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Article

Oxidative Stress in *Staphylococcus aureus* Treated with Silver(I) Ions Revealed by Spectrometric and Voltammetric Assays

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We were focused on the studying oxidative stress in bacterial culture of *Staphylococcus aureus* induced by silver ions. The effect of the seven concentrations of $AgNO_3$ (10, 25, 50, 75, 150, 225 and 300 μM) in bacterial culture was studied. Further, antioxidant activity of bacterial lysates of *Staphylococcus aureus* alone and incubated with different concentrations of $AgNO_3$ under conditions of increased oxidative stress caused by the addition of hydrogen peroxide (4, 5, 6, 7, 8, 9 and 10 mM) was observed. Oxidative stress in bacterial lysates was studied spectrophotometrically using two different methods for the determination of antioxidant activity ABTS (2.2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2.2-diphenyl-1-picrylhydrazyl). Oxidative stress was also studied electrochemically using cyclic voltammetry. Increasing oxidative stress was clearly noticeable with increasing concentrations of silver ions and hydrogen peroxide. Our results indicate the possibility of the application of above-mentioned analytical techniques in microbiology for determination of oxidative stress in bacterial cultures.

Keywords: *Staphylococcus aureus*; Silver; Oxidative Stress; Hydrogen Peroxide, Electrochemical Analysis; Screen Printed Electrode; Differential Pulse Voltammetry; Cyclic Voltammetry

1. INTRODUCTION

The bacterial growth is greatly inhibited due to silver(I) ions [1,2]. Silver(I) ions are stored in vacuoles and cell walls as granules inhibiting cell division, interacting with nucleic acids [3] and thiol

groups in enzymes and proteins essentials for the vital cell functions [4]. For these reason, the silver(I) ions are used to control bacterial growth in medical and non-medical applications [1,5-7]. The mechanism of action of silver(I) ions are inactivation of membrane proteins, interference with electron transport system and inhibition of respiratory enzymes to promote the generation of reactive oxygen species, especially superoxide-radical, and consequent bactericidal activity [2,8]. The silver nanoparticles have been also applied as antimicrobial agents. These particles have a low toxicity to human cells, and a far lesser probability to cause microorganism resistance than antibiotics [9]. Silver nanoparticles reduce the expression of some enzymes and inhibiting respiratory chain. When silver nanoparticles enter into bacteria cells, they condense DNA to prevent DNA from replicating and cells from reproducing [6]. Under aerobic conditions silver(I) ions exhibit higher bactericidal activity against *Staphylococcus aureus*, a model strains for gram positive bacteria.

Oxidative stress is one of the markers, which enables monitoring of toxic effects of generally heavy metals including silver(I) ions on microorganisms [2,10]. This toxic effect is based on the binding of silver(I) ions to the bacterial cell wall and membranes, which leads to inhibition of the respiratory process [6,11,12]. Due to ability of silver(I) ions to induce excessive production of ROS that affect almost all biomolecules, they are also able to cause metabolic toxicity. Organisms have the protective mechanisms that can effectively eliminate formed free radicals and thus eliminate their toxic effects [2,13]. On the other hand, these protective mechanisms have limited capabilities based on the proteosynthetic and generally biosynthetic abilities. Moreover, excess of ROS enables the monitoring of oxidative stress based on determination of antioxidant capacity [14,15].

Numerous methods for determination of antioxidant activity have been developed in the field of chemical analysis and biological evaluation of antioxidant characteristics [16-29]. Their diversity is given by the fact that low molecular weight antioxidants may act by different mechanisms, most often by the direct reaction with free radicals (quenching, trapping). A more precise definition of the chemical mechanism of their effect is often issue. Therefore, the procedures evaluating the antioxidant activity are based on chemically different principles [27,29,30].

Spectrophotometric methods are the most used methods in the determination of antioxidant activity/capacity. We studied oxidative stress in *S. aureus* extracts influenced by silver ions and hydrogen peroxide. In our study for the measurement of oxidative stress we used two different methods– ABTS (2.2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2.2-diphenyl-1-picrylhydrazyl). By these methods we determined the amount of radicals in *S. aureus* lysates. The results of spectrophotometric studies were completed with electrochemical analysis. Oxidative stress in *S.aureus* lysates was also studied by cyclic voltammetry using printed electrode and flow cell.

2. EXPERIMENTAL PART

2.1 Cultivation of Staphylococcus aureus

Staphylococcus aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. Strains were stored as a spore suspension in 20% (*v/v*) glycerol at -20°C. Prior to use in this study, the strains were thawed

and the glycerol was removed by washing with distilled water. The composition of cultivation medium was as follows: meat peptone 5 g/l, NaCl 5 g/l, bovine extract 1.5 g/l, yeast extract 1.5 g/l (HIMEDIA, Mumbai, India), sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted at 7.4 before sterilization. Sterilization of media was carried out at 121 °C for 30 min. in sterilizer (Tuttnauer 2450EL, Israel). The prepared cultivation media were inoculated with bacterial culture into 25 ml Erlenmeyer flasks. After inoculation, bacterial cultures were cultivated for 24 hours on a shaker at 600 rpm and 37 °C. Bacterial culture cultivated under these conditions was diluted by cultivation medium to OD_{600} = 0.1 and used in the following experiments.

2.2 Chemicals, preparation of deionised water and pH measurement

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, USA) in ACS purity unless noted otherwise. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.3 Determination of protein mass spectra for identification of bacteria Staphylococcus aureus

500 µl *S. aureus* culture, grown overnight, was centrifuged at 14,000 × g for 2 min., the supernatant was discarded and the pellet was suspended in 300 µl of de-ionized water; then, 900 µl of ethanol was added. After centrifugation at 14,000 × g for 2 min, the supernatant was discarded and the pellet was air-dried. The pellet was then dissolved in 25 µl of 70 % formic acid and 25 µl of acetonitrile and mixed. The samples were centrifuged at 14,000 × g for 2 min and 1 µl of the clear supernatant was spotted in duplicate onto the MALDI target (MTP 384 target polished steel plate; Bruker Daltonics, Bremen, Germany) and air-dried at room temperature. Then, each spot was overlaid with 1 µl of α-cyano-4-hydroxy cinnamic acid (HCCA) matrix solution saturated with organic solvent (50 % acetonitrile and 2.5 % trifluoroacetic acid, both *v*/*v*) and air-dried completely prior to matrix-assisted laser desorption-ionization time-of-flight mass spectrometric (MALDI-TOF MS) measurement. The spectra were taken in the m/z range of 2,000 Da to 20,000 Da, and each was the result of the accumulation of at least 1000 laser shots obtained from ten different regions of the same sample spot. Spectra were analysed with the Flex Analysis software (Version 3.4). Prior to analysis, the mass spectrometer was externally calibrated with a peptide mix of bombesin, angiotensin I, glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) (18-39), ubiquitine, and cytochrome c. Spectra with peaks outside the allowed average were not considered. Modified spectra were loaded into the MALDI BioTyper™ 3.1 Version (Bruker Daltonik GmbH, Bremen, Germany). Software settings for MSP creation were set to: maximal mass error of each single spectrum: 2,000; desired mass error for the MSP: 250. Reference spectra were created automatically by the software and all created spectra were added to the main spectra library as unassigned MSPs. Spectra loaded into MALDI BioTyper™ 3.1 Version were measured at the default settings. Unknown spectra were compared with the created reference library by using a score value, the common decadal logarithm for matching results. Results

were analysed following the score value system according to Bruker Daltonik GmbH (Bremen, Germany). Values from 3.00 to 2.30 indicate reliable species identification.

