**Poznan University of Technology**
**Faculty of Chemical Technology**
**Institute of Chemical Technology and Engineering**

PhD thesis

# Immobilized oxidoreductases as tools for decolorization of dyes from aqueous solutions


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# List of abbreviations

$A_{BET}$ – surface area
AB147 – Acid Blue 147
AB25 – Acid Blue 25
AB83 – Acid Blue 83
ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ADH – alcohol dehydrogenase
AG1 – Acid Green 1
AO52 – Acid Orange 52
AO7 – Acid Orange 7
APTES – (3-aminopropyl)triethoxysilane
ARS – Alizarin Red S
AV109 – Acid Violet 109
BC – bacterial cellulose
BG4 – Basic Green 4
BPA – bisphenol A
BR5 – Basic Red 5
BV3 – Basic Violet 3
CE – carboxyl esterase
C.I. – color index
CLSM – confocal laser scanning microscopy
CLEAs – crosslinked enzyme aggregates
CLECs – crosslinked enzyme crystals
COD – chemical oxygen demand
CS – chitosan
DNA – deoxyribonucleic acid
DR28 – Direct Red 28
EC – enzymes classification
EDC – *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide chloride
EDS – energy dispersive X-ray spectroscopy
ELS – electrophoretic light scattering
FTIR – Fourier transform infrared spectroscopy
GA – glutaraldehyde
GO – graphene oxide
GBG – guaiacylglycerol-β-guaiacyl ether
HAA – hydroxyanthranilic acid
HBT – 1-hydroxybenzotriazole
HPEI – hyperbranched polyethyleneimine
HRP – horseradish peroxidase
$K_m$ – Michaelis-Menten constant

MR3 – Mordant Red 3
NADH – nicotinamide adenine dinucleotide
NADPH – nicotinamide adenine dinucleotide phosphate
NHS – *N*-hydroxysuccinimide
PANI – polyaniline
PDA – polydopamine
PDLG – poly(D,L-lactide-*co*-glycolide)
PDLLA – poly(D,L-lactide)
PE – polyethylene
PEO – poly(ethylene oxide)
PES – polyethersulfone
PET – poly(ethyl terephthalate)
PMMA – poly(methyl methacrylate)
PP – polypropylene
PPO – poly(*p*-phenylene oxide)
PS – polystyrene
PVA – poly(vinyl alcohol)
RB19 – Reactive Blue 19
RB198 – Reactive Blue 198
RB221 – Reactive Blue 221
RB4 – Reactive Blue 4
RB5 – Reactive Black 5
RO16 – Reactive Orange 16
RR120 – Reactive Red 120
$S_p$ – pore diameter
SEM – scanning electron microscopy
TEM – transmission electron microscopy
UV-Vis – ultraviolet-visible light spectrophotometry
$V_{max}$ – maximal reaction velocity
$V_p$ – total pore volume
XRD – X-ray diffraction

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# Scientific activity

## Publications:

1. Ł. Klapiszewski, J. Zdarta, **K. Antecka**, K. Synoradzki, K. Siwińska-Stefańska, D. Moszyński, T. Jesionowski, Magnetite nanoparticles conjugates with lignin: A physicochemical and magnetic study, Applied Surface Science, 2017, 422, 94–103, Impact Factor: 6.707, 5-year Impact Factor: 5.905, MES: 140.

2. J. Zdarta, **K. Antecka**, R. Frankowski, A. Zgoła-Grześkowiak, H. Ehrlich, T. Jesionowski, The effect of the operational parameters on the biodegradation of bisphenols by *Trametes versicolor* laccase immobilized on *Hippospongia communis* spongin scaffolds, Science of the Total Environment, 2018, 615, 784–795, Impact Factor: 7.963, 5-year Impact Factor: 7.842, MES: 200.

3. J. Zdarta, **K. Antecka**, A. Jędrzak, K. Synoradzki, M. Łuczak, T. Jesionowski, Biopolymers conjugated with magnetite as support materials for trypsin immobilization and protein digestion, Colloids and Surfaces B: Biointerfaces, 2018, 169, 118–125, Impact Factor: 5.268, 5-year Impact Factor: 4.957, MES: 100.

4. **K. Antecka**, J. Zdarta, K. Siwińska-Stefańska, G. Sztuk, E. Jankowska, P. Oleskowicz-Popiel, T. Jesionowski, Synergistic degradation of dye wastewaters using binary or ternary oxide systems with immobilized laccase, Catalysts, 2018, 8(9), 402, Impact Factor: 4.146, 5-year Impact Factor: 4.399, MES: 100.

5. T. Jesionowski, **K. Jankowska**, A. Jędrzak, T.J. Szalaty, S. Żółtowska-Aksamitowska, Ł. Klapiszewski, A. Kołodziejczak-Radzimska, M. Stasiewicz, M. Wysokowski, J. Zdarta, Zaawansowane funkcjonalne materiały wytwarzane z użyciem substancji pochodzenia naturalnego, Przemysł Chemiczny, 2018, 97, 2026–2036, Impact Factor: 0.485, 5-year Impact Factor: 0.361, MES: 40.

6. **K. Jankowska**, F. Ciesielczyk, K. Bachosz, J. Zdarta, E. Kaczorek, T. Jesionowski, Laccase immobilized onto zirconia-silica hybrid doped with $Cu^{2+}$ as an effective biocatalytic systems for decolorization of dye, Materials, 2019, 12(8), 1252, Impact Factor: 3.623, 5-year Impact Factor: 3.920, MES: 140.

7. J. Zdarta, **K. Jankowska**, M. Wyszowska, E. Kijeńska-Gawrońska, A. Zgoła-Grześkowiak, M. Pinelo, A.S. Meyer, D. Moszyński, T. Jesionowski, Robust biodegradation of naproxen and diclofenac by laccase immobilized using electrospun nanofibers with enhanced stability and reusability, Materials Science and Engineering C, 2019, 103, 109789, Impact Factor: 7.328, 5-year Impact Factor: 6.654, MES: 140.

8. F. Ciesielczyk, S. Żółtowska-Aksamitowska, **K. Jankowska**, J. Zembrzuska, J. Zdarta, T. Jesionowski, The role of novel lignosulfonate-based sorbent in a sorption mechanism of active pharmaceutical ingredient: Batch adsorption tests and interaction study, Adsorption, 2019, 25(4), 865–880, Impact Factor: 1.949, 5-year Impact Factor: 2.639, MES: 70.

9. I. Sulym, A. Kubiak, **K. Jankowska**, D. Sternik, K. Terpilowski, Y. Sementsov, M. Borysenko, A. Derylo-Marczewska, T. Jesionowski, Superhydrophobic MWCNTs/PDMS-nanocomposite materials: Preparation and characterization, Physicochemical Problems of Mineral Processing, 2019, 55(6), 1394–1400, Impact Factor: 1.256, 5-year Impact Factor: 1.199, MES: 70.

10. **K. Jankowska**, J. Zdarta, A. Grzywaczyk, E. Kijeńska-Gawrońska, A. Biadasz, T. Jesionowski, Electrospun poly(methyl methacrylate)/polyaniline fibres as a support for laccase immobilisation and use in dye decolourisation, Environmental Research, 2020, 184, 109332, Impact Factor: 6.498, 5-year Impact Factor: 6.824, MES: 100.

11. J. Zdarta, **K. Jankowska**, K. Bachosz, E. Kijeńska-Gawrońska, A. Zgoła-Grześkowiak, E. Kaczorek, T. Jesionowski, A promising laccase immobilization using electrospun materials for biocatalytic degradation of tetracycline: Effect of process conditions and catalytic pathways, Catalysis Today, 2020, 348, 127–136, Impact Factor: 6.766, 5-year Impact Factor: 6.071, MES: 140.

12. J. Zdarta, M. Staszak, **K. Jankowska**, K. Kaźmierczak, O. Degórska, L.N. Nguyen, E. Kijeńska-Gawrońska, M. Pinelo, T. Jesionowski, The response surface methodology for optimization of tyrosinase immobilization onto electrospun polycaprolactone–chitosan fibers for use in bisphenol A removal, International Journal of Biological Macromolecules, 2020, 165, 2049–2059, Impact Factor: 6.953, 5-year Impact Factor: 6.737, MES: 100.

13. **K. Jankowska**, A. Grzywaczyk, A. Piasecki, E. Kijeńska-Gawrońska, L.N. Nguyen, J. Zdarta, L.D. Nghiem, M. Pinelo, T. Jesionowski, Electrospun biosystems made of nylon 6 and laccase and its application in dyes removal, Environmental Technology & Innovation, 2021, 21, 101332, Impact Factor: 5.263, 5-year Impact Factor: 5.116, MES: 70.

14. **K. Jankowska**, J. Zdarta, A. Grzywaczyk, O. Degórska, E. Kijeńska-Gawrońska, M. Pinelo, T. Jesionowski, Horseradish peroxidase immobilised onto electrospun fibres and its application in decolourisation of dyes from model sea water, Process Biochemistry, 2021, 102, 10–21, Impact Factor: 3.757, 5-year Impact Factor: 3.665, MES: 70.

15. J. Zdarta, L.N. Nguyen, **K. Jankowska**, T. Jesionowski, L.D. Nghiem, A contemporary review of enzymatic applications in the remediation of emerging estrogenic compounds, Critical Reviews in Environmental Science and Technology, 2021, DOI: 10.1080/10643389.2021.1889283, Impact Factor: 8.302, 5-year Impact Factor: 10.404, MES: 200.

16. J. Zdarta, **K. Jankowska**, K. Bachosz, O. Degórska, K. Kaźmierczak, L.N. Nguyen, L.D. Nghiem, T. Jesionowski, Enhanced wastewater treatment by immobilized enzymes, Current Pollution Reports, 2021, 7, 167–179, Impact Factor: 3.286, 5-year Impact Factor: 6.000, MES: 20.

17. **K. Jankowska**, Z. Su, S.B. Sigurdardóttir, M. Staszak, M. Pinelo, J. Zdarta, T. Jesionowski, Tailor-made novel electrospun polystyrene/poly(D,L-lactide-*co*-glycolide) for oxidoreductases immobilization: Improvement of catalytic properties under extreme reaction conditions,

Bioorganic Chemistry, 2021, 114, 105036, Impact Factor: 5.275, 5-year Impact Factor: 5.252, MES: 100.

## Book chapters:

1. J. Zdarta, **K. Antecka**, T. Jesionowski, Immobilizacja enzymów w ochronie środowiska, G. Schroeder, P. Grzesiak (Eds.): Środowisko i Przemysł Tom VII, Cursiva, 2017, 15–80.

2. K. Bachosz, **K. Jankowska**, J. Zdarta, T. Jesionowski, Enzymatyczna konwersja biomasy, G. Schroeder, P. Grzesiak (Eds.): Środowisko i Przemysł Tom VIII, Cursiva, 2018, 67–106.

3. **K. Jankowska**, K. Bachosz, J. Zdarta, T. Jesionowski, Application of enzymatic-based bioreactors, M. Ochowiak, S. Woziwodzki, P.T. Mitkowski, M. Doligalski (Eds.): PAIC 2019, Practical Aspects of Chemical Engineering, Springer Nature Switzerland AG, 2020, 1–12.

## Domestic conferences contributions:

Oral presentations

1. **K. Antecka**, J. Zdarta, A. Zgoła-Grześkowiak, H. Ehrlich, T. Jesionowski, Nowatorskie układy immobilizowanej lakazy jako efektywne systemy do biodegradacji bisfenoli, Ogólnopolska Konferencja Biotechnologów i Mikrobiologów BioMillenium 2017, Gdańsk, Poland, 06–08.09.2017.

2. **K. Antecka**, J. Zdarta, A. Jędrzak, K. Bachosz, Ł. Klapiszewski, T. Jesionowski, Celulaza immobilizowana na funkcjonalnym materiale biokompozytowym, NanoBioMateriały – teoria i praktyka, Toruń, Poland, 06–08.06.2018.

3. J. Zdarta, **K. Antecka**, W. Smułek, H. Ehrlich, T. Jesionowski, Lipaza immobilizowana na szkieletach gąbek morskich jako efektywny katalizator w procesie transestryfikacji trójglicerydów, NanoBioMateriały – teoria i praktyka, Toruń, Poland, 06–08.06.2018.

4. **K. Jankowska**, J. Zdarta, S. Jędrzejak, E. Kijeńska-Gawrońska, T. Jesionowski, Electrospun materials as supports for enzyme immobilization, 7$^{th}$ International Seminar on Modern Polymeric Materials for Environmental Applications, Cracow, Poland, 15–17.05.2019.

5. **K. Jankowska**, F. Ciesielczyk, K. Bachosz, J. Zdarta, T. Jesionowski, Układy tlenkowe $ZrO_2$-$SiO_2$ oraz $ZrO_2$-$SiO_2$/$Cu^{2+}$ ze zimmobilizowaną lakazą do biodegradacji barwników z roztworów wodnych, IV Interdyscyplinarna Konferencja Nano(&)BioMateriały – od teorii do aplikacji, Toruń, Poland, 06–07.06.2019.

6. K. Bachosz, **K. Jankowska**, K. Synoradzki, J. Zdarta, T. Jesionowski, Bioconversion of xylose to xylonic acid by co-immobilized dehydrogenases with cofactor regeneration, Interfacial Phenomena in Theory and Practice – The XIV$^{th}$ Summer School for Postgraduate Students, Sudomie, Poland, 24–28.06.2019.

7. J. Zdarta, **K. Jankowska**, K. Bachosz, E. Kijeńska-Gawrońska, T. Jesionowski, Immobilizowane enzymy w procesach usuwania wybranych zanieczyszczeń środowiskowych, 62 Zjazd Naukowy Polskiego Towarzystwa Chemicznego, Warsaw, Poland, 02–06.09.2019.

8. **K. Jankowska**, Z. Su, J. Zdarta, O. Degórska, M. Pinelo, T. Jesionowski, The effect of process conditions onto removal of chlorophenols from aqueous solutions by laccase immobilized onto cellulose acetate fibers, National Scientific Conference "1st Summer Scientific On-line School", on-line, 08.08.2020.

9. **K. Jankowska**, F. Ciesielczyk, J. Zdarta, Usuwanie 17$\alpha$-etynyloestradiolu z roztworów wodnych z wykorzystaniem układu $CaSiO_3$-lakaza, V Konferencja Naukowa ENZYMOS Enzymy w nauce i przemyśle, on-line, 05.02.2021.

Poster presentations

1. **K. Antecka**, J. Zdarta, A. Jędrzak, M. Łuczak, T. Jesionowski, Degradacja białek z wykorzystaniem trypsyny immobilizowanej na nośnikach magnetycznych, BioOrg 2017 II Ogólnopolskie Seminarium Chemii Bioorganicznej, Organicznej i Biomateriałów, Poznań, Poland, 02.12.2017.

2. J. Zdarta, **K. Antecka**, R. Frankowski, A. Zgoła-Grześkowiak, H. Ehrlich, T. Jesionowski, Degradacja wybranych zanieczyszczeń środowiskowych z wykorzystaniem lakazy immobilizowanej na szkielecie gąbki morskiej, BioOrg 2017 II Ogólnopolskie Seminarium Chemii Bioorganicznej, Organicznej i Biomateriałów, Poznań, Poland, 02.12.2017.

