PHOTO-BIOREACTOR DESIGN AND VALIDATION FOR REPRODUCIBLE STUDIES OF ANTIBACTERIAL EFFECTS OF PHOTOACTIVE NANOPARTICLES

[1]Markéta ŠLAPAL BAŘINKOVÁ, [1]David RUTHERFORD, [1]Bohuslav REZEK

[1]Faculty of Electrical Engineering, Czech Technical University in Prague, Prague, Czech Republic, EU,
barinmar@fel.cvut.cz

https://doi.org/10.37904/nanocon.2023.4813

Abstract

Photoactive nanoparticles have recently gained a lot of attention as alternative antimicrobial agents since their antimicrobial properties can be enhanced by illumination. The stable and reproducible application of these conditions is crucial in order to assess the antimicrobial effect of nanoparticles, illumination and the combination of these conditions on cells. Furthermore, the reproducibility of cell growth data is also essential to avoid measurement variability influencing statistical analysis. In this work we designed custom 3D-printed laboratory accessory for a bioreactor that ensured stable illumination of cell suspensions and enabled reproducible growth curves under the same growth conditions. The reproducibility of measurements was tested on *Escherichia coli* bacteria grown under illumination, with the addition of commercial ZnO nanoparticles, and with the combination of both factors. All tests showed a notable improvement in the reproducibility of bacterial growth curves and standard deviation of the measured data reduced below the documented instrument precision.

Keywords: 3D printing, bioreactors, bacteria, ZnO nanoparticles, photocatalysis, reproducibility

1. INTRODUCTION

Antimicrobial resistance (AMR) is one of the big hurdles facing humankind in the following years. The prior misuse of current antibiotics has led to an epidemic of bacteria resistant to conventional antibiotics, necessitating new ways to combat infection[1]. Apart from more regulation of the use of antibiotics and their release into the environment, research into novel antimicrobial substances is also a promising path to overcoming antimicrobial resistance, including nanoparticles. Nanoparticles can affect bacterial cells through numerous mechanisms that include ion release, reactive oxygen species (ROS) production, and mechanical interaction with cell membranes. It was hoped that the fact nanoparticles act upon bacteria through multiple pathways would be enough to prevent the development of AMR against nanoparticles [2]. However, the development of resistance has recently been shown on silver nanoparticles [3,4], pointing to the need for a more thorough examination of the effects of nanoparticles on bacterial cells.

In addition to being excellent antimicrobial agents another benefit of nanoparticles is the option of enhancing their properties via various physical methods. For example, the zinc oxide (ZnO) nanoparticles used in this work are photoactive and electron-hole pairs are formed under illumination of sufficient energy. The resulting electron-hole pairs can enhance ROS generation when surrounded by water molecules. ZnO has an energy band gap of typically 3.3 eV and thus requires wavelengths shorter than 375 nm to be excited (i.e., near UV), however, activation of ZnO by visible light (i.e., wavelengths > 390 nm) has also been reported [5-7]. This is due to the presence of dopants or crystal lattice defects in the nanoparticles, which enable their activation even under illumination with lower, sub band gap energies.

One of the most effective methods to characterize the antimicrobial effects of nanoparticles and illumination on the inhibition of bacteria growth is monitoring bacterial growth in real time[8]. Bioreactors may be used, as

they enable the incubation of cell suspensions at fixed temperatures for optimal growth and measure the change in optical densities over time at defined intervals[9, 10]. This allows for long measurements without the need to stop incubation to remove and measure samples, thus also reducing the risk of contamination. Although these systems have many advantages, they lack means for well controlled and defined illumination of the reaction mixtures during growth.