2.4 Spectrophotometric measurements of antimicrobial activity of silver ions

2.4.1 Zones of inhibition

To determine the antimicrobial effect of silver(I) ions (0, 10, 25, 50, 75, 150, 225 and 300 μM), the measurement of the inhibition zones on Petri dishes was done. Agar surface in Petri dish was covered with a mixture of 100 ml of 24 hour grown culture of *S. aureus* and 3 ml of LB medium (Luria Bertani medium). Excess volume of the mixture of the Petri dishes was aspirated. From the fabrics, which were made by Výzkumný ústav pletařský in Brno, were cut out squares in size 1x1 cm. In Eppendorf tubes these were mixed with different concentration of silver(I) ions. Soaked squares were then laid crosswise on a Petri dish, two squares per dish. Petri dishes were insulated against possible external contamination and placed in a thermostat preset at 37 °C for 24 hours. After 24 hour incubation, for each dish the zones of inhibition were measured and photographed.

2.4.2 Growth curves

A second procedure for the evaluation of the antimicrobial effect of tested compounds and their combinations was measuring the absorbance using the apparatus Multiskan EX (Thermo Fisher Scientific, Germany) and subsequent analysis in the form of growth curves. The same culture as in the previous procedure was diluted with LB medium using Specord spectrophotometer at 210 (Analytik Jena, Germany) at a wavelength of 600 nm to absorbance 0.1. In the microplate *S. aureus* culture was mixed with various concentrations of silver(I) ions, hydrogen peroxide or combination of these substances or *S. aureus* alone as a control for measurements. The concentrations of silver(I) ions were 0, 10, 25, 50, 75, 150, 225 and 300 μM. The concentrations of hydrogen peroxide were 0, 4, 5, 6, 7, 8, 9 and 10 mM. Total volume in the microplate wells was always 300 μl. Measurements were carried out at time 0, then each half-hourly for 24 hours at 37 °C and a wavelength of 620 nm. The values achieved were analysed in graphic form as growth curves for each variant individually.

2.5 Preparation of S.aureus lysates for spectrophotometric and electrochemical measurements

After inoculation, bacterial cultures were cultivated on a shaker for 24 hours at 600 rpm and 37 °C. Then bacterial cultures were mixed with silver(I) ions to reach final concentrations (0, 10, 25, 50, 75, 150, 225 and 300 μM) and incubated for further 3 hours at 37 °C. After incubation with silver(I) ions samples were collected by centrifugation (5000 rpm, 15 min.). Bacterial cells were three times washed with phosphate buffer (pH = 7). Finally, cells were resuspended in phosphate buffer (pH = 7, volume 1.5 ml) and sonicated using the ultrasound needle (Hielscher, Germany) for 2 minutes. Homogenates were vortexed (5 min, BioSan, Riga, Latvia) and centrifuged (1600 rpm, 30 min.). Obtained supernatants of bacterial lysates were used for the spectrophotometric and electrochemical determination of oxidative stress. For experiments with hydrogen peroxide (final concentrations: 0, 4,

5, 6, 7, 8, 9 and 10 mM) and hydrogen peroxide in combination with silver(I) ions, hydrogen peroxide was added 15 minutes prior to measurement.

2.6 Spectrophotometric measurements of oxidative stress

Spectrophotometric measurements of total protein content, oxidative stress (ABTS, DPPH) were carried using an automated chemical analyser BS-400 (Mindray, Shenzhen, China). Reagents and samples were placed on cooled sample holder (4 ± 1 °C) and automatically pipetted directly into plastic cuvettes. Incubation proceeded at 37.0 ± 0.1 °C. Mixture was consequently stirred. The washing steps of pipetting needle with distilled water (18 mΩ) were done in the midst of the pipetting. For detection itself, the following range of wave lengths can be used - 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740 and 800 nm. The instrument was operated using the BS-400 software (Mindray).

2.6.1 Determination of total proteins by the pyrogallol red

The pyrogallol red protein assay (Skalab) is based upon formation of a blue protein-dye complex in the presence of molybdate under acidic conditions (pH=2.5). A 150 µl volume of reagent mixture (1:1) R1+R2 (50 mM succinic acid, 3.47 mM sodium benzoate, 0.06 mM sodium molybdate, 1.05 mM sodium oxalate and 0.07 mM pyrogallol red) is pipetted into a plastic cuvette with subsequent addition of 8 µl of sample. Absorbance is measured at λ = 605 nm after 10 minutes of incubation. Resulting value is calculated from the absorbance value of the pure reagent mixture and from the absorbance value after 10 minutes of incubation with the sample.

2.6.2 Determination of oxidative stress using the DPPH method

The DPPH test is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors. The $DPPH^{\bullet}$ radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolourized after reduction with antioxidant (AH) or a radical ($R^{\bullet}$) in accordance with the following scheme: $DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$, $DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$ [31]. 150 µl of R1 reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl - DPPH) was pipetted into plastic cuvette. Subsequently, volume of 15 µl of sample was added. This method is based on the ability of stable free radical of 2,2-diphenyl-1-picrylhydrazyle to react with donors of hydrogen. DPPH has strong absorption in UV-VIS spectrum, absorbance was measured for 12 min at λ = 505 nm. To assess the production of free radicals absorbance difference of the reagent without sample and reagent with sample after ten-minute incubation was taken. Then, the absorbance difference was recalculated per gram of proteins determined in the sample. After subtraction of the blank value the biggest absorbance difference was taken as 100% of amount of free radicals. The higher was the amount of free radicals, the higher was oxidative stress.

2.6.3 Determination of oxidative stress using the ABTS method

The ABTS radical method is one of the most used assays for the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the one-

electron oxidation of the synthetic chromophore 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS): ABTS – e- ABTS+. This reaction is monitored spectrophotometrically by the change of the absorption value. Procedure for the determination was adopted from publication of Sochor et al. [32]. 150 µl of R1 reagent (7 mM ABTS (2,2-azinobis(3-ethylbenzothiazolin-6-sulphonic acid and 4.95 mM potassium peroxydisulphate) was pipetted into plastic cuvette. Subsequently, volume of 3 µl of sample was added. Absorbance was measured for 12 min at λ = 660 nm. To assess the production of free radicals absorbance difference of the reagent without sample and reagent with sample after ten-minute incubation was taken. Then, the absorbance difference was recalculated per gram of proteins determined in the sample. After subtraction of the blank value the biggest absorbance difference was taken as 100% of amount of free radicals. The higher was the amount of free radicals, the higher was oxidative stress.