3. **K. Jankowska**, J. Zdarta, A. Grzywaczyk, E. Kijeńska-Gawrońska, T. Jesionowski, Electrospinning method in biodegradation of pollution from aqueous solutions, 7th International Seminar on Modern Polymeric Materials for Environmental Applications, Cracow, Poland, 15–17.05.2019.

4. J. Zdarta, **K. Jankowska**, K. Bachosz, E. Kijeńska-Gawrońska, T. Jesionowski, Degradacja substancji farmaceutycznych z wykorzystaniem immobilizowanych enzymów, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg 2019, Poznań, Poland, 07.12.2019.

5. A. Grzywaczyk, **K. Jankowska**, K. Kaźmierczak, E. Kijeńska-Gawrońska, J. Zdarta, T. Jesionowski, Materiał elektroprzędzony PMMA/PANI jako nowatorski nośnik w immobilizacji enzymów, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg 2019, Poznań, Poland, 07.12.2019.

6. **K. Jankowska**, E. Kijeńska-Gawrońska, K. Bachosz, J. Zdarta, T. Jesionowski, M. Pinelo, Optymalizacja procesu otrzymywania materiałów elektroprzędzonych jako nośników w immobilizacji oksydoreduktaz: Charakterystyka i zastosowanie, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg 2019, Poznań, Poland, 07.12.2019.

7. **K. Jankowska**, J. Zdarta, A. Grzywaczyk, M. Pinelo, T. Jesionowski, Improving of horseradish peroxidase activity by immobilization onto nylon 6 electrospun fibers, National Scientific Conference "1st Summer Scientific On-line School", on-line, 08.08.2020.

8. **K. Jankowska**, J. Zdarta, M. Pinelo, T. Jesionowski, Removal of dyes from aqueous solutions by system made of laccase/electrospun fibers/membrane, Ogólnopolska Konferencja Naukowa "Nauka Okiem Młodego Naukowca" - V edycja, on-line, 05.06.2021.

## International conferences contributions:

Oral presentations

1. J. Zdarta, **K. Antecka**, T. Jesionowski, Laccase-electrospun fibers as an effective tools for environmental protection, 1st International Conference on Applied Catalysis & Chemical Engineering, Dubai, United Arab Emirates, 08–10.04.2019.

2. **K. Jankowska**, J. Zdarta, S. Jędrzejak, E. Kijeńska-Gawrońska, T. Jesionowski, Biodegradation of organic pollutants using advanced biocatalytic systems, 17th International Conference on Chemistry and the Environment, Thessaloniki, Greece, 16–20.06.2019.

3. J. Zdarta, **K. Jankowska**, K. Bachosz, E. Kijeńska-Gawrońska, T. Jesionowski, Laccase-electrospun materials as biocatalytic systems for application in environmental protection, 5th Edition of Global Conference on Catalysis, Chemical Engineering & Technology, London, Great Britain, 16–18.09.2019.

4. **K. Jankowska**, J. Zdarta, F. Ciesielszyk, K. Bachosz, T. Jesionowski, Oxide systems with immobilized laccase as tools for dyes decolorization processes, 5th Edition of Global Conference on Catalysis, Chemical Engineering & Technology, London, Great Britain, 16–18.09.2019.

5. **K. Jankowska**, Z. Su, K. Bachosz, M. Pinelo, J. Zdarta, T. Jesionowski, Electrospun materials for immobilization of oxidoreductases: Improving of their catalytic activity, Catalysis Virtual 2020, on-line, 17–18.07.2020.

6. **K. Jankowska**, A. Grzywaczyk, K. Bachosz, J. Zdarta, T. Jesionowski, M. Pinelo, A new approach for decolorization of textile dyes using laccase immobilized onto electrospun fibers, 12th International Congress on Membranes and Membrane Processes, on-line, 06–11.12.2020.

Poster presentations

1. **K. Antecka**, J. Zdarta, K. Siwińska-Stefańska, T. Jesionowski, Functional oxide systems as support materials for laccase immobilization and dyes degradation, Protein Formulation and Characterization, Warsaw, Poland, 23.05.2018.

2. **K. Antecka**, J. Zdarta, K. Siwińska-Stefańska, T. Jesionowski, Immobilized laccase as an effective tool for biodegradation of hazardous pollutants, 4th Symposium

on Biotransformations for Pharmaceutical and Cosmetic Industry, Trzebnica, Poland, 25–27.06.2018.

3. J. Zdarta, **K. Antecka**, A. Jędrzak, K. Synoradzki, M. Łuczak, T. Jesionowski, Magnetic biocomposites as support for trypsin immobilization: Application for protein digestion, European Congress on Biotechnology, Geneva, Switzerland, 01–04.07.2018.

4. **K. Antecka**, J. Zdarta, A. Zgoła-Grześkowiak, H. Ehrlich, T. Jesionowski, Degradation of bisphenols using immobilized laccase supported onto biopolymer marine sponge scaffolds: Effect of operational parameters on removal efficiency, European Congress on Biotechnology, Geneva, Switzerland, 01–04.07.2018.

5. A. Kołodziejczak-Radzimska, **K. Antecka**, J. Zdarta, K. Siwińska-Stefańska, T. Jesionowski, Bimodal adsorption/biodegradation of dyes by oxide systems with immobilized laccase, Ten$^{th}$ International Symposium Effects of Surface Heterogeneity in Adsorption, Catalysis and Related Phenomena, Lublin, Poland, 27–31.08.2018.

6. **K. Jankowska**, J. Zdarta, A. Grzywaczyk, E. Kijeńska-Gawrońska, T. Jesionowski, Electrospinning as an advanced technique for production of enzyme supports, 17$^{th}$ International Conference on Chemistry and the Environment, Thessaloniki, Greece, 16–20.06.2019.

7. **K. Jankowska**, K. Bachosz, J. Zdarta, T. Jesionowski, Electrospun nanomaterials for enzyme immobilization and biodegradation of environmental pollution, French-Polish Chemistry Congress, Paris, France, 04–06.07.2019.

8. I. Sulym, **K. Jankowska**, J. Zdarta, D. Sternik, Y. Sementsov, A. Derylo-Marczewska, F. Ciesielczyk, T. Jesionowski, Study of morphological properties of MWCNTs/PDMS polymer nanocomposites, Advanced Materials and Technologies, Palanga, Lithuania, 19–23.08.2019.

9. **K. Jankowska**, J. Zdarta, T. Jesionowski, M. Pinelo, Electrospun materials for enzyme immobilization processes and their application, Process and Systems Engineering Center (PROSYS) Research Seminar, Kongens Lyngby, Denmark, 25.10.2019.

10. **K. Jankowska**, M. Pinelo, J. Zdarta, T. Jesionowski, Removal of chlorophenols from aqueous solutions using membrane/electrospun fibers/laccase system, Catalysis Virtual 2020, on-line, 17–18.07.2020.

11. **K. Jankowska**, Z. Su, J. Zdarta, T. Jesionowski, M. Pinelo, Electrospun material for immobilization of oxidoreductases on membranes: polystyrene/poly(D,L-lactide-*co*-glycolide) enabled higher activities at extreme operational conditions, 12$^{th}$ International Congress on Membranes and Membrane Processes, on-line, 06–11.12.2020.

## Research projects:

1. “The novel biocatalytic systems produced by the enzyme immobilization onto multifunctional materials synthesized by the electrospinning method”; 2018/29/N/ST8/01026; PRELUDIUM 15; National Science Centre, Poland; **K. Jankowska** – principal investigator; 01.03.2019–28.02.2022.

2. “The removal of phenolic pollutants from wastewaters and biomass using membrane immobilized oxidoreductases”; The Iwanowska Programme; Polish National Agency for Academic Exchange; **K. Jankowska** – principal investigator; 01.09.2019–31.08.2020.

3. “The removal of selected environmental pollutants from aqueous solutions using immobilized laccase”; 2020/36/T/ST8/00213; ETIUDA 8; National Science Centre, Poland; **K. Jankowska** – principal investigator; 01.10.2020–30.09.2021.

4. “Biodegradation of estrogens using advanced immobilized oxidoreductases-based biocatalytic systems”; 2019/35/D/ST8/02087; SONATA 15; National Science Centre, Poland; J. Zdarta – principal investigator; **K. Jankowska** – investigator; 01.09.2020–31.08.2023.

## Internships:

1. **Warsaw University of Technology**, Faculty of Materials Science and Engineering, Warsaw, Poland, 20–27.11.2017.

2. **Warsaw University of Technology**, Faculty of Materials Science and Engineering, Warsaw, Poland, 07–14.05.2018.

3. **Warsaw University of Technology**, Faculty of Materials Science and Engineering, Warsaw, Poland, 22–26.10.2018.

4. **Technical University of Denmark**, Department of Chemical and Biochemical Engineering, Kongens Lyngby, Denmark, 01.09.2019–31.08.2020.

5. **Technical University of Denmark**, Department of Chemical and Biochemical Engineering, Kongens Lyngby, Denmark, 01.01.2021–30.06.2021.

## Awards and scholarships:

1. **Scientific scholarship awarded by the Rector of the Poznan University of Technology** for the PhD students, 2019/2020, 2020/2021.

2. **Travel Grant**, 17$^{th}$ International Conference on Chemistry and the Environment, Thessaloniki, Greece, 16–20.06.2019.

3. **Scholarship holder of the City of Poznań** for outstanding young researchers from Poznań scientific community, 2020.

# List of publications chosen as the basis for the PhD procedure

According to: Ustawa o stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki (Dz.U. 2003 Nr 65 poz. 595) - 2. Rozprawa doktorska może mieć formę maszynopisu książki, książki wydanej lub spójnego tematycznie zbioru rozdziałów w książkach wydanych, spójnego tematycznie zbioru artykułów opublikowanych lub przyjętych do druku w czasopismach naukowych, określonych przez ministra właściwego do spraw nauki na podstawie przepisów dotyczących finansowania nauki (...).

| No. | Publication | IF | 5-year IF | MES points | Individual input (%) |
|---|---|---|---|---|---|
| 1. | **K. Antecka**, J. Zdarta, K. Siwińska-Stefańska, G. Sztuk, E. Jankowska, P. Oleskowicz-Popiel, T. Jesionowski, Synergistic degradation of dye wastewaters using binary or ternary oxide systems with immobilized laccase, Catalysts, 2018, 8(9), 402.<br><br>**Katarzyna Jankowska** conceived and designed the degradation of dye experiments, developed the results of oxide systems analysis, wrote the manuscript and answered the reviewers. | 4.146 | 4.399 | 100 | 50 |
| 2. | **K. Jankowska**, F. Ciesielczyk, K. Bachosz, J. Zdarta, E. Kaczorek, T. Jesionowski, Laccase immobilized onto zirconia-silica hybrid doped with $Cu^{2+}$ as an effective biocatalytic systems for decolorization of dye, Materials, 2019, 12(8), 1252.<br><br>**Katarzyna Jankowska** was responsible for preparing and analyzing zirconia-silica hybrid material, writing the manuscript and discussion with reviewers. | 3.623 | 3.920 | 140 | 50 |

| | | | | | |
|---|---|---|---|---|---|
| 3. | **K. Jankowska**, A. Grzywaczyk, A. Piasecki, E. Kijeńska-Gawrońska, L.N. Nguyen, J. Zdarta, L.D. Nghiem, M. Pinelo, T. Jesionowski, Electrospun biosystems made of nylon 6 and laccase and its application in dyes removal, Environmental Technology & Innovation, 2021, 21, 101332.<br><br>**Katarzyna Jankowska** produced material for enzyme immobilization, designed dyes degradation process, developed the results, wrote the manuscript and discussed with reviewers. | 5.263 | 5.116 | 70 | 50 |
| 4. | **K. Jankowska**, J. Zdarta, A. Grzywaczyk, O. Degórska, E. Kijeńska-Gawrońska, M. Pinelo, T. Jesionowski, Horseradish peroxidase immobilised onto electrospun fibres and its application in decolourisation of dyes from model sea water, Process Biochemistry, 2021, 102, 10–21.<br><br>**Katarzyna Jankowska** was an author of the idea of the research, carried out the dye decolorization process, described the obtained results and wrote the manuscript. | 3.757 | 3.665 | 70 | 60 |
| 5. | **K. Jankowska**, J. Zdarta, A. Grzywaczyk, E. Kijeńska-Gawrońska, A. Biadasz, T. Jesionowski, Electrospun poly(methyl methacrylate)/polyaniline fibres as a support for laccase immobilisation and use in dye decolourisation, Environmental Research, 2020, 184, 109332.<br><br>**Katarzyna Jankowska** evaluated the immobilization process of enzymes onto produced electrospun material, examined decolorization of dye and wrote the manuscript. | 6.498 | 6.824 | 100 | 50 |

| | | | | | |
|---|---|---|---|---|---|
| 6. | **K. Jankowska**, Z. Su, S.B. Sigurdardóttir, M. Staszak, M. Pinelo, J. Zdarta, T. Jesionowski, Tailor-made novel electrospun polystyrene/poly(D,L-lactide-*co*-glycolide) for oxidoreductases immobilization: Improvement of catalytic properties under extreme reaction conditions, Bioorganic Chemistry, 2021, 114, 105036.<br><br>**Katarzyna Jankowska** fabricated electrospun material for enzyme immobilization, carried out immobilization process, described results of analysis and wrote the manuscript. | 5.275 | 5.252 | 100 | 50 |
| | **Summary:** | **28.562** | **29.176** | **580** | |

# 1. Abstract

Oxidoreductase enzymes (EC 1), due to their oxidation properties, can convert phenolic compounds to their derivatives. These enzymes play an important role in environmental protection, mainly due to the possibility to degrade pharmaceuticals, phenols, dyes and other hazardous pollutants from surface waters and wastewaters. Therefore, it is essential to develop systems that allow the use of these biomolecules for water and wastewater treatment. Based on the above-mentioned information, a study was undertaken within the presented doctoral thesis with the aim to obtain novel biocatalytic systems based on immobilized oxidoreductases for decolorization of dyes from aqueous solutions. The crucial steps of the study included: production of support materials for enzyme immobilization made of oxide systems and electrospun fibers, immobilization of oxidoreductases, characterization of the obtained biocatalysts and their application for decolorization of selected dyes from aqueous solutions. Analyzes, performed using scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction method (XRD), energy dispersive X-ray spectroscopy (EDS), ultraviolet-visible light spectrophotometry (UV-Vis), zeta potential measurement (ELS) as well as toxicity study using the *Artemia salina* model organism allowed to characterize the produced biosystems on every stage of their synthesis and examine their practical utility.

In **Publication no. 1**, entitled "***Synergistic degradation of dye wastewaters using binary or ternary oxide systems with immobilized laccase***" (**K. Antecka**, J. Zdarta, K. Siwińska-Stefańska, G. Sztuk, E. Jankowska, P. Oleskowicz-Popiel, T. Jesionowski, Catalysts, 2018, 8(9), 402), $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$ oxide materials were synthesized, characterized and applied as supports for immobilization of laccase from *Trametes versicolor* by adsorption. The biocatalytic systems with attached oxidoreductases were used for decolorization of dyes including C.I. Mordant Red 3, C.I. Reactive Black 5 and C.I. Reactive Blue 19. The crucial stage of this work was the determination of the mechanism of dyes removal by examination of percentage participation of the adsorption process of dye molecules onto oxide materials and their bioconversion by immobilized laccase. Moreover, the effect of various process conditions, such as pH, temperature and dye concentration, on the efficiency of dye decolorization was investigated.