In the current study, we focused on the development of custom laboratory equipment, which would enable such well-defined illumination of cell suspensions grown in falcon tubes using bioreactors. Fused filament fabrication (FFF) 3D printing has been on the rise in the last several years due to the rapid development of 3D-printing technologies and consequent rise in the accessibility of 3D printing. It opens the doors for cost effective, simple to modify prototyping, often within the single institution where custom equipment is needed. However, the use of FFF 3D-printing in creating laboratory equipment, specifically for microbiology, is sparsely documented. This is most likely due to the fact that in this particular field, the sterilization of labware (usually through autoclaving or dry heat sterilization) is the key. Although some filament materials with a higher heat tolerance do exist (e.g., polyethylene terephthalate - PET or acrylonitrile butadiene styrene - ABS)[11], they are also more difficult to work with than the standard PLA (polylactic acid) filament, which may change its structure during heat sterilization. A study conducted in 2022 by Diep et al. has shown the possibilities of work with PLA-printed labware with the option of sterilization of the equipment using alcohol[12]. While this method will degrade the plastic eventually, the printing of spare parts is still likely to be a much more cost-effective process than commissioning the production of these custom pieces from a company.

The problem we faced during the design of the photo-bioreactor was two-fold. Firstly, a structural mechanism was needed for the repeatable and well-defined illumination of the cell suspension in the rotating bioreactor from an external illumination source. Secondly, a cap for the falcon tube itself was needed, which would enable this illumination while not changing the cell growth when compared to standard falcon tube caps. Therefore, two pieces of the custom photo-bioreactor setup were developed to overcome both these problems through 3D modelling and 3D printing. The equipment was then tested for its functionality and reproducibility using *Escherichia coli* (*E. coli*) cells and commercial ZnO nanoparticles.

2. METHODOLOGY

2.1 Photo-bioreactor Prototyping

The first cap prototypes were created by simply gluing a quartz window over a hole created in a standard falcon tube cap. While this method was simple to implement, the sterilization of these caps and the reproducibility of growth curves obtained from them was poor. 3D modelling and printing was therefore utilized in order to create a more robust method of cell illumination. Fusion 360 software was used to create 3D models of both a falcon tube cap and a bioreactor cover for stabilizing the position and direction of illumination from the illumination source. The prototypes were then printed using an Ender 3 FFF 3D printer (Creality) and a standard PLA filament. The printed prototypes were then tested, measured and additional changes were made if necessary. The bioreactor with the printed illumination elements is dubbed photo-bioreactor further on.

2.2 Bacteria Cultures

A standard operating procedure was used for the growth of all cell cultures throughout this work. A frozen stock of gram-negative *Escherichia coli* (*E. coli*, CCM 3954) in glycerin was thawed at room temperature, diluted using a 10-step 10-fold dilution series using sterile saline solution (0.9 % NaCl, Penta). 500 µl of each dilution step was then spread onto a Mueller Hinton agar (MHA, Roth) plate, allowed to dry, before being placed into an incubator at 37 °C overnight. A single colony was removed from one plate using a sterile inoculation loop and resuspended into 10 mL of Mueller Hinton broth (MHB, Oxoid) and placed into an incubator at 37 °C with shaking at 150 rpm to obtain a monoclonal cell suspension. The following day, the culture was then adjusted

to MF = 1 (equivalent to approx. $1x10^8$ colony forming units per milliliter, cfu/ml) in MHB using a DEN-1B McFarland densitometer (Biosan) and diluted in MHB using a three-step ten-fold dilution series for a final concentration of roughly 10^4 cfu/ml. This cell suspension (called MF3 from herein) was then used for further experiments.

When testing bacteria growth during exposure to nanoparticles, a ZnO suspension was prepared by dispersing a ZnO nanopowder (Sigma; nominal size <50 nm, 6 % Al doped) in HPLC grade water (Roth) to a final concentration of 1 mg/mL. The suspension was sonicated at 35 kHz for 30 minutes prior to use. Subsequently, 2 mL of the ZnO suspension were added to 18 mL of the MF3 cell suspension for a final ZnO concentration of 100 µg/ml. Control cells were prepared in a similar manner using 2 mL of HPLC grade water instead of nanoparticles.

The prepared MF3 cell suspensions (with or without nanoparticle suspensions) were placed into RTS-1 personal bioreactors (Biosan) and grown for 20 hours at 37 °C and 2000 rpm with a change in spin direction every 1 s. The optical density of the cell suspension was read every 30 minutes at a wavelength of 850 nm (OD @ 850 nm). For experiments without illumination the bioreactor was covered with aluminum foil to prevent ambient light entering the suspensions and illuminated suspensions were closed using custom caps and illuminated using an LED light source (Schott KL 2500) at 100 % power.