2.7 Electrochemical determination of silver(I) ions using sensor array

Differential pulse voltammetric (DPV) measurements were performed using PalmSens (PalmSens, The Netherlands) potentiostat connected with sensor array (BUT, Czech Republic) through control box (BUT, Czech Republic). For smoothing and baseline correction the PalmSens software supplied by PalmSens was employed. As the supporting electrolyte acetate buffer (0.2 M CH_3COOH; 0.2 M CH_3COONa) was used. Applied volume of sample which was pipetted on one sensor array position was 50 µl. DPV conditions was as follows: start potential -0.2 V, end potential 0.5 V, amplitude 0.05 V, time of accumulation 60 s.

2.8 Cyclic voltammetry

Printed electrodes (three-electrode system) and the flow cell were used in this study. The sample was injected (20 µl) into the flow cell. Change of oxidative signal was recorded with potentiostat PGSTAT 101 (Methrohm, Switzerland). The experimental conditions were as it follows: scan rate 50 mV/s; potential step 0.005 V; start potential 0 V; upper vertex potential 1 V, lover vertex potential -1 V and stop potential 0 V. Screen-printed sensor was fabricated using Aurel C880 semiautomatic screen-printer (Aurel Automation, Italy) and fired using fast BTU furnace fire for thick-film processing (BTU, USA). The conductive layer was fabricated from AgPdPt based paste (ESL 9562-G). The protective layer was fabricated from a dielectric paste (ESL 4917). AE was fabricated from Pt based paste (ESL 5545). All cermet pastes were obtained from ESL ElectroScience Europe, UK and fired at 850 °C according to the recommended values in products datasheets. WE was screen-printed using special carbon paste electrodes for electrochemical sensors of (DuPont BQ221) from DuPont Company (DuPont, USA) and cured at 130 °C for 10 minutes according to datasheet. RE was screen-printed using special polymer Ag/AgCl paste (DuPont 5874, Ag: AgCl ratio 65:35) and dried at 120 ° C for 5 minutes [33-35].

3. RESULTS AND DISCUSSION

3.1 Mass spectrometric characterization of the used bacterial strain

The aim of our study was to investigate oxidative stress in *S. aureus* culture influenced by silver(I) ions. To obtain reliable results, we verified each step. We verified the purity of *S. aureus* culture, how much of the added silver was bound to *S. aureus* and both minimal and total silver inhibitory concentration. First, we verified whether we have a pure culture of *S. aureus* without admixture of other microorganisms using MALDI-TOF technique. MALDI-TOF is a technique that combines a soft, matrix-assisted, ionization process and a TOF analyser to separate the generated ions [36,37]. In MALDI-TOF mass spectra, the mixture of a biological sample with an energy-absorbing matrix allows the genesis of mostly intact-single-charged biomolecules. This tool is routinely used to identify bacterial species in clinical samples [25,38,39] and has been extensively used in biology to search biomarkers and to monitor protein post-translational modifications [40]. The invention of MALDI-TOF mass spectra enormously contributed to the understanding of protein chemistry and cell biology [41]. MALDI-TOF mass spectra applied for the classification of *S. aureus* strains. All isolated strains were clearly identified as *S. aureus* by their spectral fingerprints [42-44].

MALDI-TOF MS-based identification has been shown to be a fast and accurate technology in the identification of a variety of *S. aureus* [42,44-46]. The obtained mass spectrum is shown in Fig. 1A. A correct species diagnosis was calculated in *S. aureus* strains with a mean score of > 2.30 according to a score cut-off value of 2.0 into the MALDI BioType 3.1 (Fig. 1B). The minimum score was 1.958 and the maximum score was 2.322 (Fig. 1C). Therefore, we can ensure that our study was conducted with pure cultures of *S. aureus*, and eliminated any possibility of contamination.

3.2 Electrochemical determination of silver(I) ions

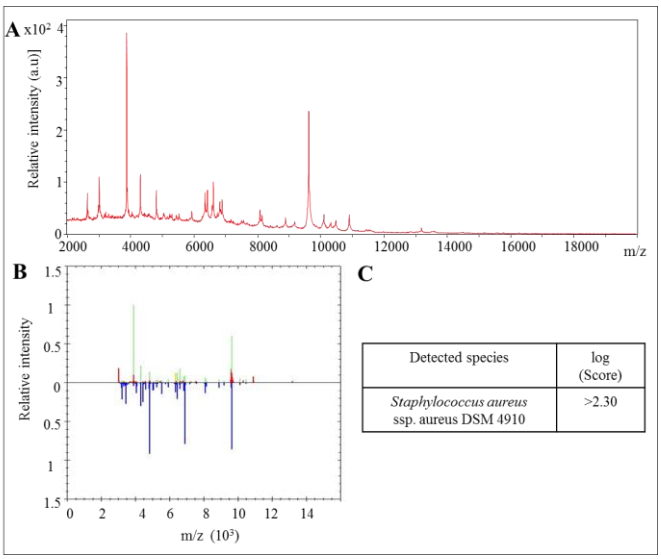

Figure 1. (**A**) MALDI/TOF mass spectra protein fingerprints for the identification of *Staphylococus aureus*. Data were collected in the m/z 2000–20000 range after processing 1 ml of *Staphylococus aureus.* (**B**) Comparison of spectra between biological samples and MSP library by MALDI Biotyper3 and (**C**) species identification by MALDI Biotyper3 using HCCA as a matrix.

To verify how much of the applied silver was bound to *S.aureus* and how much of the silver remained in the medium an electrochemical method was used. For electrochemical determination of heavy metal ions differential pulse voltammetry (DPV) is typically used [47] and can be coupled to carbon paste electrodes [48,49]. To be able to determine the concentration of a predetermined substance, it is firstly necessary to measure and construct the calibration curve, which always precedes own measurements of samples [50,51]. The calibration curve is shown in Fig. 2A with typical DP voltammogram in inset. We used this method for quantification of silver(I) ions and found that the largest quantity of applied metal was bound into bacteria *S.aureus*. Metals in the medium were measured in higher concentrations than free metals (Fig. 2B).