The next work presented in **Publication no. 2** "***Laccase immobilized onto zirconia-silica hybrid doped with $Cu^{2+}$ as an effective biocatalytic systems for decolorization of dye***" (**K. Jankowska**, F. Ciesielczyk, K. Bachosz, J. Zdarta, E. Kaczorek, T. Jesionowski, Materials, 2019, 12(8), 1252) describes the synthesis of $ZrO_2$-$SiO_2$ and doping of this material by copper ions to enhance the properties of this system as support for laccase immobilization. These oxide systems were then used for laccase immobilization by adsorption. Based on the results of zeta potential analysis and low-temperature $N_2$ adsorption/desorption measurements, the physicochemical and electrokinetic properties of $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ were determined. Moreover, the effect of the presence of $Cu^{2+}$ in the oxide system on the activity and stability of immobilized laccase was investigated and the storage stability as well as reusability were determined. The most important step of this investigation was the decolorization process of C.I. Reactive Blue 19 at various process conditions by two proposed biocatalytic systems made of $ZrO_2$-$SiO_2$-laccase and $ZrO_2$-$SiO_2$/$Cu^{2+}$-laccase.

Aside from the application of oxide materials as supports for oxidoreductases immobilization and application in dye decolorization process, the present dissertation also focused on polymeric fibers produced by electrospinning technique. Therefore, in the work showed in **Publication no. 3** "***Electrospun biosystems made of nylon 6 and laccase and its application in dyes removal***" (**K. Jankowska**, A. Grzywaczyk, A. Piasecki, E. Kijeńska-Gawrońska, L.N. Nguyen, J. Zdarta, L.D. Nghiem, M. Pinelo, T. Jesionowski, Environmental Technology & Innovation, 2021, 21, 101332), nylon 6 electrospun fibers were obtained and laccase was immobilized by two immobilization methods, including adsorption and covalent binding. These systems were applied for decolorization process of two dyes: azo dye C.I. Reactive Black 5 and anthraquinone dye C.I. Reactive Blue 4, at various process conditions. The efficiencies of dyes decolorization using the two proposed systems with attached laccase were compared.

**Publication no. 4** entitled "***Horseradish peroxidase immobilised onto electrospun fibres and its application in decolourisation of dyes from model sea water***" (**K. Jankowska**, J. Zdarta, A. Grzywaczyk, O. Degórska, E. Kijeńska-Gawrońska, M. Pinelo, T. Jesionowski, Process Biochemistry, 2021, 102, 10–21) presents the application of electrospun fibers from nylon 6 for horseradish peroxidase immobilization. In this study, horseradish peroxidase was immobilized by adsorption and covalent binding methods to compare the properties

of various immobilized oxidoreductases towards dye removal. In this report, the most crucial stage was associated with the selection of conditions of horseradish peroxidase immobilization, i.e. enzyme immobilization technique, amount and type of linker, time of process, pH and temperature. It was presented that the proposed biosystems show the properties which make them suitable for potential application in removal of dyes from model sea wastewaters.

A different approach for fabrication of electrospun fibers was presented in **Publication no. 5** "***Electrospun poly(methyl methacrylate)/polyaniline fibres as a support for laccase immobilisation and use in dye decolourisation***" (**K. Jankowska**, J. Zdarta, A. Grzywaczyk, E. Kijeńska-Gawrońska, A. Biadasz, T. Jesionowski, Environmental Research, 2020, 184, 109332). The production of hybrid poly(methyl methacrylate)/polyaniline fibers and their application in enzyme immobilization and degradation of dyes were shown. In this investigation, the crucial part was the selection of the most suitable conditions of laccase immobilization, such as enzyme immobilization technique, enzyme concentration, time of process, its pH and temperature. This was dictated by the search for the best immobilization conditions, which resulted in obtaining biocatalytic systems with the highest catalytic activity. Finally, the biosystems with the best catalytic properties proved to be effective tools for decolorization of C.I. Reactive Blue 19 from a model aqueous solution.

Finally, in the publication "***Tailor-made novel electrospun polystyrene/poly(D,L-lactide-co-glycolide) for oxidoreductases immobilization: Improvement of catalytic properties under extreme reaction conditions***" (**K. Jankowska**, Z. Su, S.B. Sigurdardóttir, M. Staszak, M. Pinelo, J. Zdarta, T. Jesionowski, Bioorganic Chemistry, 2021, 114, 105036), the properties of a complex platform system composed of electrospun fibers made of polystyrene/poly(D,L-lactide-*co*-glycolide) and surface modifiers ((3-aminopropyl)triethoxysilane (APTES) with glutaraldehyde (GA) and polydopamine (PDA)) towards immobilization of laccase were presented. The key aspect of this work was the detailed characterization of the type of interactions between the support and enzyme as well as determination of catalytic activity and stability of immobilized biomolecule.

In the presented dissertation, biosystems based on oxide materials and/or electrospun fibers with immobilized oxidoreductases were obtained and thoroughly analyzed. The attention was paid to conditions of enzyme immobilization process and their effect on

catalytic activity of the attached enzymes. Moreover, it was important to investigate the effect of immobilization methods on the properties of biomolecules in terms of stability of immobilized oxidoreductases their activity and amount of enzyme attached to the supports. Furthermore, the proposed types of biocatalysts were compared and tested in terms of their possible application, especially in dyes decolorization at various process conditions. The obtained results allowed to conclude that oxidoreductases immobilized onto oxide materials and electrospun fibers possess outstanding catalytic properties and high potential in application for environmental protection. In addition, the conducted research provides the basis for production of advanced biosystems and their application in various areas of science and industry.

# 2. Streszczenie

Oksydoreduktazy (EC 1), ze względu na swoje właściwości utleniające, mogą przekształcać związki fenolowe w ich pochodne. Enzymy te odgrywają ważną rolę w ochronie środowiska, głównie ze względu na możliwość degradacji farmaceutyków, fenoli, barwników i innych niebezpiecznych zanieczyszczeń z wód powierzchniowych i ścieków. Dlatego konieczne jest opracowanie systemów pozwalających na wykorzystanie tych biomolekuł do oczyszczania wody i ścieków. W odniesieniu do powyższych informacji podjęto się badań zrealizowanych w ramach rozprawy doktorskiej, których celem było uzyskanie nowych układów biokatalitycznych z wykorzystaniem immobilizowanej oksydoreduktazy i ich zastosowanie w usuwaniu barwników z roztworów wodnych. Kluczowymi etapami badań były: wytworzenie nośników do immobilizacji enzymów powstałych z układów tlenkowych i włókien elektroprzędzonych, immobilizacja oksydoreduktaz, charakterystyka wytworzonych biokatalizatorów oraz dekoloryzacja wybranych barwników z roztworów wodnych. W oparciu o analizę rezultatów skaningowej mikroskopii elektronowej (SEM), konfokalnej laserowej mikroskopii skaningowej (CLSM), transmisyjnej mikroskopii elektronowej (TEM), spektroskopii w podczerwieni z transformacją Fouriera (FTIR), dyfrakcji rentgenowskiej (XRD), energodyspersyjnej mikroanalizy rentgenowskiej (EDS), spektrofotometrii UV-Vis (UV-Vis), jak i pomiarom potencjału dzeta, a także testy toksyczności z wykorzystaniem organizmów modelowych *Artemia salina*, dokonano charakterystyki produkowanych biosystemów i zbadano ich potencjał użytkowy.

W **Publikacji nr 1** zatytułowanej „***Synergistic degradation of dye wastewaters using binary or ternary oxide systems with immobilized laccase***” (**K. Antecka**, J. Zdarta, K. Siwińska-Stefańska, G. Sztuk, E. Jankowska, P. Oleskowicz-Popiel, T. Jesionowski, Catalysts, 2018, 8(9), 402) opisano syntezę materiałów tlenkowych $TiO_2$-$ZrO_2$ i $TiO_2$-$ZrO_2$-$SiO_2$, ich charakterystykę i zastosowanie jako nośników do immobilizacji lakazy z *Trametes versicolor* metodą adsorpcji. Systemy biokataliczne z unieruchomioną oksydoreduktazą zostały następnie wykorzystane jako narzędzia biokatalityczne w procesie dekoloryzacji barwników, jakimi były C.I. Mordant Red 3, C.I. Reactive Black 5 i C.I. Reactive Blue 19. Kluczowym etapem pracy było określenie mechanizmu usuwania barwników poprzez zdefiniowanie procentowego udziału adsorpcji cząsteczek barwników na materiałach tlenkowych oraz biokonwersji substratów przez immobilizowaną lakazę w całościowym procesie dekoloryzacji

barwników. Ponadto zbadano wpływ różnych warunków procesu, takich jak pH, temperatura i stężenie barwnika na skuteczność usuwania zanieczyszczeń.

**Publikacja nr 2** "***Laccase immobilized onto zirconia-silica hybrid doped with $Cu^{2+}$ as an effective biocatalytic systems for decolorization of dye***" (**K. Jankowska**, F. Ciesielczyk, K. Bachosz, J. Zdarta, E. Kaczorek, T. Jesionowski, Materials, 2019, 12(8), 1252) opisuje syntezę materiału tlenkowego $ZrO_2$-$SiO_2$ oraz domieszkowanie tego układu jonami miedzi celem poprawy jego właściwości pod kątem immobilizacji lakazy poprzez adsorpcję. W oparciu o analizę rezultatów potencjału dzeta i niskotemperaturowej adsorpcji/desorpcji azotu określono parametry fizykochemiczne i elektrokinetyczne materiałów $ZrO_2$-$SiO_2$ i $ZrO_2$-$SiO_2$/$Cu^{2+}$. Ponadto zbadano wpływ obecności jonów miedzi w układzie tlenkowym na aktywność i stabilność unieruchomionej lakazy, testując także stabilność układów podczas przechowywania oraz definiując możliwość ich wielokrotnego użycia. Najważniejszym etapem prac było przeprowadzenie procesu odbarwiania C.I. Reactive Blue 19 w różnych warunkach procesowych przez dwa proponowane układy biokatalityczne: $ZrO_2$-$SiO_2$-lakaza i $ZrO_2$-$SiO_2$/$Cu^{2+}$-lakaza.

Oprócz zastosowania materiałów tlenkowych jako nośników do immobilizacji oksydoreduktaz oraz w procesie odbarwiania barwników, w badaniach w ramach pracy doktorskiej zwrócono również uwagę na włókna polimerowe wytwarzane techniką elektroprzędzenia. W **Publikacji nr 3** „***Electrospun biosystems made of nylon 6 and laccase and its application in dyes removal***" (**K. Jankowska**, A. Grzywaczyk, A. Piasecki, E. Kijeńska-Gawrońska, L.N. Nguyen, J. Zdarta, L.D. Nghiem, M. Pinelo, T. Jesionowski, Environmental Technology & Innovation, 2021, 21, 101332) na materiale elektroprzędzonym wytworzonym z nylonu 6 unieruchomiono lakazę dwiema metodami: za pomocą adsorpcji oraz wiązania kowalencyjnego. Systemy z przyłączonym enzymem zostały zastosowane w procesie dekoloryzacji dwóch barwników: barwnika azowego C.I. Reactive Black 5 i barwnika antrachinonowego C.I. Reactive Blue 4, w różnych warunkach.

**Publikacja nr 4** pt. „***Horseradish peroxidase immobilised onto electrospun fibres and its application in decolourisation of dyes from model sea water***" (**K. Jankowska**, J. Zdarta, A. Grzywaczyk, O. Degórska, E. Kijeńska-Gawrońska, M. Pinelo, T. Jesionowski, Process Biochemistry, 2021, 102, 10–21) przedstawia zastosowanie elektroprzędzonych włókien z nylonu 6 do immobilizacji peroksydazy chrzanowej za pomocą adsorpcji i wiązania

kowalencyjnego celem porównania właściwości immobilizowanych oksydoreduktaz w usuwaniu barwników. W pracy ważnym etapem był dobór warunków procesu immobilizacji peroksydazy chrzanowej, w tym m.in. rodzaju techniki immobilizacji enzymu, ilości modyfikatora powierzchni, czasu trwania procesu, czy pH i temperatury. Wykazano, że proponowane biosystemy wykazują właściwości, które predestynują je do zastosowania w usuwaniu barwników, nawet z modelowego roztworu wody morskiej.

Odmienne podejście do wytwarzania włókien elektroprzędzonych przedstawiono w **Publikacji nr 5** „***Electrospun poly(methyl methacrylate)/polyaniline fiber as a support for laccase immobilisation and use in dye decolourisation***" (**K. Jankowska**, J. Zdarta, A. Grzywaczyk, E. Kijeńska-Gawrońska, A. Biadasz, T. Jesionowski, Environmental Research, 2020, 184, 109332). W niej opisano wytwarzanie włókien z poli(metakrylanu metylu) jak i polianiliny oraz ich zastosowanie w immobilizacji enzymów i degradacji barwników. W badaniach kluczowym elementem był dobór najodpowiedniejszych warunków procesu immobilizacji lakazy, takich jak rodzaj metody immobilizacji, stężenie biokatalizatora, czas trwania procesu oraz jego pH i temperatura. Etap ten był podyktowany poszukiwaniem optymalnych warunków immobilizacji, skutkujących uzyskaniem układów biokatalitycznych o najwyższej aktywności katalitycznej, a biosystemy o najlepszych właściwościach katalitycznych okazały się skutecznymi narzędziami do odbarwiania C.I. Reactive Blue 19 z modelowego roztworu wodnego.

Natomiast w **Publikacji nr 6** "***Tailor-made novel electrospun polystyrene/poly(D,L-lactide-co-glycolide) for oxidoreductases immobilization: Improvement of catalytic properties under extreme reaction conditions***" (**K. Jankowska**, Z. Su, S.B. Sigurdardóttir, M. Staszak, M. Pinelo, J. Zdarta, T. Jesionowski, Bioorganic Chemistry, 2021, 114, 105036) scharakteryzowano właściwości systemów opartych o włókna elektroprzędzone z polistyrenu/poli(D,L-laktydu-*ko*-glikolidu), które zostały poddane modyfikacji z wykorzystaniem (3-aminopropylo)trietoksysilanu (APTES) z aldehydem glutarowym (GA) i/lub polidopaminy (PDA) jako potencjalnych platform w immobilizacji oksydoreduktaz. Kluczowym aspektem prezentowanej pracy była wnikliwa charakterystyka rodzaju połączeń między nośnikiem oraz lakazą, jak i zdefiniowanie aktywności i stabilności unieruchomionych enzymów.

W rozprawie doktorskiej przedstawiono biosystemy oparte na materiałach tlenkowych lub włóknach elektroprzędzonych oraz immobilizowanych oksydoreduktazach. Szczególną uwagę zwrócono na warunki procesu immobilizacji enzymów i ich wpływ na aktywność katalityczną przyłączonych biomolekuł. Ponadto, istotną kwestią było zbadanie wpływu zastosowanej metody immobilizacji na stabilność, aktywność i ilość unieruchomionego enzymu. Dodatkowo, porównano i przetestowano zaproponowane typy biokatalizatorów pod kątem możliwości ich zastosowania w dekoloryzacji barwników w różnych warunkach procesowych. Uzyskane wyniki pozwoliły stwierdzić, że oksydoreduktazy immobilizowane na materiałach tlenkowych i włóknach elektroprzędzonych posiadają wyróżniające się właściwości katalityczne i znaczny potencjał w ochronie środowiska. Warto również podkreślić, iż przeprowadzone badania i otrzymane rezultaty mogą stanowić podstawę do produkcji zaawansowanych biosystemów z możliwością ich stosowania w różnych dziedzinach nauki i przemysłu.