All materials and media were sterilized prior to use using an autoclave (in the case of media and laboratory equipment) or using dry heat sterilization (in the case of glass equipment and inoculation loops). 3D printed materials were sterilized using isopropanol and UV treatment for 30 minutes prior to use. Remnants of the alcohol were consequently washed using sterilized water and left to air dry.

A total of four experiments were conducted for monitoring cell growth:

- a triplicate of measurements using the first (glued) cap prototype to test the reproducibility of measurements (illumination, no ZnO),
- a comparison between cell growth with a commercial falcon tube cap and the second (3D-printed) cap to test the functionality of the new cap (no ZnO, no illumination),
- a triplicate measurement using the second (3D-printed) cap prototype to test the improvement of reproducibility with these caps (illumination, no ZnO),
- a triplicate of measurements for 4 distinct exposure conditions to test the function of the new caps when exposing cells to ZnO and illumination (4 culture tubes - 1 for each combination of illumination and ZnO suspension addition).

2.3 Data Analysis

All growth curves were filtered using a moving average filter with a window of size 3. Means and standard deviations of experiments conducted in triplicate were computed and plotted. The reproducibility of measurements was assessed by looking at the mean and standard deviation in all time points and finding the maximum standard deviation of optical density as well as the time at which it occurred. All data analysis was conducted in MATLAB.

3. RESULTS AND DISCUSSION

3.1 First falcon tube cap prototype (glue)

The first cap prototype created for the illumination of the cell suspensions was created by simple attachment of a quartz glass window (chosen for its optical properties) positioned over a hole cut into a standard falcon tube cap using glue. The cap can be seen in the **Figures 1e-f**. The growth curves of cells grown under illumination using this cap in three separate experiments can be seen in the **Figures 1a-d**. It is clear that there is variance in the growth curves within the replicates, where the maximum standard deviation of the bacterial

growth curves reaches 0.79 OD (the measurement precision of the instrument is ± 0.3 OD as reported by the manufacturer). This high deviation is problematic since the variance may mask effects caused by the changes in growth conditions during actual experiments. In addition, the sterilization of these caps proved problematic as they could not be autoclaved because the glue would liquify at elevated temperatures and weaken the seal whereas alcohol sterilization gradually degraded the seal between the lid and quartz glass window, therefore a new design was needed.

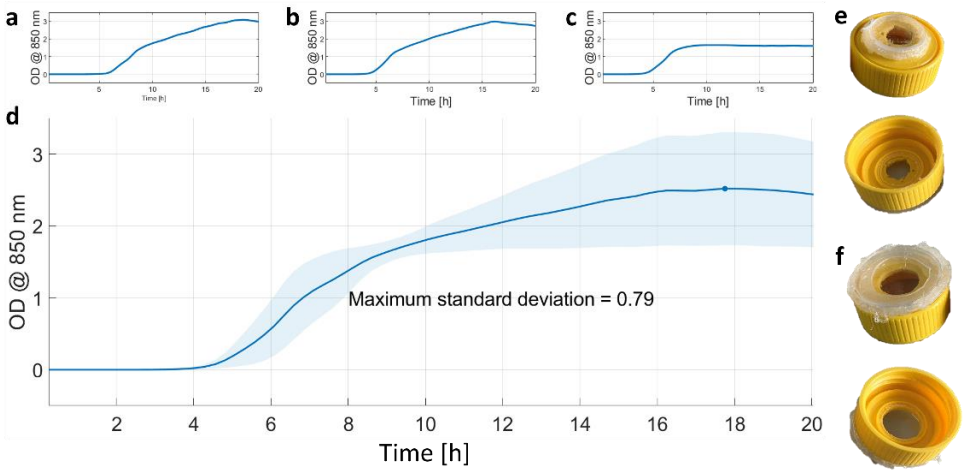

Figure 1 Testing the first (glued) cap prototype. **a-c** growth curves of individual experiments; **d** mean and standard deviation of the individual experiment (i.e., **a-c**), the blue dot denotes the position of the maximum of standard deviation; **e-f** photos of the first cap prototypes.