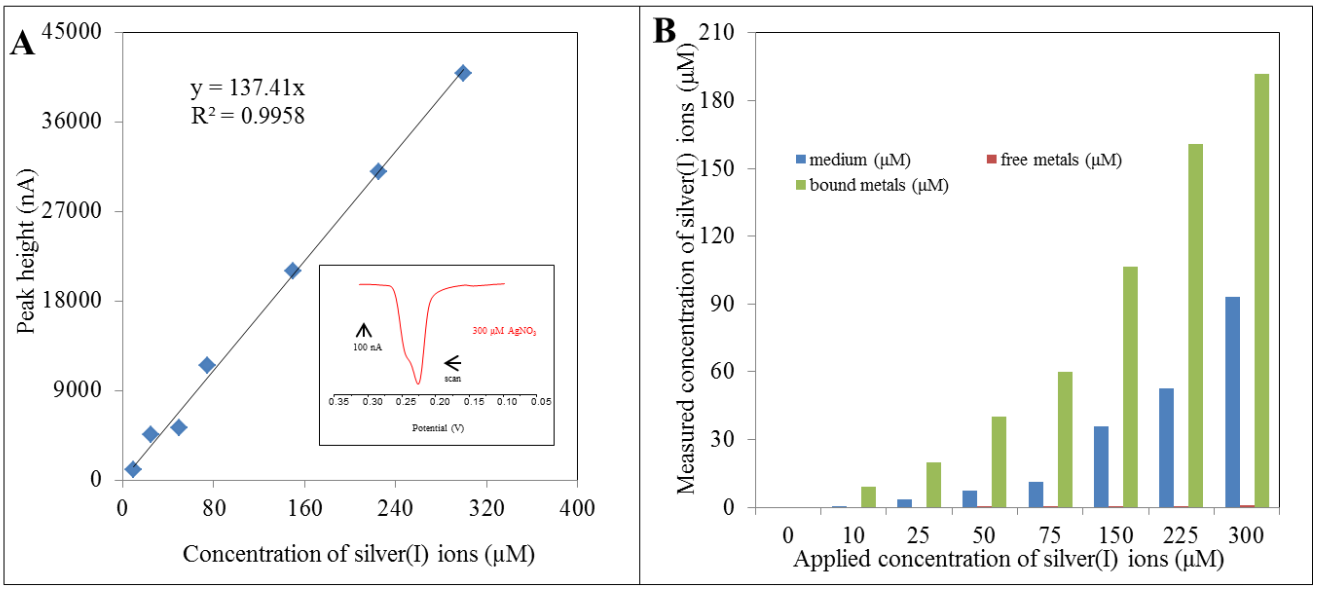

Figure 2. (**A**) Electrochemical analysis of *Staphylococus aureus*. Calibration curve of silver(I) ions concentrations (0, 10, 25, 50, 75, 150, 225 and 300 µM); in inset: DP voltammogram of 300 µM silver(I) ions. DPV parameters were as follows: start potential -0.2 V, end potential 0.5 V, amplitude 0.05 V, time of accumulation 60 s. (**B**) Electrochemical analysis of *Staphylococus aureus* and silver ions in medium, free metals and bound metals. Comparison of added amount of silver(I) ions concentrations (0, 10, 25, 50, 75, 150, 225 and 300 µM) and measured quantity.

3.3 Inhibition of bacterial growth revealed by growth curves and antioxidant activity determination

It is generally known that silver(I) ions inhibit the growth of microorganisms, thus, these ions are suitable substance for incorporation into a variety of materials where there is requirement for antimicrobial activity [52,53]. One of the ways to determine the antibacterial effect of silver ions on bacteria is the growth curves method [54-56]. Using the growth curves method for *S. aureus*, we determined the minimal inhibition concentration (MIC) [57] and total inhibition concentration (TIC) [58]. Measurements were made both in seven added concentrations of silver(I) ions (concentration of $AgNO_3$ were 0, 10, 25, 50, 75, 150, 225 and 300 µM). Minimal inhibition concentration (MIC) of *S. aureus* and $AgNO_3$ resulted from concentration of 10 µM (Fig. 3A), total inhibition concentration was

then achieved with silver(I) ions at concentration of 150 µM. Increasing inhibition of *S. aureus* growth which was caused by the addition of silver(I) ions was also confirmed by the dependency graph of the growth rate of bacteria on the applied concentration of $AgNO_3$ (Fig. 3B). In experimental groups of *S. aureus* with the addition of various concentrations of silver(I) ions, growth curve regression equations were determined from the initial six hours. From these equations, the bar chart was compiled for each variant, which tells us the growth speed of *S. aureus* in the first six hours after the addition of $AgNO_3$ concentrations (Fig. 3B).

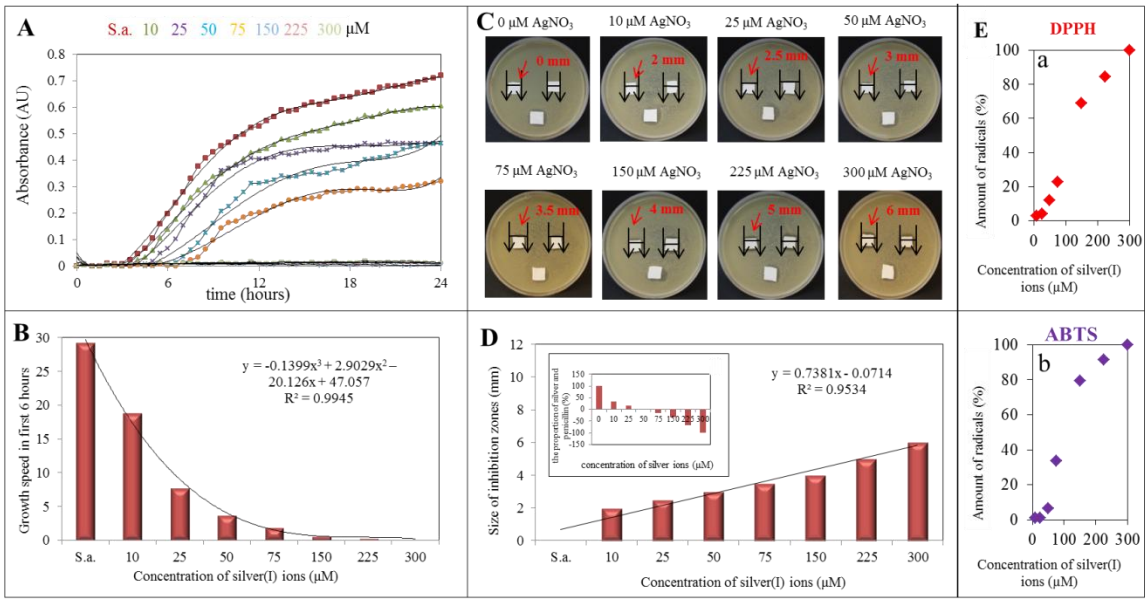

Figure 3. (**A**) Spectrophotometric analysis of the *S. aureus* growth with applied silver(I) ions concentrations 0, 10, 25, 50, 75, 150, 225 and 300 µM in 24 hours. (**B**) Spectrophotometric analysis of the *S. aureus* growth speed with applied silver(I) ions concentrations 0, 10, 25, 50, 75, 150, 225 and 300 µM in 6 hours. (**C**) Zones of inhibition of the *Staphylococus aureus* with applied silver(I) ions concentrations 0, 10, 25, 50, 75, 150, 225 and 300 µM. (**D**) The dependence of the size of the inhibition zones on the applied concentration of silver(I) ions (0, 10, 25, 50, 75, 150, 225 and 300 µM); in inset: The percentage of silver and penicillin. Comparison of the size of the zones of inhibition caused by treatment with various concentrations of silver(I) ions and a constant concentration of penicillin (300 µM). (**E**) Spectrophotometric analysis of the *S. aureus* oxidative stress using analytic methods (a – DPPH, b – ABTS). Dependence of amount of radicals (%) on applied silver(I) ions concentrations (0, 10, 25, 50, 75, 150, 225 and 300 µM).