# 3. Introduction

## 3.1. Oxidoreductases – general information and application

Oxidoreductases belong to the first class of enzymes (EC1), which catalyse oxidation and redox reactions of a wide range of substrates containing e.g. phenolic compounds, their derivatives, amines, alcohols, proteins and even metal ions (Fomenko et al., 2008; Cosgrove et al., 2018; Dong et al., 2018; Zerva et al., 2021). These biomolecules are widely distributed in the environment, as confirmed by their presence in various bacteria, fungi, plants and even animal and human organisms (Xu, 2005). For example, alcohol dehydrogenase (ADH) is produced by human liver and is responsible for the conversion of ethanol to aldehydes and ketones (Kaiser et al., 1993). However it is also produced by yeast, such as *Saccharomyces cerevisiae,* which plays important role in restoring the redox balance of these microorganisms by conversion of acetaldehyde to ethanol (Raj et al., 2014). The other widely known oxidoreductase, laccase, was found in plants, such as in the Chinese tree *Rhus vernicifera* (Olshansky et al., 2018) and also in bacteria of the *Streptomyces* genus and *Trametes versicolor* white-rot fungi (Margot et al., 2013), in case of which it takes part in regulating the concentration of phenolic compounds. Another oxidoreductase, which is horseradish peroxidase, obtained mainly from horseradish roots, is responsible for e.g. resistance to infections and lignification of cell wall (Veitch, 2004; Khurshid et al., 2012). The widespread distribution of this class of enzymes shows that they play an important role in catalytic reactions occurring in single cells as well as in complex systems. Although the main role of oxidoreductases is catalyzing oxidation and redox reactions, each of the subclasses within the first class of enzymes is responsible for a special type of reaction. Table 1 presents the division of oxidoreductases with their donor, acceptor or specific reaction and examples of biocatalysts.

The catalytic properties of oxidoreductases are strictly related to building of their polypeptide backbone and active sites. Whereas the structure of the biomolecule (mostly tertiary or quaternary) affects its stability and selectivity (Robinson, 2015), the active sites are responsible for redox reactions. For example, laccase has four copper atoms forming the active site, which are involved in the transfer of electrons between substrates and products and reduction of molecular oxygen (Arregui et al., 2019). Meanwhile bacterioferritin

possesses a binuclear iron centre and haem b in its structure, which play a crucial role in redox reaction of iron taken up from soils by bacteria, and its storage in organisms (Rivera, 2017). The other oxidoreductase, which possesses iron ions in its structure, is horseradish peroxidase, however in this case the active site in this enzyme plays a different role than in bacterioferritin: it involves redox reactions with the presence of hydrogen peroxide and water molecules, resulting formation of oligomers (Veitch, 2004).

***Table 1.*** *Division of oxidoreductases into subclasses, based on Younus (2019).*

| Subclass | Donor (D), acceptor (A) or specific reactions | Example |
|---|---|---|
| EC 1.1 | CH–OH group (D) | alcohol dehydrogenase |
| EC 1.2 | aldehyde, oxo groups (D) | pyruvate dehydrogenase |
| EC 1.3 | CH–CH group (D) | fumarate reductase (quinol) |
| EC 1.4 | CH–$NH_2$ group (D) | aspartate dehydrogenase |
| EC 1.5 | CH–NH group (D) | pteridine reductase |
| EC 1.6 | NADH, NADPH compounds (D,A) | monodehydroascorbate reductase (NADH) |
| EC 1.7 | nitrogenous compounds (D) | azobenzene reductase |
| EC 1.8 | sulfur group (D) | sulfite dehydrogenase |
| EC 1.9 | heme group (D) | cytochrome c oxidase |
| EC 1.10 | phenolic compounds (D) | laccase |
| EC 1.11 | peroxide (A) | horseradish peroxidase |
| EC 1.12 | hydrogen (D) | hydrogen dehydrogenase |
| EC 1.13 | incorporation of molecular oxygen to single donors | nitronate monooxygenase |
| EC 1.14 | incorporation of molecular oxygen to paired donors | tyrosine hydroxylase |
| EC 1.15 | superoxide radicals (A) | superoxide reductase |
| EC 1.16 | oxidation metal ions | bacterioferritin |
| EC 1.17 | CH and $CH_2$ groups (D/A) | ethylbenzene hydroxylase |
| EC 1.18 | iron–sulfur proteins (D) | nitrogenase |
| EC 1.19 | flavodoxin (D) | nitrogenase (flavodoxin) |
| EC 1.20 | phosphorus, arsenic (D) | phosphonate dehydrogenase |
| EC 1.21 | conversion of X–H and Y–H to X–Y bond | aureusidin synthase |

Due to the specific properties of oxidoreductases, which are governed by their structures and active site composition, the wide range of reactions which they catalyze, and their omnipresence, these enzymes have a wide application potential. Hitherto many researchers have studied the possible use of oxidoreductases using their native or immobilized forms. The proposed areas of application of oxidoreductases are presented in Figure 1.

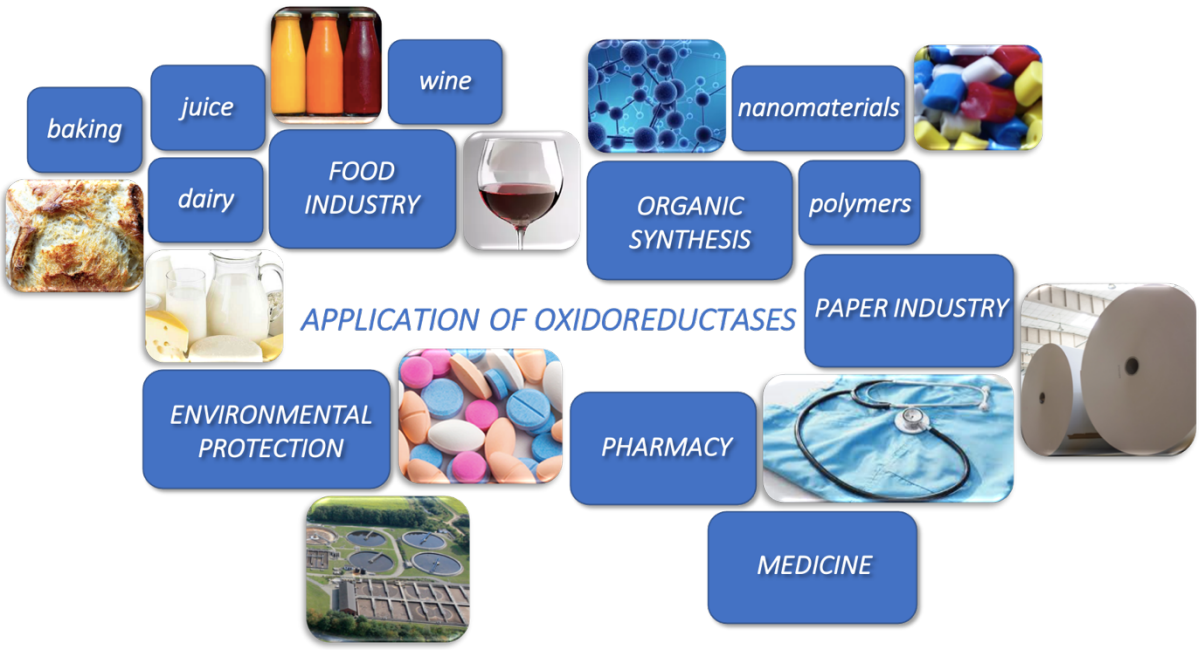


***Figure 1.*** *Possible application of oxidoreductases in various branches of science, everyday life and industry.*

Food industry is one of the biggest areas of oxidoreductases application. They are used in this branch mainly for improvement of food quality. In baking, glucose oxidase is the most commonly used enzyme. It catalyses various reactions, such as crosslinking of glutenin, albumin and globulin from wheat. The final products are characterized by improved viscoelastic properties, which highly affect the volume and the crispness of bread. In a work presented by Rasiah et al. (2005) it was confirmed that the addition of glucose oxidase to wheat caused the crosslinking of albumin and globulin, which finally enhanced the crumb properties of baking. The other enzyme applied in food industry is lipoxygenase. Zhang et al. (2013) used a purified recombinant lipoxygenase from *Bacillus subtilis* in bread production. Compared to the control sample, bread after treatment with lipoxygenase was characterized by improved volume up to 17% and loaf height to 10.3%. Enhancing of enzyme-

treated bread properties was explained by increasing of disulfide bonds which contribute to retention of gas inside of baking products. However, the addition of oxidoreductases in the baking process should be highly controlled due to the fact that an excess of the enzyme may result in a loss of flavour and decrease of nutritional values. Moreover, oxidoreductases are applied in dairy industry, especially glucose dehydrogenase. It may convert lactose to lactobionic acid, which is used as a food additive due to its antioxidant and moisturizing properties (Oh et al., 2020). On the other hand, application of oxidoreductases in beverages and wine industry may resolve the most common problems, such as browning, tart flavour and high turbidity. These problems are caused by the presence of undesirable phenolic compounds in juices, wines and beers, which can be oxidized by laccases, thus creating dimers and oligomers that may be easily separated by filtration processes (Bilal and Iqbal, 2020).

Oxidoreductases are also characterized by a great application potential in synthesis of polymers and nanomaterials. They can convert phenols in oxidative coupling reactions, which finally results in the production of oligomeric and polymeric compounds. The advantage of application of enzymes in phenols polymerization over chemical synthesis, is mainly associated with the possibility to carry out the reaction under mild conditions (Ghoul, 2012). In this case, laccases and polyphenoloxidases are mainly used. For example, laccases are applied in enzymatic polymerization of aniline to polyaniline by one-electron oxidation of aniline monomers, obtaining anilino radicals, which can undergo radical-radical coupling and further oxidation processes (Walde et al., 2019). Moreover, as presented by Choi and Lee (2020), NAD(P)H-dependent reductases are able to reduce metal ions in cells of microorganisms (*Fusarium oxysporum* and *Bacillus licheniformis*) to inorganic nanomaterials made of silver and zirconia.

The next branch of industry which applies oxidoreductases is the paper industry. This area of industry strongly affects ecology, mainly by exploitation of huge fields of forests and production of highly toxic compounds as co-products in paper processing. Lignolytic enzymes, such as manganese peroxidase, lignin peroxidase and laccase, can be used in process of lignin degradation, which is one of the most common waste generated from paper mills (Singh et al., 2016; de Gonzalo et al., 2016). Due to this fact, the application of oxidoreductases can decrease the energy input for lignin processing and minimize the use of hazardous compounds for wood treatment (Singh and Gupta, 2020). Therefore,

oxidoreductases applied in paper processing allow to decrease the harmfulness of wastewaters. In many recent published studies, it was demonstrated that laccase applied in the paper industry has a dual function. First of all, it can delignify the pulp mixture by selective oxidation of lignin hydroxyl and benzylic groups. Moreover, it is also possible to decrease the toxicity of wastewaters from paper mills, especially by oxidation of chlorophenol compounds generated during the bleaching process of papers (Singh et al., 2016; Upadhyay et al., 2016; Navas et al., 2019). Furthermore, laccase is able to remove undesirable color from paper wastewaters. As presented by Pedroza-Rodriguez and Rodriguez-Vazquez (2013), whole *Trametes versicolor* fungi, producing laccase, were immobilized in a bubble column reactor. After passing wastewaters from the paper industry through this reactor, approx. 80% of color was removed. Among oxidoreductases applied in the paper industry, attention should also be paid to polysaccharide monooxygenase, a copper-dependent biomolecule, which is able to break down polysaccharide chains by oxidation reaction with the presence of $O_2$ or $H_2O_2$ (Labourel et al., 2020). For example, polysaccharide monooxygenase from *Pleurotus ostreatus* white-rot fungus allowed to degrade even 46.5% of lignin from an aqueous solution (Li et al., 2019).

Oxidoreductases also play an important role in pharmacy and medicine. It should be highlighted that the crucial application of such enzymes in these fields is associated with the catalysis of synthesis of various pharmaceuticals. For example, laccase can be used for direct synthesis of vindoline or cephalosporin antibiotics (Mikolasch et al., 2007; Sagui et al., 2009), whereas methane monooxygenase converts methane to methanol, which is used as a solvent in production of vitamins or synthetic hormones (Amao and Watanabe, 2004). Moreover, the role of oxidoreductases in cancer therapy should be highly emphasized. For example, xanthine oxidoreductase is a marker in breast, kidney or liver cancers, due to the fact that it catalyzes the synthesis reaction of carcinogenic substances in human cells (Battelli et al., 2016).

## 3.1.1. Laccase and horseradish peroxidase

The information presented above allow to state that oxidoreductases are the most commonly used enzymes at an industrial scale. This is mainly caused by their outstanding catalytic properties, which enable their application in food or paper industries, organic synthesis,

medicine and pharmacy and even environmental protection. Although oxidoreductases include numerous compounds, special attention was paid to two enzymes from this group, namely laccase (EC 1.10.3.2) and horseradish peroxidase (EC 1.11.1.7). Therefore they are the main subject of research in the framework of the presented doctoral dissertation. Their structures were presented in Figure 2.

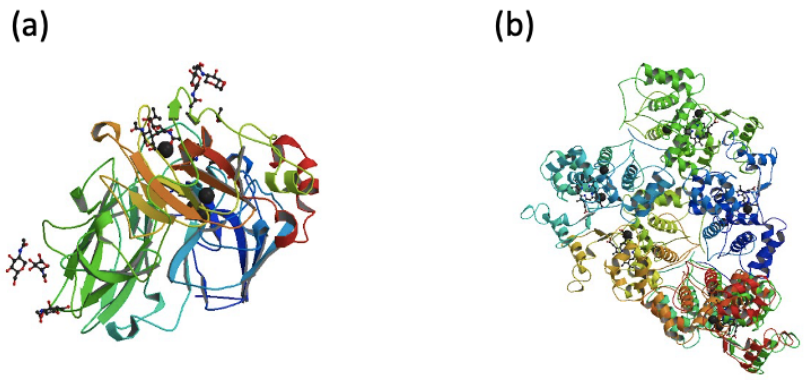


***Figure 2.*** *Structure of (a) laccase from Trametes versicolor and (b) horseradish peroxidase from Amoracia rusticana, based on Berman et al. (2003).*

Laccase (oxygen oxidoreductase), also called „blue oxidoreductase", is a widely distributed enzyme in environment – it can be found in various bacteria, fungi and plants. This oxidoreductase was isolated for the first time in 1883 from the Japanese lacquer tree *Rhus vernicifera* by Yoshida (1883). It should be noted that laccases vary from each other in terms of size, optimal working conditions and other properties, depending on their origin. For example, bacterial laccases are proteins with average size lower than 60 kDa and possess pH and temperature optima at approx. 6 and 50 °C, respectively. The most commonly known bacterial laccases are produced by *Streptomyces coelicolor*, *Aquisalibacillus elongatus* or *Pseudomonas aeruginosa*. In case of "blue oxidoreductase" obtained from fungi, its average size is between 60–80 kDa. It is characterized by the highest catalytic activity at pH in the range of 4.5–6 and temperature between 25 and 30 °C. It should be stated that laccases produced by fungi, such as *Cerrena unicolor*, *Trametes versicolor* or *Trametes vilosa*, arouse the greatest interest, compared to oxidoreductase obtained from other sources, which is caused by their relatively low price, availability and high activity. The biggest laccase proteins are usually produced by plants (e.g. *Rhus vernicifera*), and their size may have reach 130 kDa. Their optimum working pH is slight below 7 and temperature does not exceed 30 °C

(Gochev and Krastanov, 2007; Brijwani et al., 2010; Wan et al., 2010; Dwivedi et al., 2011; Giardina et al., 2015; Chauchan et al., 2017; Janusz et al., 2020). The biggest advantage of laccase is that it is able to catalyze the oxidation of e.g. phenolic substrates, such as mono-, di- and polyphenols, and *N*-heterocyclic compounds, substrates with aromatic amine groups, diamines or phenothiazines. For this reason laccase is used in conversion of these substrates in various areas, such as food industry, wood industry, environmental protection or organic synthesis (Zdarta et al., 2018a; Arregui et al., 2019).