3.2 Photo-bioreactor cover prototype

Firstly, a cover for the photo-bioreactor prototype was 3D modelled and printed. This cover was designed to sit on the RTS-1 bioreactor and hold the optical fiber from the light source above the falcon tube in a well-defined, reproducible position while the falcon tube rotates in the bioreactor. The hole in the cover was created to the dimensions of the fiber which held the fiber firmly in place perpendicular to the surface of the suspension at a fixed distance of 1 cm from the top of the falcon tube cap. After multiple design tests, the final 3D model and a photo of the cover in use is shown in the **Figures 2a-b**.

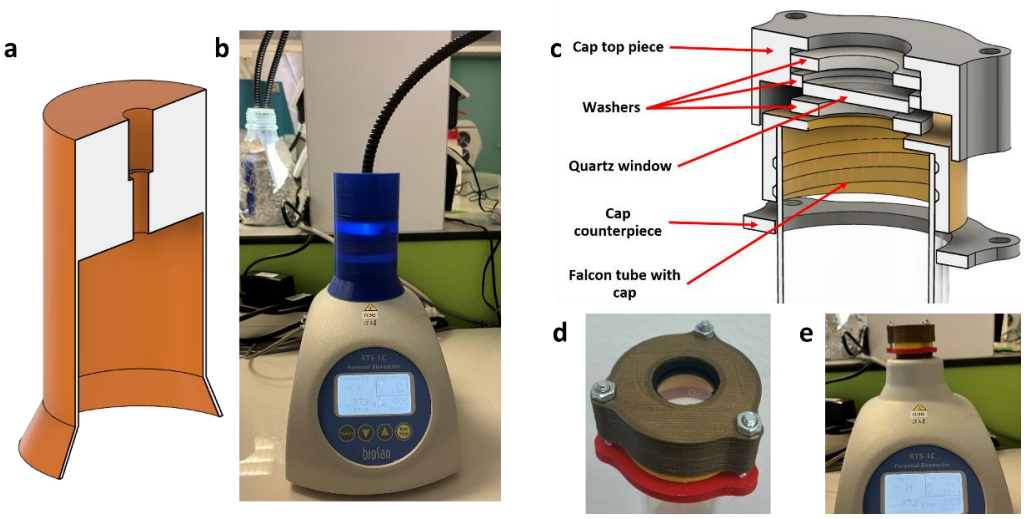

Figure 2 3D models and 3D-printed equipment. **a, b** - 3D model cross-section and photo of the bioreactor cover for guiding the optical fiber; **c, d, e** - 3D model cross-section and photos of the custom falcon tube cap.

3.3 Second falcon tube cap prototype (3D-printed)

The cap for the falcon tube was re-designed using 3D modelling to hold in place a quartz glass window and enable reproducible cell growth during the illumination of the falcon tubes (**Figure 2c**). A conventional falcon tube cap with a round hole cut into the top with a quartz glass window held in place using 3 rubber washers that blocked the influx of contaminants and make the custom cap as airtight as possible. The window and washers are then held in place using two 3D-printed elements (i.e., a cap top piece and counterpiece), which are then fixed using 3 screws and bolts (**Figures 2d-e**).

3.4 Falcon Tube Cap Testing

The first step was to uncover whether the use of the cap itself had any effect on the growth of the cells as a result of differences in air flow, etc. A fresh MF3 culture was, therefore, divided between two sterile falcon tubes, where one was closed using a standard cap (see the **Figure 3b**) and the other using the 3D-printed cap (**Figure 3c**). Cells were grown without illumination or addition of nanoparticles, to keep culturing conditions as uniform as possible. The resulting growth curves are seen in the **Figure 3a**, and we observed a maximum difference of 0.047 OD between the data which is well below the documented instrument precision (±0.3 OD). Thanks to this, we can say that any differences seen in future growth curves are caused by the conditions under which the cells are cultured, rather than a change in equipment used.