The antimicrobial properties of silver and other heavy metals on microorganisms (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, etc.) have been tested by many studies [59]. To compare the results of antimicrobial properties of silver(I) ions on the bacterium *S. aureus*, obtained by growth curves method, a method of determining the inhibition zones on Petri dishes was used [60-62]. With this method, after 24 hours of cultivation in thermostat at 37 °C, zones of inhibition with increasing intensity in response to increased concentrations of added silver(I) ions were determined (Fig. 3C). Size of the inhibition zones thus ranged from 2 mm to 6 mm, with the widest inhibition zone of a combination of *S. aureus* with 300 µM $AgNO_3$. From the measured size of the

inhibition zones the dependence of the applied concentration of silver(I) ions on the size of these zones was compiled. With upward trend of this graph, we therefore confirmed that the size of the inhibition zones increased with increasing concentration of $AgNO_3$ (Fig. 3D). Moreover, it is shown in inset in Fig. 3D a comparison of the size of the inhibition zones after exposure to different concentrations of silver(I) ions and a constant concentration of penicillin. By comparing the inhibition zones of different concentrations of silver(I) ions with the inhibition zones of control antibiotics penicillin (applied in constant concentration 300 μM) we concluded that the same size of inhibition zones of silver(I) ions has been already achieved at concentration of 50 μM, thus they showed greater antibacterial effects than antibiotics.

Microorganisms, such as *Staphylococcus* species are used as starter cultures in fermented meat products, reduce the level of volatile substances produced by oxidation of lipids. For these, and other micro-organisms is necessary to determine their antioxidant capacity using spectrophotometric methods [50,63]. In this study, we focused on determination of oxidative stress in bacterial lysates caused by silver(I) ions and hydrogen peroxide. Effect of silver(I) ions on oxidative stress in bacteria *S.aureus* is shown in Fig. 3Ea and Eb. Oxidative stress in *S. aureus* lysates prepared after incubation with different concentrations of silver(I) ions was examined by two different methods for the determination of antioxidant activity DPPH and ABTS. The amount of radicals ($DPPH^{\bullet}$ or $ABTS^{+\bullet}$) and their loss after the addition of *S. aureus* lysates was observed spectrophotometrically (Fig. 3Ea and Eb, respectively). Oxidative stress was expressed by the percentual amount of free radicals. The results of measurement of oxidative stress obtained by both methods are similar, i.e. with the increasing silver concentration oxidative stress in *S. aureus* lysates was also increased. The lowest oxidative stress was observed at lower concentrations of added silver (10-50 μM). The largest increase in oxidative stress was observed at silver concentration ranging from 75 to 225 μM, when there was a substantial growth inhibition of *S. aureus*. The highest oxidative stress reached *S. aureus* lysates at silver concentration of 300 μM.

3.4 Hydrogen peroxide and oxidative stress

Next, effect of hydrogen peroxide on oxidative stress in *S. aureus* lysates was investigated using spectrophotometric and electrochemical methods (Fig. 4). Seven different concentrations of hydrogen peroxide (4, 5, 6, 7, 8, 9, 10 mM) was added to the bacterial culture of *S. aureus*. Using the growth curves method it was found that hydrogen peroxide caused *S. aureus* growth inhibition like silver(I) ions, but at much higher concentrations. Minimal inhibition concentration of *S. aureus* and H_2O_2 resulted from concentration of 4 mM, total inhibition concentration was then achieved with hydrogen peroxide at concentration of 10 mM (Fig. 4A). Similarly to the previous experiment with silver(I) ions only, the dependence of bacterial growth speed in the first six hours of measurement was compiled (Fig. 4B). Oxidative stress in *S. aureus* lysates with addition of different concentrations of hydrogen peroxide was again examined by two different methods for the determination of antioxidant activity DPPH and ABTS (Figs. 4C and D). The results of measurement of oxidative stress obtained by both methods are similar. With increasing hydrogen peroxide concentration oxidative stress in *S. aureus* lysates was increased as well, which was also confirmed electrochemically (Fig. 5A) by

electrochemical index determined according to the previously published papers [64-66]. Voltammograms obtained by analysis of each experimental group are shown in Figs. 5B, C, D, E, F, G, H and I.