Laccases are called "blue oxidoreductases" due to the presence of 4 copper atoms in their active centers: a single Type-1 atom, a single Type-2 atom and two Type-3 atoms. Moreover, it should be emphasized that each of them plays a different role in the catalytic cycle. The mononuclear Type-1, which has an absorption band at 610 nm, is responsible for the characteristic blue color of this biomolecule and electron transfer in oxidation of the substrate. This copper ion is responsible for direct $O_2$ oxidation, therefore, the laccase activity is strictly related to the presence of oxygen in reaction environment. In the next stage, products in forms of radicals are produced, whereas free electrons are rapidly transported *via* the cysteine-histidine (Cys-His) pathway to Type-2 and Type-3 copper atoms. These atoms form the T2/T3 trinuclear copper cluster, in which reduction of molecular oxygen to water takes place (Abdel-Hamid et al., 2013; Arregui et al., 2019). The overall reaction scheme catalyzed by laccase is presented in Figure 3.

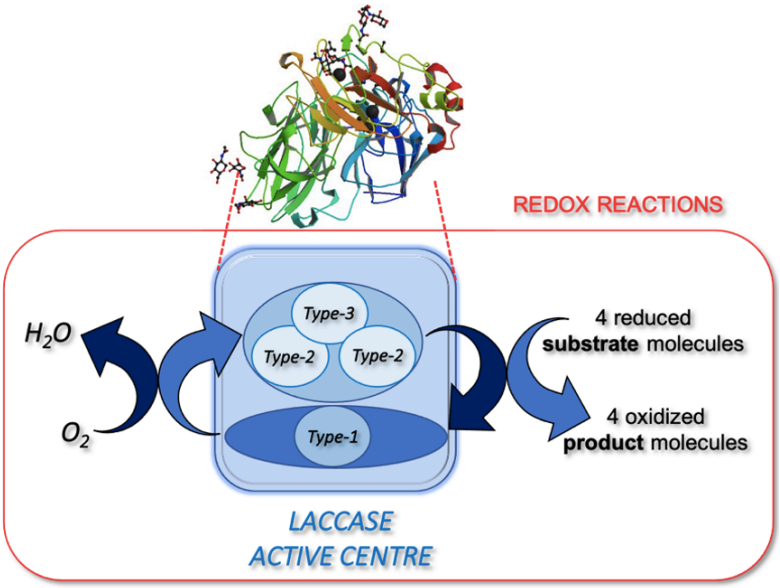


***Figure 3.*** *Scheme of redox reactions catalyzed by laccase indicating the presence of copper atoms (Type-1, Type-2 and Type-3).*

Moreover, reactions, which are catalyzed by laccase, could be divided into three groups: without a mediator, with a mediator and coupling reactions. These reactions depend on type of substrates and their redox potentials. Schemes of these catalytic cycles were presented in Figure 4.

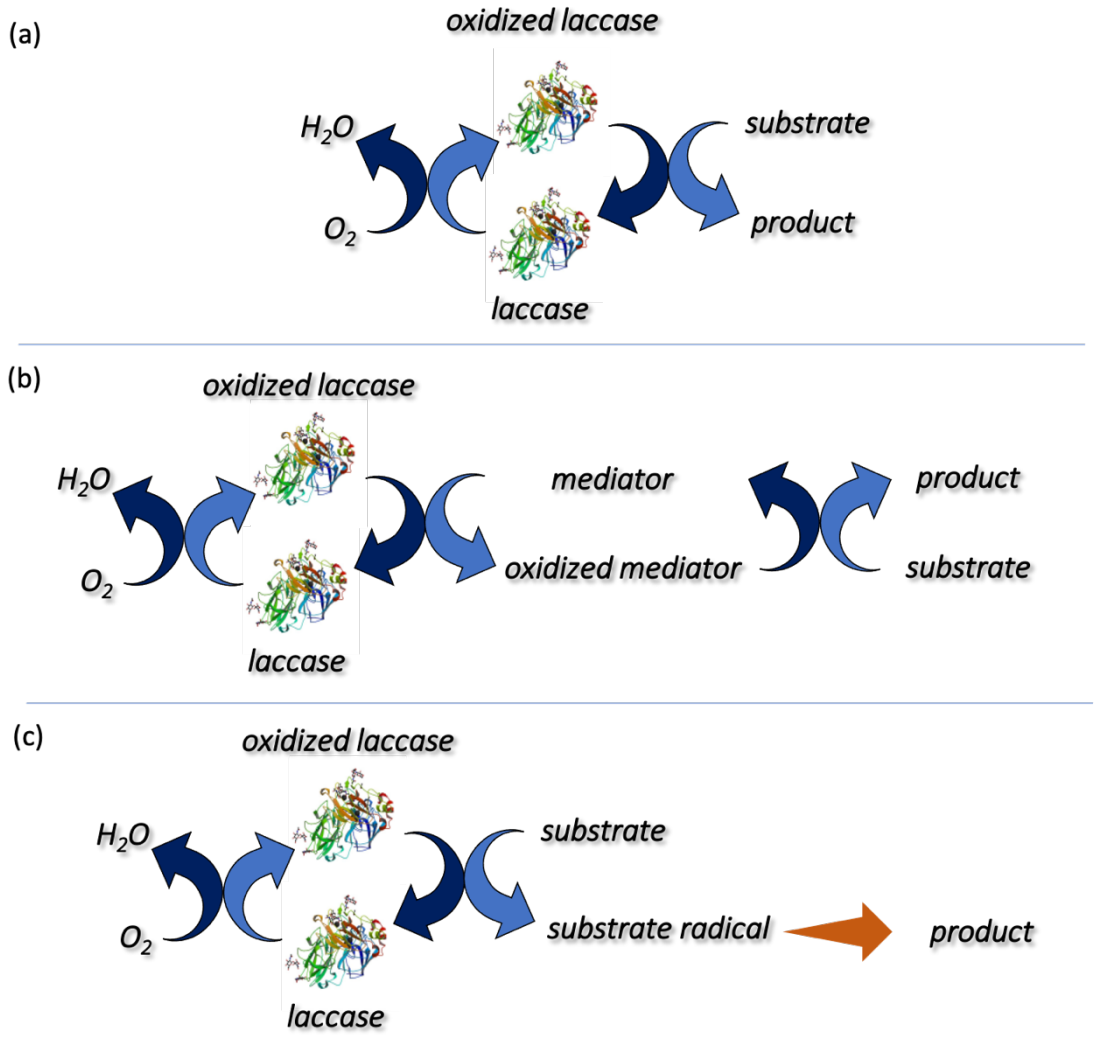


***Figure 4.*** *Schemes of reactions catalyzed by laccase: (a) without the presence of mediator, (b) with the presence of mediator and (c) coupling reaction, based on Polak and Jarosz-Wilkołazka (2007).*

During the reactions presented in Fig. 4(a), small organic compounds such as mono-, di-, polyphenols and their derivatives with methoxy, amine or sulfo groups, which also exhibit a relatively low redox potential, are oxidized directly by laccase. The mechanism of these reactions is based on the hydrogen proton detachment from the hydroxyl group of phenolic compounds and their further conversion to a phenoxy radical. Such radicals are unstable, they

may be oxidized to quinones and subsequently they can undergo polymerization (Claus 2004; Polak and Jarosz-Wilkołazka, 2007).

The next type of reactions catalyzed by laccase must be conducted with the presence of a mediator (see Fig. 4(b)). In contrast to the first type of reactions, big phenolic and non-phenolic molecules with high redox potential, e.g. estrogens, diclofenac and lignin (Baiocco et al., 2003; Lloret et al., 2010), can be oxidized by laccase assisted by low molecular weight compounds. These chemicals, which usually possess functional groups such as nitro or hydroxyimine, play the role of electron transfer agents between the substrate and copper atoms in the structure of laccase. The form of the mediator oxidized by laccase can further oxidize substrates, which are inaccessible for the enzyme due to their size or high redox potential. After that, such a chemical in oxidized form is further reduced to its initial state. The most commonly used mediators are guaiacylglycerol-β-guaiacyl ether (GBG), 1-hydroxybenzotriazole (HBT), hydroxyanthranilic acid (HAA), and 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Polak and Jarosz-Wilkołazka, 2007; Hilgers et al., 2018).

The coupling reactions belong to third group of reactions catalyzed by laccase. The basis of these type of reactions is direct oxygenation of the organic compounds which, in consequence, causes the formation of intermediates in the form of radicals. Due to the fact that the obtained radicals are unstable, they may undergo polymerization to new conjugated structures, such as dimers, oligomers and polymers. Moreover, the obtained new products are the result of coupling reactions between the formed radicals based on bonds such as C–C and C–O, or aromatic amines by N–N and C–N bonds. The coupling reactions can be also divided into two types: (i) homomolecular and (ii) heteromolecular. The first one involves the same type of molecules, such as bisphenols, salicylic ester or tetrahydro-2-naphthol, whereas for the second one, the coupled molecules can be different. The examples include reactions between 3-methyl-2-benzothiazolinic hydrazone and 3-dimethylaminobenzoic acid or dihydroxylated compounds and 4-aminobenzoic acid (Polak and Jarosz-Wilkołazka, 2007). It should be also noted that, compared to the reaction presented in Fig. 4(a), it is possible to design the bonding location of substrates in coupling reactions by selection of process conditions, such as pH, temperature and type of used solvent (Zille et al., 2005).

The next enzyme with high application potential is horseradish peroxidase (EC 1.11.1.7). This biomolecule, with average size of 40 kDa, belongs to the 3rd group of peroxidases, which includes enzymes built of proteins and oligosaccharides. Moreover, horseradish peroxidase possesses two types of metal centers, which are ferroporphyrin (called the heme group) and two calcium ions. The heme group consists of four pyrrole rings connected by methane bridges with iron ions, which are five-coordinated for native counterpart of horseradish peroxidase, whereas the enzyme in the natural form is six-coordinated. Moreover, ferroporphyrin plays a crucial role in enzyme activity, because after oxidation by hydrogen peroxide, Fe(IV) becomes an electron donor for the substrate. Afterwards, cleavage of hydrogen peroxidase to water molecule occurs, whereas oxidized HRP returns to the initial state and, at the same time, new products are formed *via* condensation of aromatic rings (Michon et al., 1997; Azevedo et al., 2003). In case of calcium ions, they are located proximal and distal to the ferroporphyrin and connected to this group by hydrogen bonds. It should be noted that these ions strictly affect the stability of the three-dimensional structure of enzyme. It was shown that calcium in horseradish peroxidase increases the free energy change during reaction to even 16.7 kJ/mol, whereas enzyme without $Ca^{2+}$ allows to obtain only approx. 9.2 kJ/mol of free energy change (Azevedo et al., 2003; Laberge et al., 2003; Veitch, 2004; Ribatti, 2015). Figure 5 presents the scheme of reaction catalyzed by HRP with the presence of $H_2O_2$.

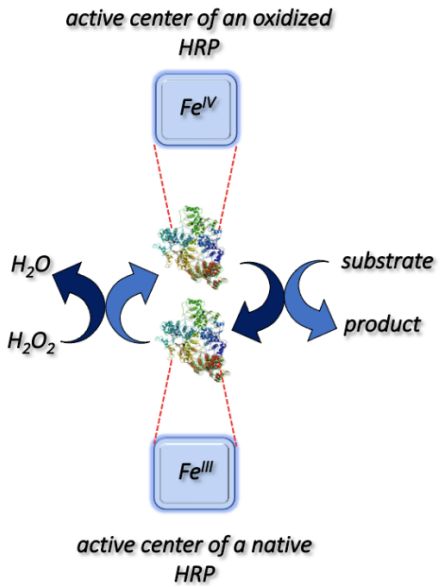


***Figure 5.*** *Scheme of reactions catalyzed by horseradish peroxidase, based on Azevedo et al. (2003).*

Similar to laccase, horseradish peroxidase catalyzes the oxidation reaction of phenolic compounds (e.g. *p*-cresol) and aromatic amines (e.g. *o*-phenylenediamine, *p*-anisidine), but it can also convert iodide. Moreover, this oxidoreductase participates in organic synthesis such as oxidative coupling, selective hydroxylation or *N*- and *O*-dealkylation, which can be used for the production of new compounds with various applications. What is more interesting, this biocatalyst possesses good activity and stability in both aqueous and non-aqueous solutions, which can further expand the range of uses for this enzyme (Dai and Klibanov, 1999). Moreover, compared to other oxidoreductases, horseradish peroxidase is relatively easy to isolate, due to the possibility of its extraction from horseradish roots in a three-step procedure including ultrasonication, precipitation by ammonium sulfate salt and chromatographic separation (Lavery et al., 2010). Although the horseradish peroxidase possesses similar oxidative properties of organic compounds as laccase, the selection of enzymes for specific reaction should be dictated by parameters of conducted process. Compared to laccase, the optimal process conditions of horseradish peroxidase are pH 7 and temperature in the range 25–40 °C (Hermanson, 2013; Alshawafi et al., 2018).

Hitherto many enzymes belonging to the oxidoreductases group have been isolated and characterized in detail in terms of their specific catalytic properties. Moreover, they were classified into subclasses, which are different from each other in terms of the type of reactions catalyzed by these biomolecules or the presence of specific types of donor and acceptor during such catalytic processes. Moreover, the information presented above regarding this group of enzymes allow to state that oxidoreductases possess huge application potential in various areas, especially food industry, paper industry, organic synthesis, pharmacy, or environmental protection. It should be noted that special attention was paid to laccase and horseradish peroxidase, which due to their specific catalytic properties may find application in industrial processes involving oxidation of wide range of organic compounds, such as phenolic, non-phenolic compounds and aromatic amines. The mechanisms of reactions catalyzed by these enzymes were also described and the crucial information regarding them, such as origin and the optimal reaction conditions, were presented. However, one of the limitations of the use of such enzymes in their native forms is the lack of their reusability and inhibition of catalytic activity at harsh reaction conditions, which decidedly increases the costs of conducted processes. The production of biosystems based on these enzymes immobilized

onto support materials can be a potential solution in order to increase the catalytic properties and applicability of these biomolecules in various branches of industry.