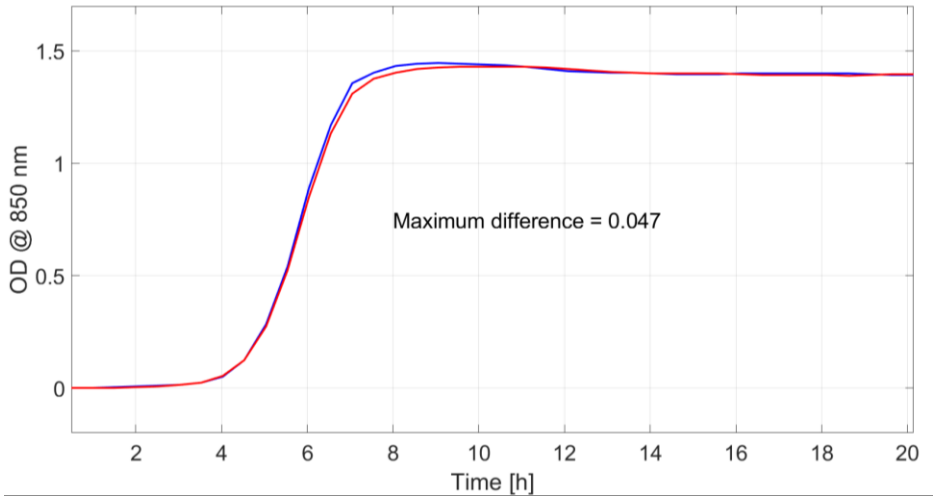

Figure 3 Growth curves of the bacterial cells curves with commercial and custom designed falcon tube caps.

The reproducibility of measurements using this new cap was then tested in order to see whether the customization of the equipment led to an improvement of growth curve reproducibility. Cells were grown under illumination in three separate experiments and the results may be seen in the **Figure 4**. Here, we can see that the reproducibility of the measurements has been greatly improved with the use of the custom caps with the maximum standard deviation decreasing from 0.79 OD (**Figure 1**) to 0.25 OD, below the documented instrument precision.

Because the main aim of the customization was to enable the illumination of cells containing ZnO nanoparticles, the cap was finally tested with all combinations of ZnO addition and illumination. The results of this analysis are shown in the **Figure 5**. Here, we can see that for all conditions, the maximum standard deviation does not exceed the instrument precision. The devised method is therefore viable for showing the effects of photoactive nanoparticles and their illumination on bacterial cells. It is also noteworthy that the position of the maximum standard deviation is always found within the log phase of bacterial growth. Here, the growth and division of cells is most turbulent, and we can therefore expect to see more deviation. The control bacteria also show the least variability of growth curves (maximum standard deviation = 0.11 OD) as opposed

to the other growth conditions (maximum standard deviations of 0.16 OD and 0.22 OD). The optical density curves were further used to analyze the effects of ZnO and illumination on *E. coli* cell suspensions via lag phase length detection and maximum growth rate computation. These results are, however, beyond the scope of this article and will be included in further publications.