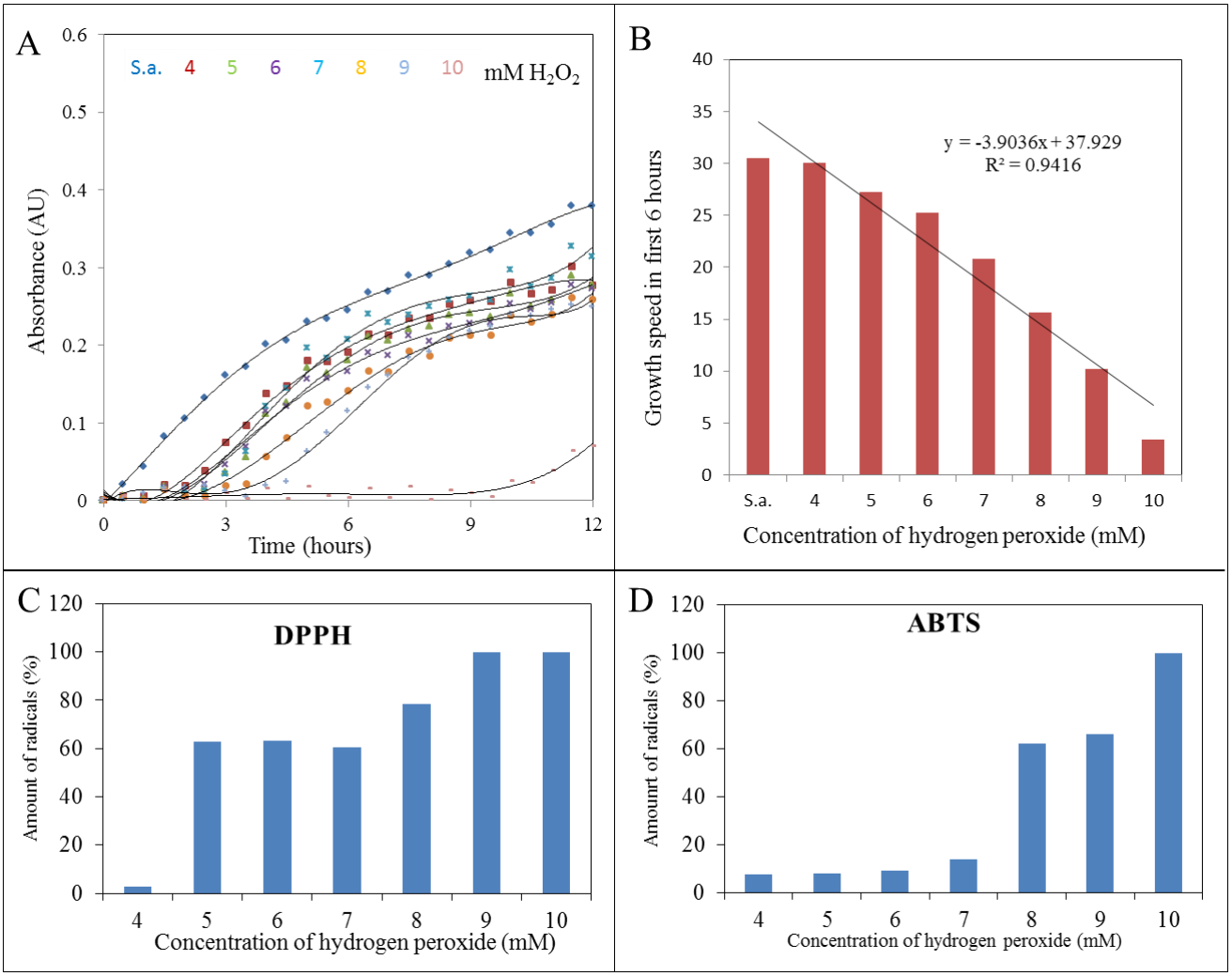

Figure 4. (**A**) Spectrophotometric analysis of the growth of *S. aureus* bacterial culture with concentrations of hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM) in 12 hours. (**B**) Spectrophotometric analysis of the growth speed of *S. aureus* bacterial culture with concentrations of hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM) in 6 hours. Spectrophotometric analysis of *S. aureus* oxidative stress using analytic methods (**C** – DPPH, **D** – ABTS). Dependence of amount of radicals (%) on used hydrogen peroxide concentrations (4, 5, 6, 7, 8, 9 and 10 mM).

Further, combined effect of silver(I) ions and hydrogen peroxide on bacterial culture *S. aureus* was investigated. Seven different concentrations of hydrogen peroxide (4, 5, 6, 7, 8, 9, 10 mM) was added to bacterial cultures containing different concentrations of $AgNO_3$ (10, 25, 50, 75, 150, 225, 300 μM) (Fig. 6). Effect of combination of silver(I) ions with hydrogen peroxide on bacterial culture *S. aureus* was first monitored by growth curves method. The obtained results pointed to a border silver ion concentration of 75 μM, at which there was a significant change in *S. aureus* growth (Fig. 6). Up to

the concentration of 50 μM there was only mild inhibition of bacterial growth, in a concentration of 50 μM there was already partial inhibition and in a concentration of 75 μM and higher there was recorded complete inhibition of *S. aureus* growth.

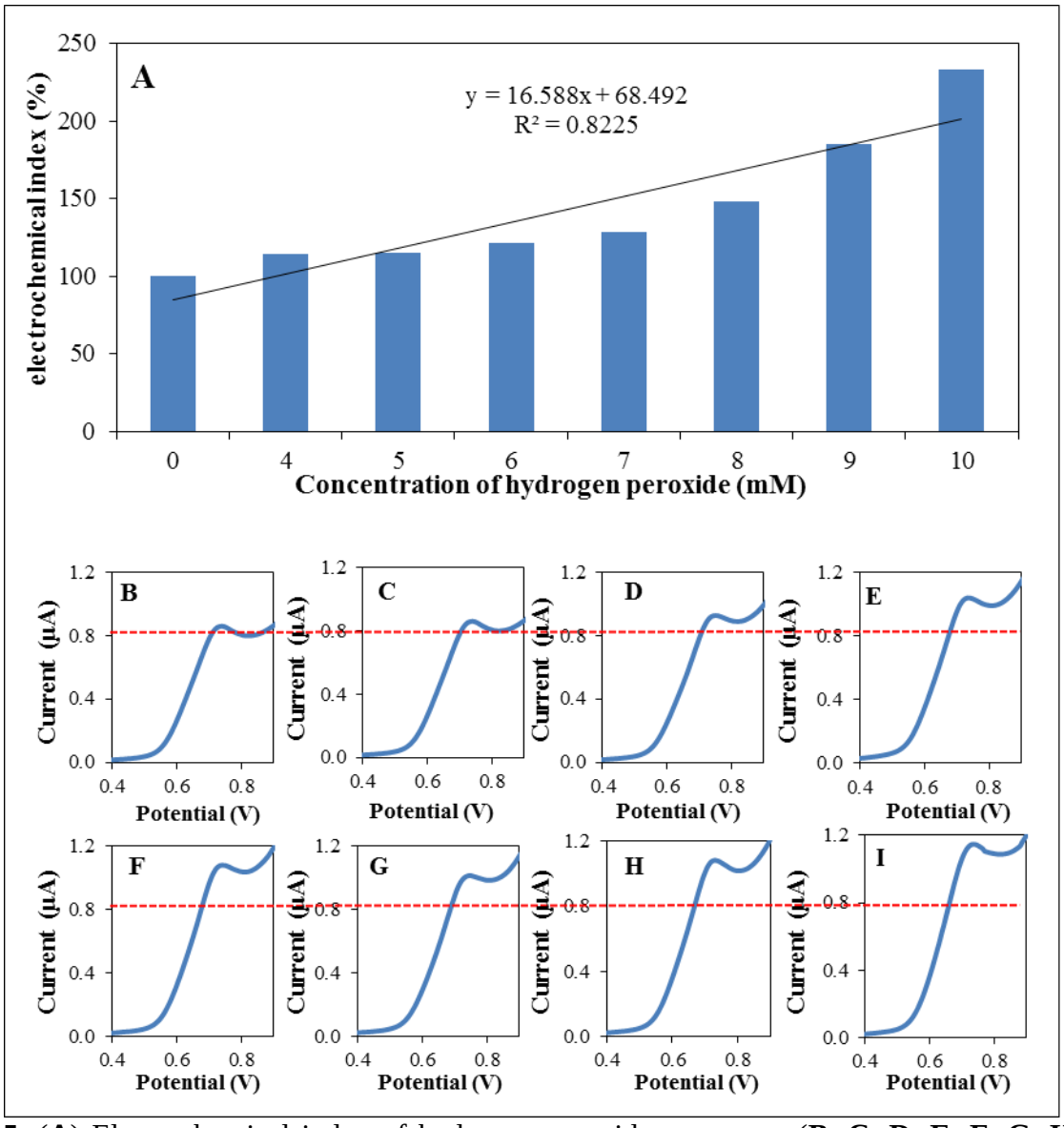

Figure 5. (**A**) Electrochemical index of hydrogen peroxide treatment. (**B, C, D, E, F, G, H and I**) Voltammograms of oxidative changes in signal of *S. aureus* according to the added concentrations of hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM).

The same results were also confirmed by spectrophotometric methods (DPPH, ABTS), when the silver ion(I) concentration of 75 μM again became a border concentration and amount of free radicals at this concentration increased rapidly (Figs. 7A and B).