## 3.2. Enzyme immobilization – information, properties and challenges

### 3.2.1. Basic information

Enzymes, besides their specific application, which is the possibility to accelerate various reactions, also possess some drawbacks and limitations. The main disadvantages of native enzymes are their low stability and low activity under reaction conditions different than optimal as well as extremely limited reusability. All these factors significantly increase the process costs and hinder the operational capabilities and controllability. There are different ways to overcome these limitations, however enzyme immobilization seems to be the best solution of these problems by increasing of the stability and prolonging activity of biomolecules attached to the support material (Datta et al., 2013). Moreover, it is worth noting that immobilized forms of enzymes exhibit relatively high selectivity and specificity to the substrate, and they can effectively catalyze specific reactions at even harsh conditions (Guzik, 2014). The idea of enzyme immobilization process and advantages of biomolecules after attachment to the support are schematically presented in Figure 6.

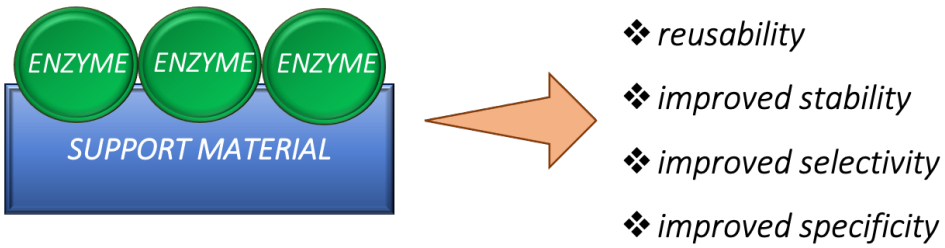


***Figure 6.*** *Idea of enzyme immobilization process and its effect on properties of the immobilized biocatalysts.*

There are many works regarding immobilization of enzymes from various catalytic groups with different applications, however there is still a need to find new supports for enzyme immobilization to establish stable connections between biomolecules and these materials. It should also be noted that enzyme immobilization techniques strictly affect attachment of the enzyme to the support and final properties of the produced biosystems

(Mohamad et al., 2015). Due to these facts, enzyme immobilization processes and application of immobilized biocatalysts arouse much interests of scientists around the world.

## 3.2.2. Support materials

The crucial factors in enzyme immobilization are the selection of support that can affect the properties of immobilized biomolecules and further application of the obtained biocatalytic system. The properties of ideal support material for enzyme immobilization are presented in Figure 7.

The support material should be stable and inert as well as insoluble in the reaction environment, which provides a heterogeneous form of the biocatalyst. This fact, which is most important from an industrial application point of view, allows to reuse the systems with enzyme, as immobilized biocatalysts retain their catalytic properties over several consecutive cycles (Patel et al., 2014; Defaei et al., 2018). It should also be highlighted that for processes at industrial scale, the price of support material might be a barrier in their widespread application in specific enzyme immobilization. Therefore, it is very important to reduce the costs of production and obtaining of such supports (Zdarta et al., 2018b).

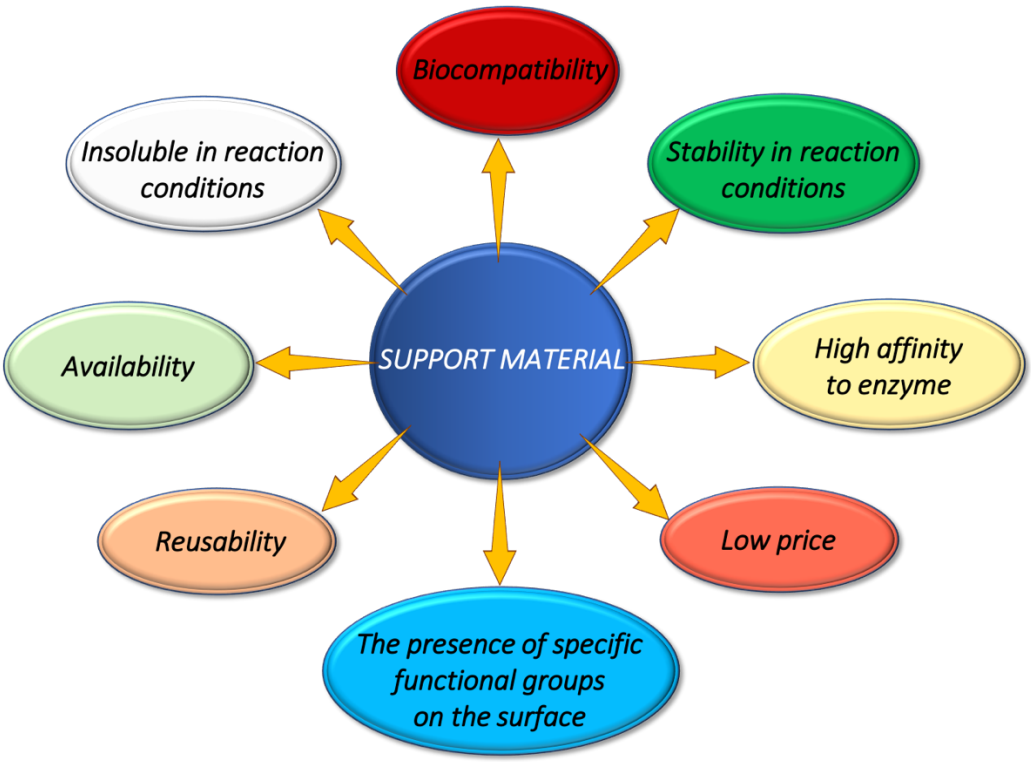


***Figure 7.*** *Properties of ideal material for support in enzyme immobilization.*

The next property, which should characterize materials suitable as support for enzyme immobilization, is high affinity to the attached biomolecule. Usually it is associated with the presence of specific functional groups on the surface of the support, such as hydroxyl, amino or carbonyl groups. These groups possess affinity to the moieties in the structure of the enzyme, which allows to create stable interactions between the support and biomolecule, and to reduce diffusional limitations in the transport of the substrate to the active site of the enzyme. In addition, the support material should be biocompatible with the immobilized protein and facilitate retention of high activity of enzymes even at harsh reaction conditions (Sheldon and van Pelt, 2013). The next important property of support material for enzyme immobilization is its porosity and surface area, appropriate for the size of a given biomolecule. The microporous supports seem to be the most suitable for enzyme immobilization due to a lack of spatial limitations, however the attached biomolecules could be eluted more easily from this type of materials. Therefore, the interests in application of microporous supports for enzyme immobilization is still growing. They may support the conformational stability of the attached protein; however the protein loading could decrease with the decrease of pore diameter. Therefore the selection of proper support with appropriate porosity for the biomolecule is crucial.

The different divisions of support materials for enzyme immobilization are possible in terms of size of porous diameter, their origin, structure or synthesis method (Datta et al., 2013; Jesionowski et al., 2014; Ali et al., 2017; Zdarta et al., 2018a). However, the most common division is based on chemical composition of supports and distinguishes materials of organic and inorganic origin (Figure 8). It should be also noted that interest in application of hybrid and composites materials in enzyme immobilization is still growing. The combining of two of more constituents of various origin in synthesis of support materials for biomolecules strictly affects the final properties of the obtained products. Formed materials may possess the same features as the used components or even additional, not observed for separate constituents. Therefore, such hybrids or composites with immobilized enzymes can be used in a wider range of applications compared to the support made of one component (Zdarta et al., 2018b). The type of materials applied as supports for oxidoreductases immobilization in this doctoral dissertation are marked in green, presented in Fig. 8.

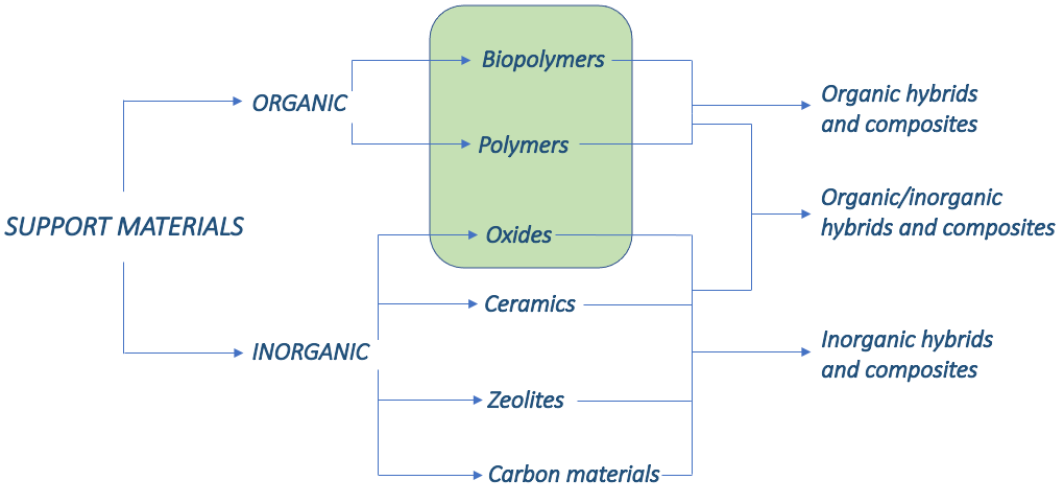


***Figure 8.*** *Division of supports materials for enzyme immobilization, based on Zdarta et al. (2018b).*

The selection of proper support materials depends on their features, which could be crucial for the properties of immobilized enzyme, such as stability and catalytic activity. For example, Pandey et al. (2020) presented the application of carbon material, which was biochar, for enzyme immobilization. This support obtained from waste biomass by pyrolysis process may be predestinated as a support for biomolecule attachment due to its properties such as porosity as well as chemical and thermal stability in reaction conditions. High pore size of this material could allow for diffusion of the enzyme inside the support, which affects effective immobilization, and it may exhibit a protective effect onto the attached biomolecule against harsh reaction conditions. Moreover, the internal structure of biochar provides mass transfer between the enzyme's active center, substrates, products, and environment of reaction. However, the lack of specific functional groups on biochar surface requires its chemical activation. One of the possible ways of chemical surface activation is acidic treatment. It allows to introduce acidic moieties on biochar's surface, such as carbonyl or carboxylic groups that can be compatible with the enzyme's functional groups, which finally increases stabilization of enzyme structure by chemical bonding with support and it could protect of biomolecule against leaching to the reaction medium (Lau et al., 2017).

Zeolites and ceramics are groups of materials that may be considered as supports for enzyme immobilization. They include aluminosilicate materials and materials based on silica and alumina oxides, respectively. Their advantages over other supports are impressive thermal and chemical stability and easy regeneration. Moreover, due to high porous structure

of these materials, it is possible to immobilize enzymes into the pores and, at the same time, retain stability of biomolecules and prevent their leakage from support (Soy et al., 2010; Mulinari et al., 2020). However, as carbon materials, they usually have to be chemically treated by modifiers to increase the compatibility with immobilized enzymes. For example, Mukhopadhyay et al. (2003) modified zeolite Y by (3-aminopropyl)trimethoxysilane (APTES) for introducing amino groups onto the surface of this mineral. Such prepared support was next used for immobilization of pepsin. It was shown that after 4 consecutive catalytic cycles the immobilized pepsin retained 2.5 IU/μg of its catalytic activity. In other work, silicon carbide ceramic powder was treated by APTES and glutaraldehyde. This modification allowed to immobilize alcohol dehydrogenase (ADH) onto this material by covalent binding between the enzyme's amine groups and carbonyl groups present on the prepared support. Moreover, it was shown that after 15 days of storage, the immobilized ADH retained around 40% of its catalytic activity (Sigurdardóttir et al., 2019).

The group of oxide materials as supports for enzyme attachment include e.g. zirconia, titania, silica, alumina and magnetic supports (Zucca and Sanjust, 2014). These materials can be used as supports for biomolecule attachment due to their various properties, such as good mechanical strength and chemical and thermal resistance, however their most important characteristic is high surface area and the presence of hydroxyl groups, through which it is possible to obtain final biocatalytic systems with high value of enzyme loading (Tran and Balkus, 2011). It should be also emphasized that the production of hybrid oxide materials and their application in enzyme immobilization became more popular. The combination of two or more oxides allows to obtain a final material with properties specific for used components. In the work presented by Patel et al. (2017), hybrid material $Fe_3O_4$-graphene oxide was produced by spray pyrolysis process. The obtained system was characterized by magnetic properties, due to the presence of $Fe_3O_4$, and a well-developed surface area of GO nanosheets. Such hybrid material was used for immobilization of two enzymes: laccase and horseradish peroxidase. It was shown that the properties of $Fe_3O_4$-GO support allowed to attach enzymes with high enzyme loading, which was over 300 mg of enzyme per 1 g of support material for both biomolecules tested. It should be highlighted that the magnetic properties of the produced support improve the separation of support with immobilized biomolecules.

The next, very wide groups of potential support materials for enzyme immobilization are polymers and biopolymers. These groups of organic compounds are interesting because they can be easily processing for obtaining various structures, such as foams, scaffolds, hydrogels, membranes or fibers. Therefore, it is easy to apply such supports for enzymes in various types of bioreactors, e.g. continuous stirred tank, bubble column reactors, packed bed or membrane reactors (Spier et al., 2011). The polymeric and biopolymeric supports are also characterized by insolubility in aqueous solutions and relatively good mechanical properties, which enables their reuse with immobilized enzymes even for several consecutive catalytic cycles without losing the stability of these materials. Moreover, they possess various functional groups, such as carbonyl, hydroxyl or amine groups, which allow effective enzyme immobilization on their surface (Bezerra et al., 2015; Nyari et al., 2016; Rodriguez-Abetxuko et al., 2020). The big advantage of biopolymers is their omnipresence; they are components of plant cells (lignin, cellulose, pectin, agar) or fungi or arthropods cells (chitin) (Bilal and Iqbal, 2019). In the work presented by Gomes et al. (2004), natural polymer chitin was applied for immobilization of lipase from *Candida rugosa*. The presence of numerous amino groups in its structure provides binding sites for enzymes, which allowed to immobilize over 250 U of lipase per 1 g of chitin. In case of synthetic polymers, it is possible to design their properties in terms of application in enzyme immobilization by building a polymeric chain from selected monomers and selecting the type of polymerization (Hanefeld et al., 2009; Fang et al., 2011). Moreover, synthetic polymers can be processed in a variety of ways, obtaining various forms, e.g. pellets, fibers or films. As was shown by Li et al. (2010), the polymeric films made of polyethylene (PE), polypropylene (PP) and poly(ethylene terephthalate) (PET) were applied as supports for β-glucosidase. It was shown that the enzyme loading on the prepared supports was equal to 26.4 U/mg, 39.8 U/mg and 41.4 U/mg, respectively.

