The Intercollegiate Faculty of Biotechnology
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Review of the doctoral dissertation of Ms. Sada Raza MSc, entitled: "Inactivation of Bacteriophages", prepared under the supervision of Associate University Prof. Jan Paczesny, PhD, DSc.

Bacteriophages (phages), or viruses that infect and kill bacteria, have been known to the scientific world for over a hundred years, dating back to their independent discoveries and descriptions by Frederick Twort and Félix d'Herelle in the early 20th century. Phages are a subject of both intensive fundamental and applied research, primarily associated with their use as promising alternatives to antibiotics in various biological control applications, as well as in different biological systems.

The great potential of bacteriophages to infect and kill bacterial cells is also a major threat to all applications based on the use of metabolically active bacteria for human-governed industrial processes, including those associated with biotechnology (precise fermentation), pharmaceutical production (e.g., insulin), and food safety (e.g., treatment of the raw meat with phage cocktails against human pathogens). As humanity increasingly relies on microbiological applications, the threat of killing valuable bacteriological strains by environmental phages needs to be urgently assessed.

The Doctoral Dissertation of Sada Raza, M.Sc., prepared under the supervision of Prof. Jan Paczesny at the Institute of Physical Chemistry in Warsaw, submitted for review, discusses the development of active materials to prevent phage infections as well as to selectively remove phages from bacterial-occupied environments, without harming bacterial cells. Even though phage contamination of (industrial) bacterial cultures is a known and real problem, the existing knowledge on this topic is rather limited, and the practical approach used routinely in various applications is based almost solely on preventing such contaminations (hygienic measures). Such sanitary measures are insufficient, and therefore, there is a pressing need to explore new, environmentally friendly solutions that can be used for this purpose.

For this reason, the proposed topic and research carried out by the PhD candidate are extremely interesting, relevant, and innovative. Moreover, this research undoubtedly has a direct, applicable, and industrial impact.

In her doctoral thesis, Ms. **Sada Raza** employed 13 strains of bacteriophages, representing different phage groups and morphotypes, as a model for her research. Combined with that, the PhD candidate used numerous physical methods, including DLS, UV-VIS and Raman Spectroscopy, FTIR, SEM, EDS, NMR, BET Analyses, XRD, and DSC. The topic of the selective bacteriophage inactivation has therefore been treated in great detail. It should be emphasized that the research approach proposed by the PhD candidate has also been well described.

The doctoral dissertation of Sada Raza, MSc., was prepared as an intermediate form between a typical research monograph and a collection of thematically related experimental scientific publications. The thesis was prepared solely in English, accompanied by the Abstract in Polish, which summarizes the major research conducted by the PhD candidate. The thesis is composed of separate chapters, including Theoretical Introduction, Aims and Scope, Materials and Methods, and three following chapters that altogether present the results of the PhD thesis (Novel Antimicrobials, Naturally Selective Antiviral Compounds, and Engineered Selectivity Through Charge-based Interactions) and discuss them in detail in the light of the current knowledge on this topic. The PhD thesis also contains a chapter presenting an overall Summary and Conclusions, as well as a chapter on References. Additionally, the doctoral dissertation is thoroughly illustrated.

Furthermore, it includes a list of publications developed as part of the doctoral project (9 publications – in 7 of these, Sada Raza is the first author) and patents (1 Polish patent). The publication list also includes three other publications co-authored by the PhD candidate but not directly related to the doctoral project. It is essential to emphasize that most of the information presented in Sada Razo's M.Sc. thesis has already been published and, therefore, successfully made public, or will be made public soon.

Detailed discussion of the individual parts of the doctoral dissertation:

Chapters such as: Abstract in English, Abstract in Polish, List of Abbreviations, and Table of Contents have been prepared in a standard way, have the form and content commonly accepted for this type of lists and scientific papers, and contain all the required and necessary information.

The **Introduction** chapter describes the research problem, specifically the role of bacteria and bacteriophages in food and industrial biotechnology. This includes bacteriophages as bacterial viruses, the environmental role of phages, the impact of physical and chemical factors

on phage inactivation, and the use of nanoparticles as antiphage solutions. This chapter concludes by highlighting the need to research new and innovative, safe, and scalable antiphage solutions, and presents the detailed aims of each of the subsequent experimental chapters.
This chapter is comprehensive, interesting, and well written.