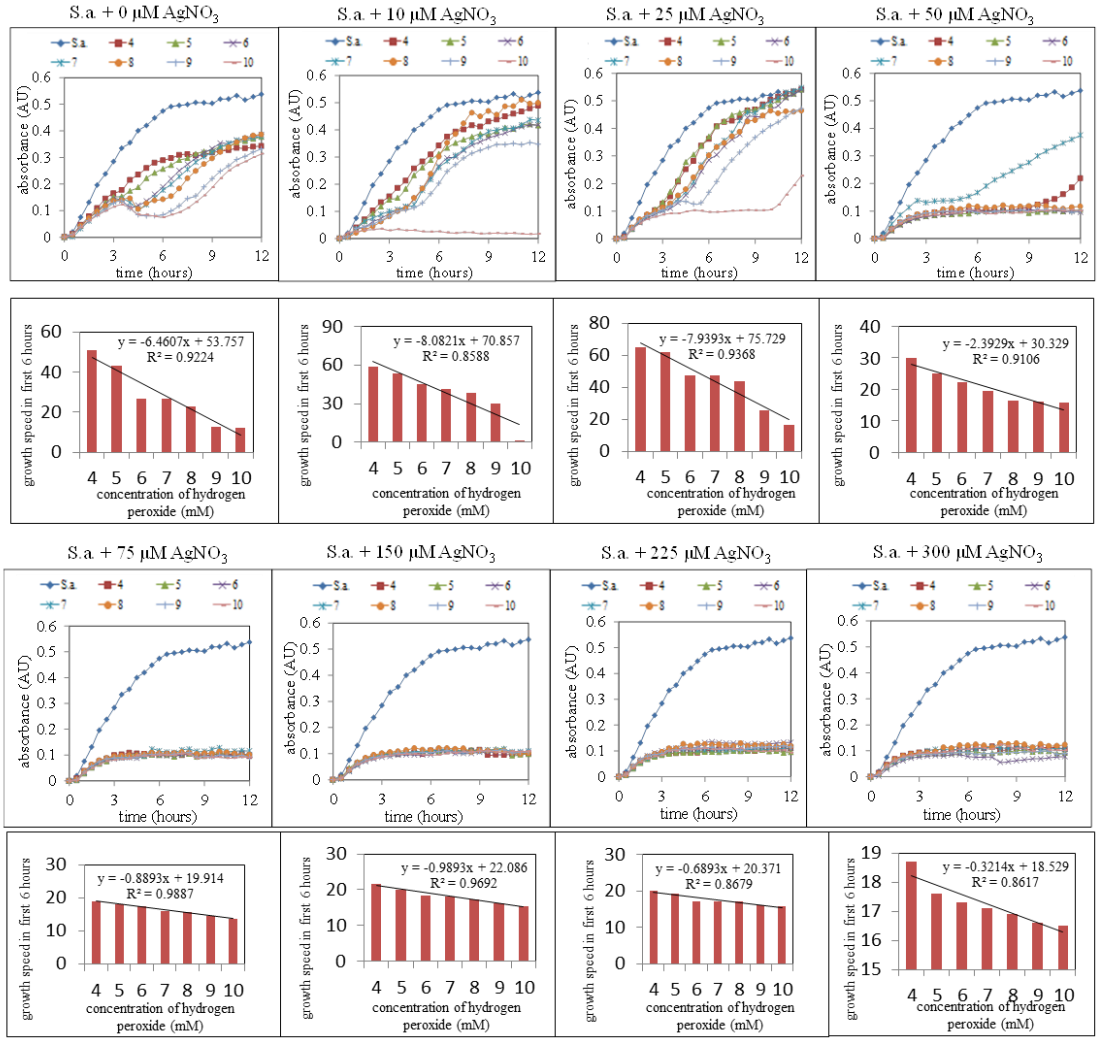

Figure 6. (**A**) Spectrophotometric analysis of the growth of *S. aureus* bacterial culture with concentrations of silver ions (0, 10, 25, 50, 75, 150, 225 and 300 μM) and hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM).

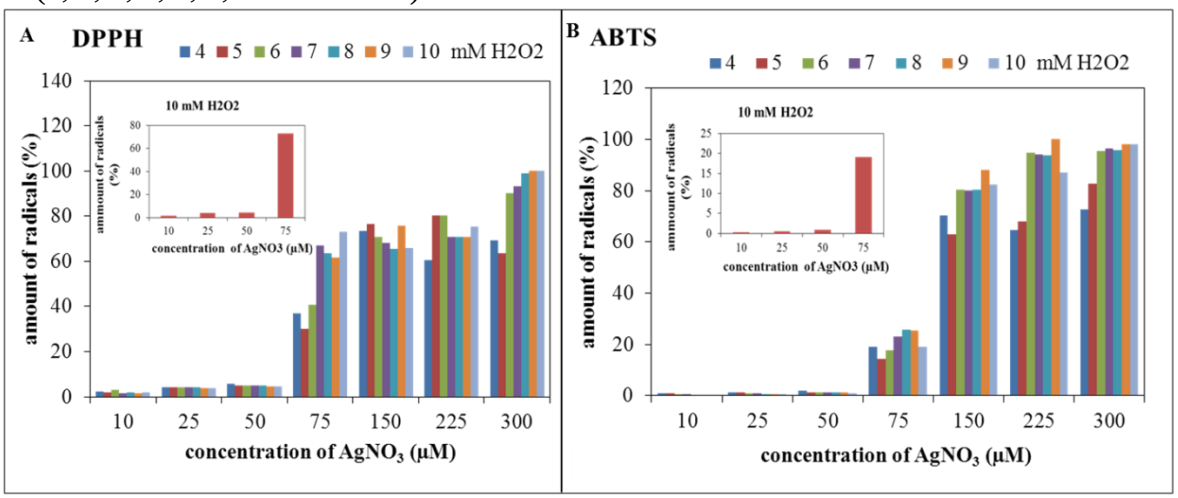

Figure 7. Spectrophotometric analysis of *S. aureus* oxidative stress using analytic methods (**A** – DPPH, **B** – ABTS). Dependence of amount of radicals (%) on used concentrations of silver(I) ions (10, 25, 50, 75, 150, 225 and 300 μM) and concentration of hydrogen peroxide (4, 5, 6, 7, 8, 9 and 10 mM).

3.5 Cyclic voltammetry as a tool for studying of oxidative stress

Cyclic voltammetry (CV) is an elementary electrochemical method implemented in all of electrochemical analyzers. For this reason it is very accessible and easy-to-use for various users [67-70]. Printed electrodes have recently become increasingly popular, especially thanks to its compact dimensions, low analyte consumption, and ease of use for a wide range of applications [33,71-87]. Oxidative stress was also studied by cyclic voltammetry, using flow cell and printed electrodes (Fig. 8). Using this method we were able to demonstrate an increase in oxidative signal in *S. aureus* lysates treated with different concentrations of silver(I) ions and hydrogen peroxide. First, pure *S. aureus* lysates were examined electrochemically in concentration of 3.7×10^7 cells/ml (Fig. 9A). Then *S. aureus* lysates measured were prepared from bacteria, where previously incubated with different concentrations of $AgNO_3$ with subsequent addition of hydrogen peroxide (Fig. 9B and C). Increase in oxidative signal was observed with rising concentrations of silver and hydrogen peroxide, which can be explained by increasing oxidative stress (Fig. 10). Furthermore we observed a shift to higher oxidation potential values with increasing concentrations of $AgNO_3$ and applied hydrogen peroxide (Fig. 9C). For a better understanding of induced oxidative stress, voltammograms were recalculated with growth curves to electrochemical index (Fig. 9B).