The important aspects of application of polymers or biopolymers as supports for enzyme immobilization is the possibility of producing hybrids or composites composed of two or more constituents. Such connection of polymers (or biopolymers) allows to obtain materials which combine the properties of used precursors. The interesting polymeric hybrids and composites can be obtained by electrospinning. This technique is rapidly developing as a simple process in which careful control of operating conditions, machine parameters and

polymer solution properties enables the production of highly porous structures of smooth non-woven fibers (Bhardwaj and Kundu, 2010; Ahmed et al., 2015). This production method of support materials allows to fabricate hybrids by dissolving polymers (or biopolymers) in the same type of solvent and next electrospinning of the obtained solution, giving uniform fibers. Moreover, it is also possible to produce the composite by core-shell method using coaxial needle for simultaneous spinning of solutions immiscible with each other (Kijeńska and Swieszkowski, 2017). Due to the possibility of obtaining fully designed properties of electrospun fibers, they are alternatives for other support materials used for enzyme immobilization by various methods including adsorption, covalent binding, and encapsulation. In the work presented by Arecchi et al. (2010), electrospun fibers made of nylon 6 were applied as supports for glucose oxidase immobilization by adsorption. It was shown that due to high porosity of the produced electrospun material, which was equal to 92.5%, it was possible to attach even $2.1x10^5$ enzyme molecules. In case of covalent binding, Ramon-Marquez et al. (2018) immobilized uricase from *Candida* sp. onto fibers made of two components: NanoMyP®-poly(methyl methacrylate) (PMMA) by covalent binding and next used them for production of uric acid biosensor. In this study, the electrospun material was obtained by coaxial spinning: inner fibers from PMMA and outer made of NanoMyP®. It was shown that these fibers had a different impact on the final properties of electrospun material: inner fibers possessed oxygen sensitive dye, which can increase the sensitivity of the produced biosensor, and outer fibers with aldehyde groups, which allowed to modify the support by ethylenediamine and glutaraldehyde and finally, effective uricase immobilization onto its surface by covalent binding. The promising method of enzyme immobilization using electrospun fibers is encapsulation. In work presented by Sakai et al. (2010) lipase from *Rhizopus oryzae* was encapsulated into polystyrene electrospun fibers from a direct suspension in *N,N*-dimethylformamide solution. It was shown that the transesterification rate measured by conversion of (*S*)-glycidol to glycidyl n-butyrate was 47-fold faster, compared to the free form of this enzyme. However, although the encapsulated enzymes retain their activity, limitation in mass transfer between the immobilized enzyme and the substrates may occur and therefore this method needs to be continuously improved.

## 3.2.3. Immobilization methods

Aside from the selection of support material for enzyme attachment, the method of biomolecule immobilization is also a key issue. The proposed division of enzyme immobilization methods is presented in Figure 9.

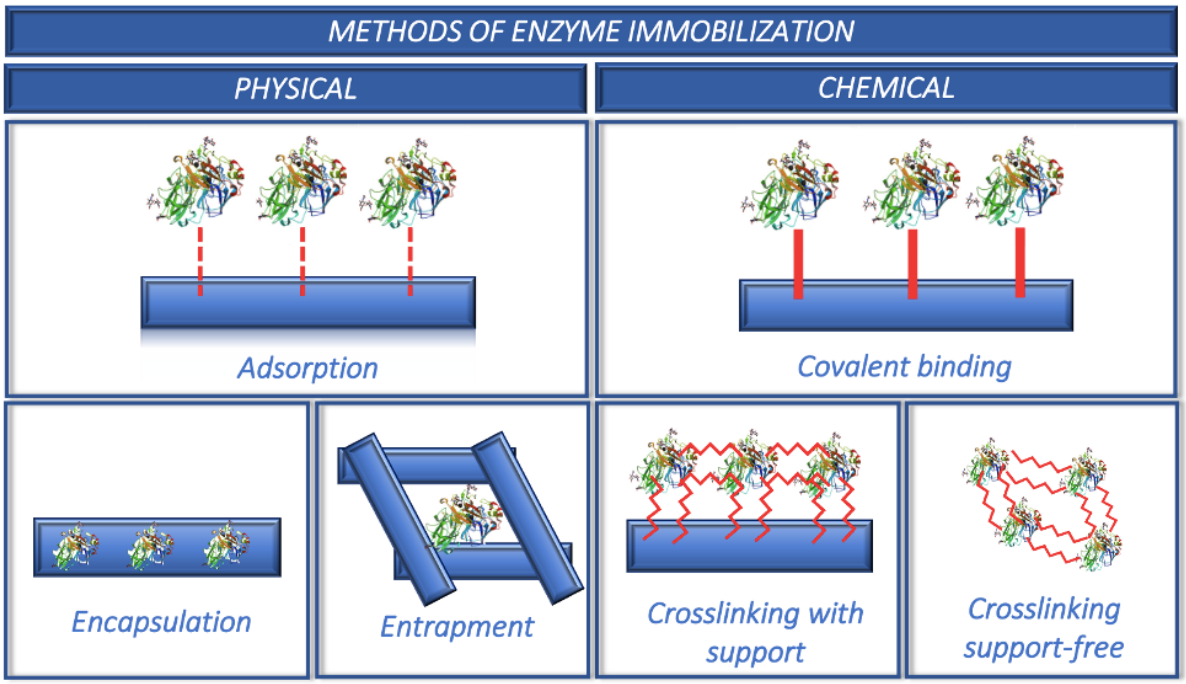


***Figure 9.*** *General division of methods of enzyme immobilization, based on Mulinari et al. (2020).*

The type of connection between the biocatalyst and support material, which could be both physical or chemical, strictly affects the stability and activity of enzymes. However, it is also correlated with type and properties of used support, such as the presence of functional groups and porosity of material. It should also be stated that enzymes can be attached to the support in various places, depending on the immobilization methods. For example, methods such as adsorption, covalent binding and crosslinking between the biomolecule and solid material consist of attaching the enzyme onto the outer surface of support, whereas in case of encapsulation and entrapment the protein is placed into a support material. Apart from these locations, the enzyme can be immobilized without the presence of support by direct crosslinking between proteins (Zdarta et al., 2018b; Zdarta et al., 2019; Mulinari et al., 2020).

The most commonly used enzyme immobilization technique is adsorption immobilization. The process of enzyme attachment by adsorption is relatively easy to carry out and low-cost due to the lack of additional chemicals, besides the enzyme and support. It should be noted that the connection between enzyme and support material is made by weak bonds, such as hydrogens bonds, van der Waals forces or electrostatic interactions (Jesionowski et al., 2014). Therefore, the applied support materials should be characterized by the presence of functional groups, e.g. hydroxyl or amine groups, which could form these types of bonds with enzyme. Moreover, properties of supports such as porosity and well-developed surface area are also highly desirable because they can provide additional protection of protein against harsh reaction conditions. Even though enzymes immobilized by adsorption can be easily released from the material, limited interference in the structure of biomolecule occurred, leading to the retention of high activity by the immobilized biocatalyst (Ali et al., 2017). Moreover, the materials used as supports for enzyme immobilization by adsorption can be reused after enzyme elution in terms of re-immobilization (Zdarta et al., 2018b). Recently, Machado et al. (2019) used rice husk silica, a by-product of rice production, for immobilization of lipase *via* adsorption. The type of bonds between the enzyme and silica were proposed, which were ionic and hydrophobic interactions. In another work, Zdarta et al. (2015) applied a novel hybrid system made of chitin and lignin for attachment of lipase from *Aspergillus niger*. It was shown that the systems with adsorbed enzyme retained 80% of their catalytic activity after 20 consecutive catalytic cycles. In a different work, Rueda et al. (2016) stated that all lipase biomolecules immobilized by adsorption onto octyl supports can be released from the material by the presence of surfactant Triton X-100 in the environment of the reaction, which indicates the weak attachment between support and biomolecule. This fact allows to state that such octyl supports could be reused for immobilization of biomolecules.

Among the immobilization methods, entrapment and encapsulation deserve special attention. The first one is based on attachment of the biomolecule inside the pores or between layers of support material, whereas encapsulation consists of enclosing the enzyme by a semi permeable cover made of various material with both organic and inorganic nature. The most significant limitation of both methods may be the reduction of mass transfer between the substrate and active sites of immobilized enzymes due to the presence of steric

hindrances (Datta et al., 2013). The crucial property of support materials in terms of enzyme entrapment is porosity. Therefore, materials with well-developed surface area, such as oxide systems, gels, polymers, minerals, and ceramics, are attractive supports applied for this type of protein immobilization. It should be also noted that sorption capacity and variable pore size could be designed for specific types and sizes of enzymes, to increase their immobilization onto the support material (Ispas et al., 2009). Mesoporous silica can be an example of such materials. Its properties, such as high adsorption capacity and uniform pore distribution, were the motivation for its application as support for laccase immobilization by entrapment. In the work presented by Mansor et al. (2016) it was shown that laccase retained over 90% of its initial activity after 10 catalytic cycles, even in the presence of mass transfer limitation. In case of encapsulation, the important parameter of permeable cover for enzyme should be also porosity, however the size of pores should be adjusted to the sizes of biomolecules, substrate and products. It can prevent enzyme leakage and, at the same time, allow transport of substrates and products between process environment and the inside of the material with the immobilized biomolecule (Nguyen and Kim, 2017). Especially, the application of electrospun fibers made of polymers (or biopolymers) seem to be a future-proof way for production of system with enzymes encapsulated into the fibers. In the work presented by Dai and co-workers (2016), laccase encapsulated into composite fibers made of poly(D,L-lactide-*co*-glycolide)/multiwalled carbon nanotubes allowed to degrade over 90% of bisphenol A even after 10 catalytic cycles. This was caused by a protective effect of fibers on the immobilized biomolecule, which retained laccase activity and limited the leakage from fibers. However, there is still a need to develop and explore this method of biomolecule immobilization in order to obtain a balance of mass transfer between substrate and products of catalytic reaction.

In addition to adsorption, encapsulation and entrapment, which belong to the physical methods of enzyme immobilization, attention should also be paid to chemical ways of biomolecule attachment. One of them is covalent binding. In this method, the selection of proper support is needed, mainly in terms of the presence of specific functional groups, compatible with biomolecule. In this case, the most commonly used supports are biopolymers and polymers, which possess moieties such as amine and carbonyl groups that allow to form strong covalent bonds with biomolecule. However, sometimes the support material does not

have specific functional groups, capable of forming covalent bonds between the support's surface and enzyme, but it still might be used in enzyme immobilization due to other properties, such as stability under reaction conditions. In this case, to allow the formation of interactions between the immobilized enzyme and support materials, various support modifiers are used, such as 3-(trimethoxysilyl)propyl methacrylate (Hosseini et al., 2018), glutaraldehyde (Vazquez-Ortega et al., 2018), polyethyleneimine (Rios et al., 2019), *N*-hydroxysuccinimide (NHS) in the presence of a peptide-coupling agent *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide (EDC) (Khaldi et al., 2018), (3-aminopropyl)triethoxysilane (Gunda et al., 2014) and polydopamine (Sureshkumar and Lee, 2011). For example, bacterial cellulose (BC) fibers, characterized by high surface-to-volume ratio and a 3-D structure, were used as supports for horseradish peroxidase immobilization. However, it was necessary to modify this material by glutaraldehyde. It is caused by the fact that although BC surface is rich in functional groups, hydroxyl groups are characterized by low reactivity in glucose units (Yu et al., 2019). Bilal et al. (2019a) applied agarose-chitosan hydrogel for immobilization of horseradish peroxidase using *N*-hydroxysuccinimide as a crosslinker. It was shown that over 90% immobilization efficiency was obtained after using this eco-friendly support, which shows the necessity of application of a crosslinking agent for effective enzyme immobilization. As was presented by Simón-Herrero et al. (2019), enzyme immobilization by covalent binding improves the binding efficiency, compared to the adsorption method. Laccase immobilized onto polyimide aerogels modified by glutaraldehyde possessed 8.0 U/g of its activity, whereas laccase attached by adsorption had 5.9 U/g activity, which decidedly shows the advantage of covalent binding over physical adsorption. It should also be noted that the formed covalent bonds directly interfere with protein structure causing improvement of its stiffening and, in consequence, enhancement of stability of the immobilized enzyme. However, changing of biomolecule structure by forming covalent bonds can cause a decrease of enzyme's activity compared to the native form of protein.

Crosslinking is the next method of enzyme immobilization that consists of production of heterogeneous biocatalysts by intermolecular crosslinking of enzymatic biomolecules. This way of enzyme binding can be carried out by two ways. The first technique is crosslinking without a support material, which is based on interactions only between enzymes.

In this case bifunctional organic compounds are used, such as hexamethylene diisocyanate or glutaraldehyde (Sheldon et al., 2009; Datta et al., 2013). Moreover, the properties of the final products depend on form of used protein. For example, before crosslinking protein aggregates can be precipitated using solutions of inorganic salts or organic solvents. The obtained enzyme aggregates are then crosslinked using bifunctional organic compounds and the final biocatalysts are formed, which are named crosslinked enzyme aggregates (CLEAs). The next forms of the enzyme, which might be formed in immobilization by crosslinking, are protein crystals. The crosslinked enzyme crystals (CLECs) are characterized by high resistance to denaturing agents; however, the obtaining of these systems is relatively expensive due to the need to crystallize native forms of enzymes prior to immobilization. Both CLEAs and CLECs biosystems are characterized by stable structures of biomolecules without using a support. However, the use of expensive and harmful chemicals as bifunctional agents in their production may negatively affect the activity of final enzymatic products (Talekar et al., 2013). The alternative way for intermolecular crosslinking of enzymes is a simultaneous crosslinking of enzyme particles with each other and between the support and the biomolecule. Due to the presence of strong interactions, stability of the enzyme under harsh reaction conditions and even after several catalytic cycles might be improved, compared to the crosslinked enzymes without additional support. The example of production of a biocatalytic system with crosslinked enzymes was presented by Lee et al. (2021). They used $CaCO_3$ support and crosslinked carboxyl esterase (CE) from *Rhizopus oryzae* by glutaraldehyde on its surface. It was concluded that due to the fact that 30% of initial activity of CE was retained after 180 days of storage at room temperature, the stability of biomolecules was improved, compared to the native form of this enzyme. Moreover, the elution of the biomolecules from support is highly limited, compared to the adsorption or covalent binding methods due to the synergistic effect of both connections of enzyme with support and between the enzymes molecules. However, this multipoint enzyme attachment can cause a decrease of biocatalyst activity by changing the enzyme structure and mass transport limitations (Chen et al., 2013).

Due to specific catalytic properties of enzymes, they found application in various branches of industry. However, their native forms might not be efficient tools for biotechnological processes, which is caused by the lack of possibility of their reuse and low

stability at changing reaction conditions. Therefore, enzyme immobilization processes can be a solution of these problems. In this Section, information regarding enzyme attachment to the supports, especially advantages of application of immobilized biomolecules over their free counterparts in various catalytic reactions, were presented. Moreover, it was shown that it is possible to design the properties of biocatalysts by selecting suitable support material and type of enzyme immobilization. In case of supports for biomolecule attachment, they should allow to immobilize sufficient amount of enzymes to obtain a final biocatalyst with high catalytic activity. Moreover, the properties of these support materials, such as porosity and the presence of specific functional groups, should strictly affect the stabilization of enzyme's structure, therefore the selection of support for enzyme immobilization is crucial in terms of final properties of the obtained heterogeneous biocatalyst. Currently, various groups of materials are applied as supports for enzyme immobilization, such as biopolymers, polymers, oxides, ceramics, zeolites or carbon materials and composites and hybrids materials. It was also shown that the important issue in enzyme immobilization process is the selection of immobilization method. The most commonly known methods are adsorption, covalent binding, encapsulation, entrapment or crosslinking, which is reflected by numerous scientific publications. The proper support and methods of enzyme attachment allow not only to retain high activity of the immobilized biomolecule and to increase of its reusability and stability under harsh reaction conditions, but also to facilitate the use of the obtained biocatalysts at various reaction environments and decrease the reaction costs. However, there is still a need to produce new support materials and create novel and stable connections between such supports and immobilized enzymes, which is caused by changing requirements regarding the conditions for conducting and optimization of technological processes.