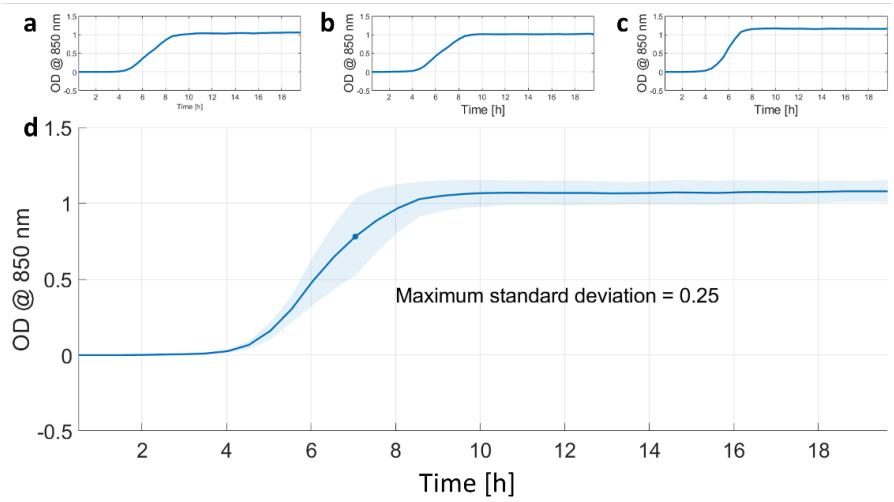

Figure 4 Growth curves of the second (3D-printed) cap prototype. **a-c** - growth curves of individual experiments; **d** - mean and standard deviation of the individual experiment, the blue dot denotes the position of the maximum of standard deviation.

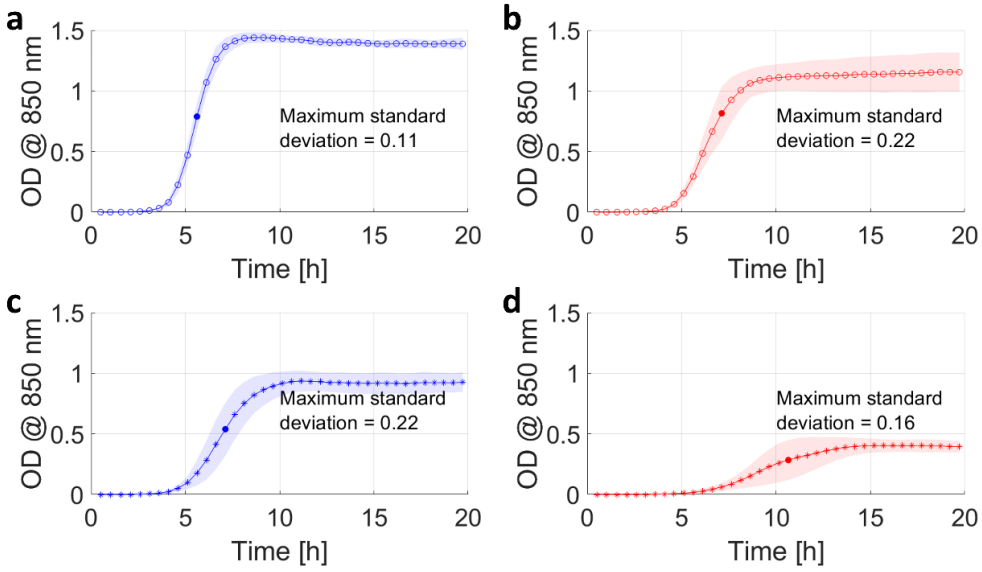

Figure 5 Growth curves for cells exposed to nanoparticles and illumination. **a** - reference (unilluminated samples without ZnO); **b** - illuminated samples without ZnO; **c** - unilluminated samples with ZnO; **d** - illuminated samples with ZnO. Full dots denote the positions of the maximum standard deviation for each condition.

4. CONCLUSION

This work successfully developed a method for the acquisition of reproducible results for bacterial growth by creating customized photo-bioreactor for consistent illumination. This allowed for real-time monitoring of a

bacterial suspension during growth in order to test the effects of illuminated photoactive nanoparticles. Using 3D modelling and printing, we were able to greatly improve the reproducibility of bacterial growth curves, allowing for more reliable data analysis. This equipment will be used in further experiments in order to uncover the effects of ZnO nanoparticles and illumination on the growth of bacterial cells.

ACKNOWLEDGEMENTS

This work was supported by the Grant Agency of the Czech Technical University in Prague, grant No. SGS23/166/OHK4/3T/13.

REFERENCES

[1] DADGOSTAR, P. Antimicrobial resistance: implications and costs. *Infection and Drug Resistance.* 2019, vol. 12, pp. 3903-10. Available from: https://doi.org/10.2147/IDR.S234610.

[2] BALDERRAMA-GONZÁLEZ, A. S.; PIÑÓN-CASTILLO, H. A.; RAMÍREZ-VALDESPINO, C. A.; LANDEROS-MARTÍNEZ, L. L.; ORRANTIA-BORUNDA, E. and ESPARZA-PONCE, H. E. Antimicrobial resistance and inorganic nanoparticles. *International Journal of Molecular Sciences.* 2019, vol. 22, no. 23, 12890. Available from: https://doi.org/10.3390/ijms222312890.