The chapter **Aims and Scope** provides a brief introduction and justification for undertaking the research, outlines the study's purpose, and offers a concise and synthetic summary of the results obtained, with a clear emphasis on the most significant findings and observations.

The chapter **Materials and Methods** contains a description of all reagents, materials, protocols, and laboratory procedures used by the PhD candidate during the formulation of the doctoral dissertation. Likewise, this chapter contains descriptions of the methods, procedures, and protocols for preparing the experiments, as well as the statistical analyses. Reading this chapter, I came up with several topics to discuss with the PhD candidate:

- **On which basis and why were the pathogenic bacterial strains selected for this project (page 37) and not beneficial bacterial strains?**
- **On which basis and why were the respective bacteriophage strains selected for this project?**
- **According to the description of phage 37, the PhD candidate used OD_{600} measurements rather than CFU/mL. Is this fully justified, considering that the chosen bacterial strains belong to different species and genera?**

Chapters 4, 5, and 6 present the **Results** of the doctoral dissertation obtained and analysed by the PhD candidate. Due to that, these chapters are the most interesting for the reader. Each of these experimental chapters also includes a detailed discussion of the results with available literature data, as well as a summary and conclusions.

Chapter 4 (**Novel Antimicrobials**) explores the development of novel antimicrobial strategies to combat various bacteriophages and bacterial strains. Silver nanoparticles synthesized using tea extracts (TeaNPs) demonstrated potent antibacterial activity, highlighting their eco-

friendly and scalable production. Additionally, iron-based approaches were examined for their ability to inhibit bacterial growth and biofilm formation. At the same time, copper-based surface coatings were tested for their antiviral and antibacterial properties. These coatings demonstrated wide antibacterial and antiviral activity, especially when applied to surfaces, thereby offering potential as preventive measures in medical and industrial applications.

This Chapter presents a progression from eco-friendly, green-synthesized nanoparticles to metal-based nanomaterials, providing valuable insights into their roles under both natural and human-controlled conditions. It also discusses bacteriophages and engineered phage-derived proteins as highly specific tools for targeting resistant pathogens. Overall, it highlights the importance of integrating nanotechnology and phage biology to develop new, effective, sustainable, and safe antimicrobial solutions for both clinical and environmental applications.

- **Tea extracts appear promising in the development of green nanoparticles; however, tea extracts may not be standardized for this specific purpose. Did the PhD candidate try to standardize (and if so, how?) these extracts before using them for nanoparticles synthesis?**

- **Are there any other plant extracts that are a good source of green nanoparticles? If so, why are tea extracts so exceptional?**

In **Chapter 5** (**Naturally selective antiviral compounds**) of the doctoral dissertation, the PhD candidate focused on the use of natural compounds expressing antiviral (anti-phage) activities. This chapter investigates naturally occurring compounds with selective activity against bacteriophages, in contrast to the nonspecific action of nanomaterials described in **Chapter 4**. The primary objective was to identify molecules that can effectively inactivate phages without harming bacterial hosts, which, as stated in the Introduction, is particularly relevant for applications in food safety, phage therapy, and biotechnology. The key compound on which this Chapter is built around is indigo carmine, a dye widely recognized as a food-safe and environmentally nonthreatening substance. It has been demonstrated that indigo carmine exhibits strong anti-phage activity against various bacteriophage strains of different origins; however, it does not show an inhibitory effect on bacterial growth. This specificity and selectivity make it an interesting candidate for targeted phage control in various platforms. The mechanism of indigo carmine action lies probably in its ability to bind to phage DNA and, consequently, inactivate it. The natural questions that should be asked are as follows:

- **Indigo carmine appears to be a safe food dye, but this largely depends on its concentration and the duration of the delivery period. Are there any data available that demonstrate the safety of the compound at high concentrations and its use over extended periods? If not, what kind of experiments should be planned to assess it?**

- **Is there any information on how indigo carmine (at the standard intake concentrations) may influence a gut microbiome in terms of phage inactivation in the gut?**

The results obtained by the PhD candidate and presented in this chapter clearly suggest that indigo carmine may be recognized as a promising, inexpensive, human-safe, and environmentally friendly antiphage agent.