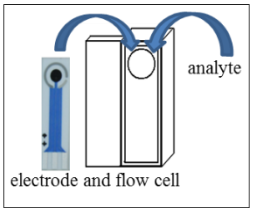

Figure 8. Printed electrode and flow cell.

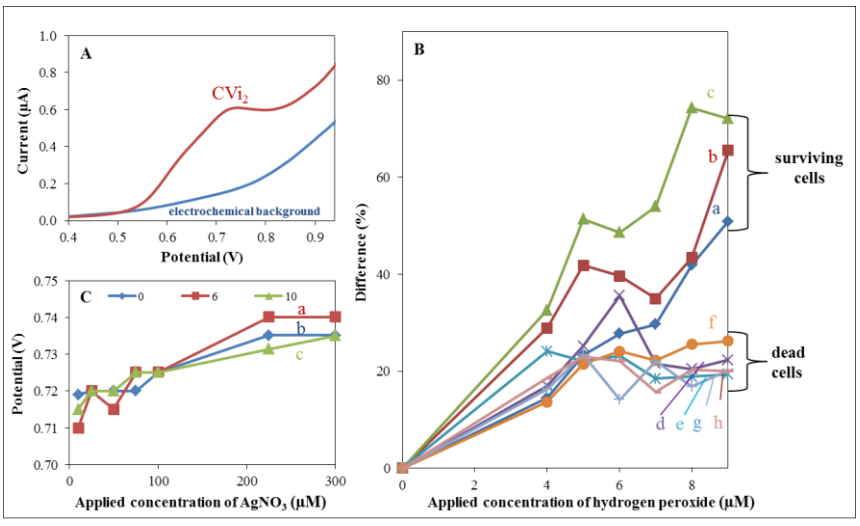

Figure 9. (**A**) Cyclic voltammograms of 0.2 M acetate buffer (pH 5) and oxidative peak of *S. aureus* (concentration of *S. aureus* corresponds 0.1 AU). **(B)** Dependence of oxidative stress of *S. aureus* on applied hydrogen peroxide concentration (0, 6, 10 mM) enriched with $AgNO_3$ (a = 0, b = 10, c = 25, d= 50, e = 75, f = 150, g = 225 and h = 300 µM). (**C**) Dependence values of the potential of oxidative stress *S. aureus* on applied concentration of silver ions and hydrogen peroxide.

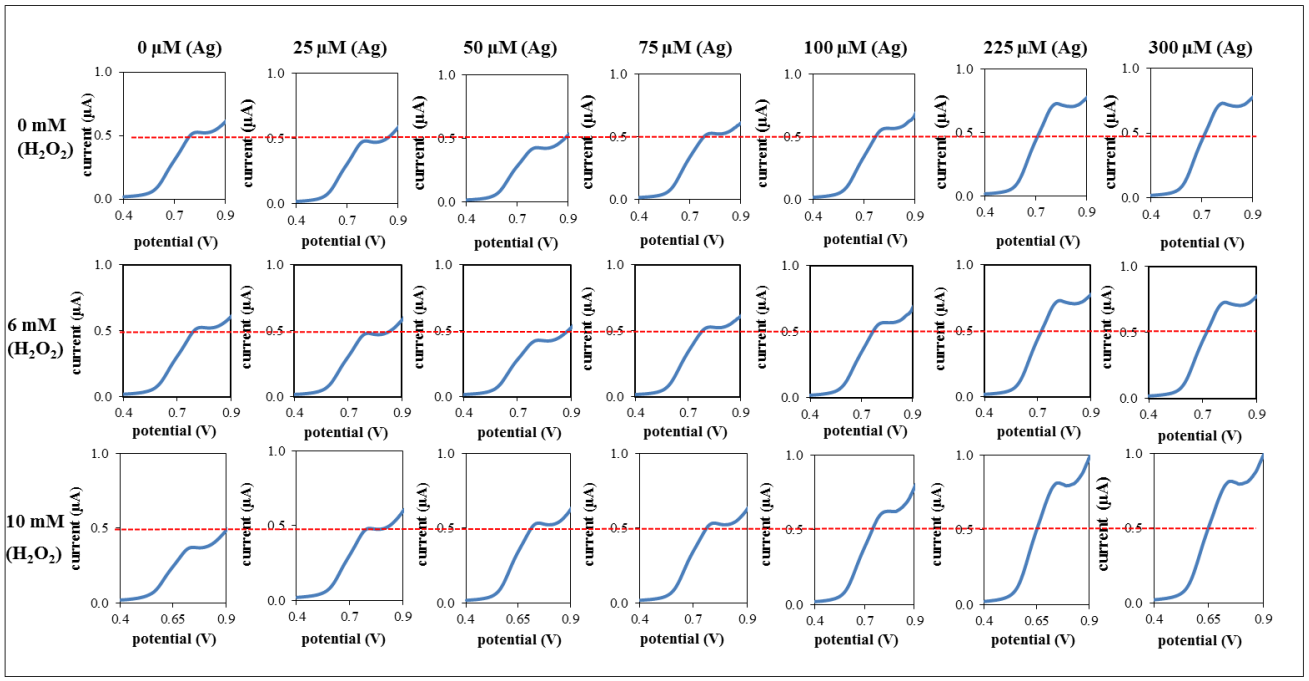

Figure 10. Cyclic voltammograms of detected *S. aureus* oxidative stress, which was developed after addition of $AgNO_3$ (0, 10, 25, 50, 75, 150, 225 and 300 µM) and hydrogen peroxide (0, 6, 10 mM).

Electrochemical index (%) was identified as a direct correlation of peak area and *S. aureus* growth curve absorbance in the sixth hour. It was observed that *S. aureus* resisted to the oxidative stress up to the concentration of 25 µM $AgNO_3$ and 10 mM hydrogen peroxide as is shown in Fig. 9B – a,b,c. Higher concentration of $AgNO_3$ was for *S. aureus* lethal (Fig. 9B – d,e,f,g).

4. CONCLUSION

In recent decades, amount of knowledge about the role of free radicals and their role in oxidative stress in organisms significantly increased. This work was focused on the study of oxidative stress in bacterial culture of *Staphylococcus aureus* induced by silver(I) ions using spectrophotometric and electrochemical methods. It was also done testing antibacterial effects of silver(I) ions on the growth of *S. aureus* bacterial culture using the measuring zones of inhibition and growth curves. Increased concentration of applied silver(I) ions led to the increase in oxidative stress which was enhanced by addition of hydrogen peroxide. Obtained results enabled further elucidation of the role of silver(I) ions in oxidative stress in bacterial culture of *S. aureus*. Our results indicate the possibility of the application of used analytical techniques in microbiology in determination of oxidative stress in bacterial cultures [88-95].

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