[3] GRAVES, J. L.; TAJKARIMI, M.; CUNNINGHAM, Q., CAMPBELL, A.; NONGA, H.; HARRISON, S. H. and BARRICK, J. E. Rapid evolution of silver nanoparticle resistance in Escherichia coli. *Frontiers in Genetics.* 2015, vol. 5, no. 6. Available from: https://doi.org/10.3389/fgene.2015.00042.

[4] PANÁČEK, A.; KVÍTEK, L.; SMÉKALOVÁ, M.; VEČEŘOVÁ, R.; KOLÁŘ, M.; RÖDEROVÁ, M.; DYČKA, F.; ŠEBELA, M.; PRUCEK, R.; TOMANEC, O. and ZBOŘIL, R. Bacterial resistance to silver nanoparticles and how to overcome it. *Nature Nanotechnology.* 2018, vol. 13**,** pp. 65-71. Available from: https://doi.org/10.1038/s41565-017-0013-y.

[5] LIPOVSKY, A.; GEDANKEN, A.; NITZAN, Y. and LUBART, R. Enhanced inactivation of bacteria by metal-oxide nanoparticles combined with visible light irradiation. *Lasers in Surgery and Medicine.* 2011, vol. 43, no. 3, pp. 236-40. Available from: https://doi.org/10.1002/lsm.21033.

[6] ZUDYTE, B. and LUKSIENE, Z. Visible light-activated ZnO nanoparticles for microbial control of wheat crop. *Journal of Photochemistry and Photobiology B: Biology.* 2021, vol. 219, 112206. Available from: https://doi.org/10.1016/j.jphotobiol.2021.112206.

[7] APONIENE, K., SEREVIČIUS, T., LUKSIENE, Z. and JURŠENAS, S. Inactivation of bacterial biofilms using visible-light-activated unmodified ZnO nanorods. *Nanotechnology.* 2017, vol. 28, no. 36. Available from: https://doi.org/10.1088/1361-6528/aa7a53.

[8] THEOPHEL, K., SCHACHT, V. J., SCHLÜTER, M., SCHNELL, S., STINGU, C. S., SCHAUMANN, R. and BUNGE, M. The importance of growth kinetic analysis in determining bacterial susceptibility against antibiotics and silver nanoparticles. *Frontiers in Microbiology.* 2014, vol. 5, NOV. Available from: https://doi.org/10.3389/fmicb.2014.00544.

[9] BANKOVSKY, VASILY. *Determination of the concentration of microorganisms in real time,* [online]. 2022. [viewed 2023-08-23]. Available from: https://biosan.lv/media/products/files/determination-of-the-concentration-of-microorganisms-in-real-time.pdf.

[10] RUTHERFORD, D., JÍRA, J., KOLÁŘOVÁ, K., MATOLÍNOVÁ, I., MIČOVÁ, J., REMEŠ, Z. and REZEK, B. Growth inhibition of gram-positive and gram-negative bacteria by zinc oxide hedgehog particles. *International Journal of Nanomedicine.* 2021, vol. 16**,** pp. 3541-54. Available from: https://doi.org/10.2147/IJN.S300428.

[11] BUTE, I., TARASOVS, S., VIDINEJEVS, S., VEVERE, L., SEVCENKO, J. and ANISKEVICH, A. Thermal properties of 3D printed products from the most common polymers. *International Journal of Advanced Manufacturing Technology.* 2023, vol. 124, no. 7-8, pp. 2739-53. Available from: https://doi.org/10.1007/s00170-022-10657-7.

[12] DIEP, T. T., RAY, P. P. and EDWARDS, A. D. Methods for rapid prototyping novel labware: using CAD and desktop 3D printing in the microbiology laboratory. *Letters in Applied Microbiology.* 2022, vol. 74, no. 2, pp. 247-57. Available from: https://doi.org/10.1111/lam.13615.