Chapter 6 (**Engineered selectively through charge-based interactions**) explores several strategies for designing new, modified nanomaterials with tailored physicochemical properties that enable the selective inactivation of bacteriophages in bacterial-phage coexistence environments. The key concept presented in this chapter is that changing the surface charge should enable discrimination between phage particles and bacterial cells, thereby inactivating the former without harming the latter. Such an approach could be recognized as a highly precise anti-phage tool for use in all applications that require exceptional specificity.

This chapter places a major focus on mixed-ligand gold nanoparticles (AuNPs), which were carefully modified by the addition of charged and uncharged ligands to modulate their activity. The PhD candidate demonstrated that AuNPs with specific positive-to-neutral ligand ratios were capable of destabilizing phage capsids and, therefore, inactivating viruses, while having no (or only a very slight effect) on bacterial cells. Such an observation highlights the potential of the nanoparticle ligand engineering approach in achieving antiviral selectivity, while also avoiding toxicity in bacterial cells.

In parallel, this chapter also evaluates polypyrrole nanoparticles (PPyNPs), which can be modified similarly to AuNPs with various ligands, resulting in a precise charge on the nanoparticles. PPyNPs were here modified in this way to enhance specificity in phage inactivation and simultaneously minimize inhibition of bacterial cells.

Chapter 6 clearly emphasizes the importance of charge-based interactions between bacterial cells and phage particles, as well as between phage particles and the surrounding

environment. Furthermore, it demonstrates how nanomaterial engineering can be used to acquire selective anti-phage properties. One important question should be posed here:

- **This precise and novel approach to selectively remove phage particles gives truly impressive possibilities, but the cost of nanoparticle production and application may bias its routine use. What are the calculated average costs per assay?**

The last chapter (**Chapter 7: Summary and Conclusions**) contains a summary of each of the previous experimental chapters, as well as presents the most important results obtained by the PhD candidate in the course of the PhD studies. This is rather a short chapter because, as I noted earlier, the experimental chapters contain discussions of the results obtained.

Ms. Sada Raza's doctoral dissertation also includes a chapter **"References**," where the author presents all the works used during the presentation of her PhD thesis. This chapter contains 315 publications, including those published recently.

Preparation of the doctoral dissertation and editorial notes:

In terms of editorial preparation, I highly rate the work of Ms. Sada Raza. The thesis is written carefully, using clear scientific language, and, in this sense, it remains interesting for the reader. The figures (charts and diagrams) complement the content of the doctoral dissertation. The minor editorial errors and imprecise wording that sometimes occur in the work are so insignificant that I will allow myself to omit them in my review. They do not affect my overall assessment of this work, and a high substantive value for a doctoral project.

Summary and Final Conclusions:

The doctoral dissertation presented to me for evaluation is an original and important contribution to the topic of selective bacteriophage inactivation in bacterial-phage full settings. **For this reason, my assessment of the doctoral dissertation is clearly positive.**

I made my assessment considering the following points:

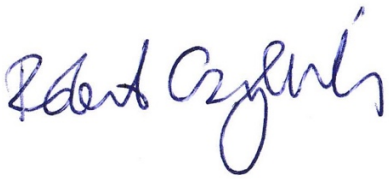
(1) The presented doctoral dissertation has been correctly and carefully planned,

(2) It presents a rational research topic and a logical sequence of scientific thinking,
(3) It has led to significant scientific discoveries and application solutions (vide publications and a granted patent)
(4) The PhD candidate used various research methods and laboratory techniques in this work to clearly formulate answers to the research questions posed before this doctoral project started
(5) The doctoral dissertation defines new research areas and objectives
(6) The results of the research were mostly published in journals with a global reach and impact

Therefore, the reviewed doctoral dissertation meets all the requirements of the Law on Higher Education and Science of 20 July 2018 (Journal of Laws of 2018, item 1668, consolidated text Journal of Laws of 2023, items 742, 1088, 1234). Therefore, I request the High Scientific Council of the Institute of Physical Chemistry of the Polish Academy of Sciences to allow Ms. Sada Raza, M.Sc., to proceed to the next stages of the doctoral procedure.

Likewise, due to the high level of the presented research work, the publication of the results of the doctoral dissertation in international journals with a global reach, and the development of practical (industrial) applications, **I request that this doctoral thesis be distinguished and that Ms. Sada Raza, M.Sc., be honoured with an appropriate award.**

Yours sincerely,



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