* and *Lemna minor* than Ag-NPs biologically synthesized. The toxicity effects of Ag-NPs for both plants were depended on media in which Ag synthesis was performed. The lowest effect of toxicity was observed for Ag-NPs synthesized in the supernatant from the culture of

Bacillus subtilis growing on LB medium. However, the highest effect of toxicity was detected for Ag-NPs synthesized in the supernatant from the culture of *Bacillus subtilis* growing on brewery effluents. The physico-chemical properties of the Ag-NPs are important factors of the their toxicity.

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Abstract. In the current study the toxicity of biologically synthesized Ag-NPs to *Selenastrum capricomutum* and *Lemna minor* was assessed. The supernatants from the *Bacillus subtilis* cultures were applied for the Ag-NPs synthesis. The following media were used for culturing of *Bacillus subtillis* (l'-1a): molasses (Mol.), brewery effluent (# 6) and Luria-Bertani (LB) medium. Silver nitrate was added to the culture supernatants to the final concentration of 1 mM. The Ag-NPs presence was monitored by UV-Vis spectrophotometer. Toxicity tests were made according to OECD Guideline 201 (2006) and ISO standard 20079 (2006). In the test Algaltoxkit^F the two parameters cell growth and content of chlorophyll a were evaluated. However, in the test with *Lemna minor* the five endpoints including: number and total area of fronds, fresh weight (f.w.), chlorophylls *a* (Chl a) and *b* (Chl b) were used to assess phytotoxicity. Ag+ ions were considered to be the most toxic for *Selenastrum capricornutum* and *Lemna minor* than Ag-NPs biologically synthesized. The toxicity of biologically synthesized Ag-NPs was lower to *S. capricomutum* and to *L. minor* for all analyzed parameters compared to the toxicity of Ag+. However, the toxicity of Ag-NPs synthesized in the supernatant from the culture of *Bacillus subtilis* growing on brewery waste was higher compared to the Ag-NPs synthesized in the supernatants from the cultures of strain growing on molasses and LB media. The variability in sensitivity of both organisms towards Ag-NPs was observed.

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