## 3.3. Immobilized oxidoreductases as a tool for dye decolorization process

Dyes are compounds widely used in various industries, such as paper, automotive and textile industry. Due to the high consumption of this type of chemicals in such areas of industry, they are present in industrial wastewaters at high concentrations. Unfortunately, dyes from these wastewaters could get into surface waters, polluting whole ecosystems.

Most of dyes, even in trace amounts, negatively affect human health and aquatic systems. Therefore, the removal of these compounds from aqueous solutions should be highly effective. The negative impact of dyes on human health and the environment is presented in Figure 10.

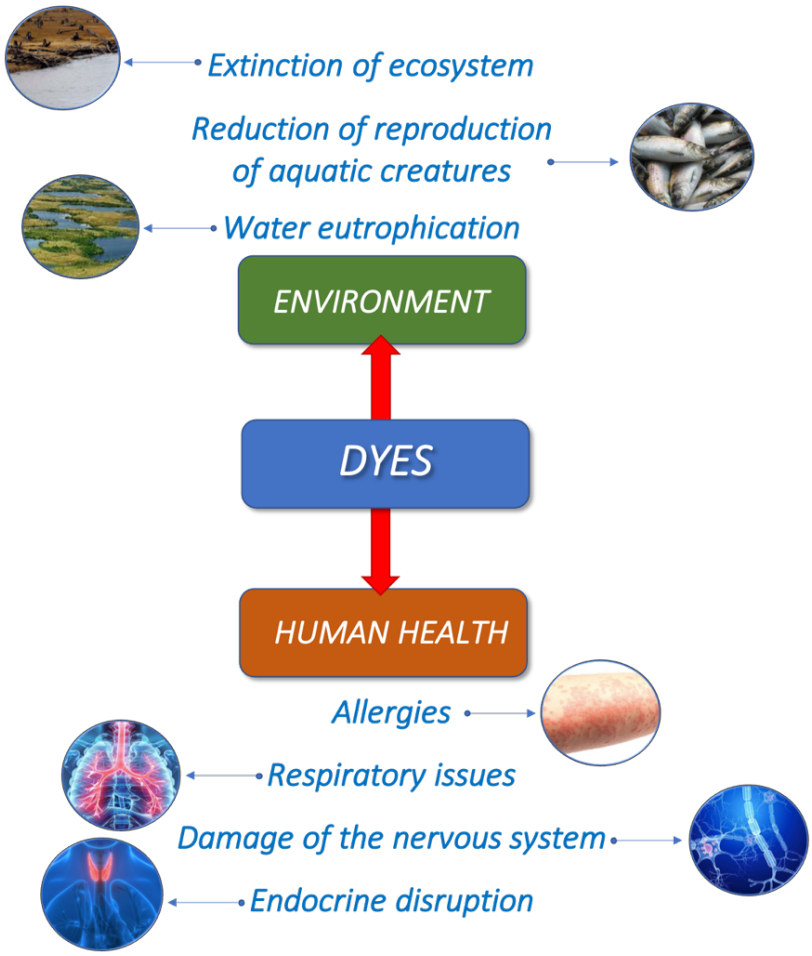


***Figure 10.*** *Effect of dyes on human organisms and the environment.*

In case of the effect of dyes on environment, it should be noted that even small amounts of these compounds in rivers, lakes, seas and other surface waters may lead to the extinction of whole aquatic systems. This is mainly caused by the high toxicity of selected dyes. Dyes, especially azo dyes (i.e. C.I. Reactive Black 5), after entering to the aquatic bodies, cause DNA damage. Moreover, due to high photo, thermal and chemical stability, some dyes are resistant to biodegradation or even decay with sunlight. Furthermore, dyes present in waters can inhibit sunlight transmission, which in consequence causes inhibition

of photosynthesis and production of molecular oxygen, essential for life of aquatic organisms (Gita el al., 2017).

Dyes also have a negative impact on human health. The most commonly used dyes in textile industry are azo dyes, such as C.I. Reactive Black 5. They can be transformed by azo reduction to benzidine by human liver azoreductase, skin and human intestinal microflora (Chung et al., 1992). It was shown that this cleavage product of C.I. Reactive Black 5 is cancerogenic and can cause allergies and skin irritation (Chung 2016). The next group of dyes commonly used in textile industry is anthraquinone dyes (Costa et al., 2012). As was presented by Leme et al. (2015), the anthraquinone dye C.I. Reactive Blue 19 was investigated in terms of its potential negative effect on human cells. The mutagenicity of RB19 was detected through base pair substitution (Ames test). Triarylmethane dyes are also hazardous compounds for humans. Biberoglu et al. (2011) presented a significant inhibition of enzyme butyrylcholinesterase by C.I. Basic Green 4. This enzyme is responsible for regulating the activity of acetylcholine – a neurotransmitter in the central nervous system (Ilyushin et al., 2013). Moreover, this type of dyes can strictly affect the endocrine system. Jiao et al. (2008) showed that C.I. Basic Green 4 prevents thyroid hormone synthesis by exhibits anti-estrogenic activity.

Due to many scientific reports regarding the negative effect of dyes, mainly belonging to azo, anthraquinone and triarylmethane groups, the selected dyes from these groups were used in studies carried out as part of the presented doctoral dissertation (see Figure 11).

Although methods such as adsorption, ozonation or photocatalysis are widely used in dyes degradation, they generate high amounts of harmful waste. For example, after adsorption of pollutants onto the adsorbent, the used adsorbents need to be disposed (Sadegh and Ali, 2018). Moreover, the biggest limitation of ozonation and photocatalysis is the occurring of products and byproducts characterized by high toxicity. Moreover, hazardous chemicals are used in the production of photocatalysts, which generate a secondary disposal problem (Mozia, 2010). It should be also noted that methods such as ozonation or photocatalysis need high energy and money input for degradation processes, therefore they become less attractive for use at the industrial scale (Ascha et al., 2015; Mecha and Chollom, 2020). It was also observed that some of dyes are resistant to these treatment techniques because of their complex structures and the presence of other

chemicals in aqueous solutions, which interfere with the degradation of dyes (Khataee and Kasiri, 2010; Tehrani-Bagha et al., 2010).

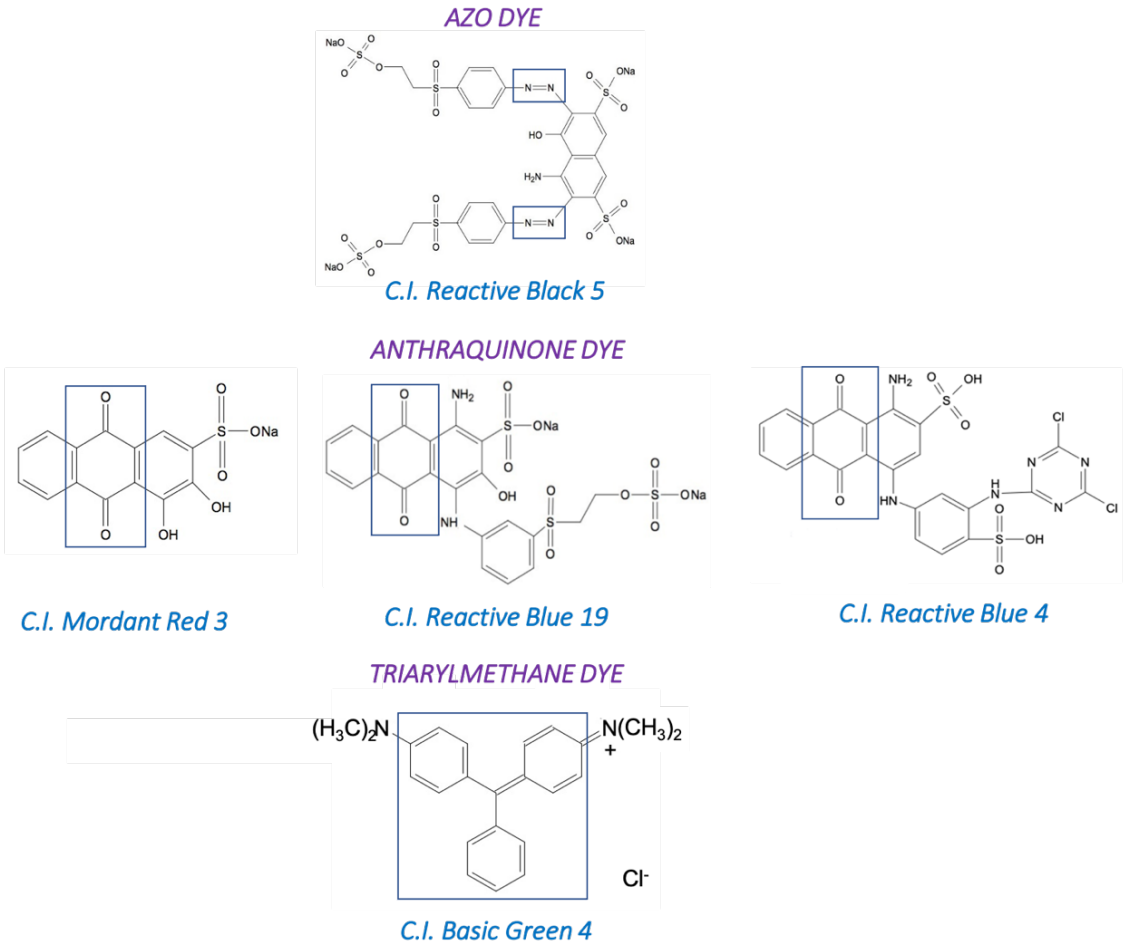


***Figure 11.*** *Dye groups most commonly applied in the industry with dyes examples and chromophores marked in their structures.*

The biocatalysis reactions catalyzed by oxidoreductases, such as laccases and peroxidases, have enormous application potential in environmental protection in terms of removal of hazardous compounds, especially dyes, from wastewaters and surface waters. This is mainly caused by the fact that they allow to degrade dyes at high efficiencies, compared to the other removal methods. It should be also noted that products of enzymatic conversion of dyes are usually characterized by lower toxicity, compared to the products obtained after dye degradation using e.g. ozonation or photocatalysis (Wang et al., 2003; Chen, 2009). These biocatalysts are also biodegradable, which results in limited second disposal problem. Moreover, the number of scientific reports on this subject is growing every

year, indicating the high interest of the scientific society in this research area, and showing the great need to produce systems with oxidoreductases, enabling effective degradation of dyes from the environment. For example, Blanquez et al. (2019) produced laccase from *Streptomyces ipomoea* and applied it for decolorization of azo dye C.I. Reactive Black 5. It was shown that decolorization efficiencies of this compound reached over 90%. In another work, two-chambered microbial fuel cell with *Pleurotus ostreatus* URM 4809, fungus producing laccase, degraded over 86% of C.I. Reactive Blue 19 dye from domestic wastewater (Simoes et al., 2019). Yuan et al. (2021) used *Antrodia* P5 white rot fungus for decolorization of C.I. Reactive Blue 4. It was proven that enzymes produced by this type of fungus, such as lignin peroxidase, manganese peroxidase and laccase, were responsible for removal of RB4 with efficiency equal to 96%.

Native oxidoreductases or whole microorganisms producing these enzymes may degrade dyes with satisfactory efficiency. However, the main disadvantages of these free enzymes are the lack of reusability and low activity under harsh reaction conditions. In the work presented by Nejad et al. (2019), laccase produced by *Phanerochaete chrysosporium* possessed the highest catalytic activity at pH 4, whereas at pH higher than 7 the catalytic activity decreased to 20%. Huang et al. (2020) used laccase from *Trametes hirsuta* MX2 for removal of dyes such as C.I. Reactive Blue 19, C.I. Acid Red 1, C.I. Basic Violet 3 and C.I. Basic Red. It was shown that laccase drastically lost its catalytic activity at pH higher than 2.5. In case of temperature stability, after 30 min of process at 70 °C, laccase completely lost its activity. It should be noted that wastewaters possess various pH and temperatures values, therefore native forms of oxidoreductases, which can lose their activity under harsh process conditions, may be unprofitable for removal of dyes from wastewaters. Immobilized oxidoreductases can be a solution of these problems. Therefore, in various publications many researchers decided to investigate the application of immobilized oxidoreductases in removal of specific dyes from aqueous solutions. Table 2 presents examples of immobilized oxidoreductases used for removal of dyes from aqueous solutions.

It can be seen that various support materials and methods of oxidoreductases immobilization were applied, which is the cause of differences among the produced biocatalysts in terms of final properties. In the work presented by Arslan (2011), poly(ethylene terephthalate) (PET) in form of fibers, was used as support for horseradish

peroxidase immobilization, and next in decolorization process of C.I. Acid Orange 52. The selection of this support was dictated by its properties, such as high surface area and resistance to high temperature and pH conditions. However, in order to introduce reactive functional groups onto the produced support material for efficient enzyme immobilization, it was necessary to modify PET using glutaraldehyde. It was shown that using this biosystem with immobilized HRP allowed to decolorize of AO52 with high efficiency equal to 98%.

***Table 2.*** *Biocatalysts made of laccases or horseradish peroxidases immobilized onto supports with various origin, applied in removal of selected dyes.*

| Enzyme | Support | Type of enzyme immobilization | Dye | Removal efficiency | Literature |
|---|---|---|---|---|---|
| Laccase from *Trametes versicolor* | Polymeric ionic liquid membrane | Entrapment | RB19 | 75% | (HajKacem et al., 2020) |
| Laccase from *Boletus edulis* | Rice husk | Adsorption | RB19 | 91% | (Tuncay and Yagar, 2020) |
| Laccase from *Trametes versicolor* | Laccase-$Cu_3(PO_4)_2 \cdot 3H_2O$ hybrid nanoflower | Crosslinking | AB83<br>AB147 | 73%<br>73% | (Patel et al., 2018) |
| Laccase from *Myceliophthora thermophila* | Epoxy-functionalized silica | Covalent binding | AB83<br>BV3 | 97%<br>78% | (Salami et al., 2018) |
| Laccase from *Trametes pubescens* | Chitosan beads | Entrapment | BR5<br>DR28<br>AG1 | 37%<br>25%<br>20% | (Zheng et al., 2016) |
| Peroxidase from horseradish | Poly(ethylene terephthalate) | Covalent binding | AO52 | 98% | (Arslan, 2011) |
| Peroxidase from horseradish | Calcium alginate gel beads | Encapsulation | AO7<br>AB25 | 75%<br>84% | (Gholami-Borujeni et al., 2011) |
| Peroxidase from horseradish | Kaolin | Adsorption | AV109 | 92% | (Sekulijca et al., 2016) |
| Peroxidase from horseradish | Calcium alginate | Crosslinking | RR120<br>RB4<br>R016 | 72%<br>87%<br>80% | (Bilal et al., 2016) |
| Peroxidase from horseradish | Calcium alginate beads | Encapsulation | RB221<br>RB198 | 93%<br>75% | (Farias et al., 2017) |

An interesting biocatalyst was presented by Bilal et al. (2016), who immobilized horseradish peroxidase by crosslinking onto sodium alginate. Due to the beads form of this biosystem, it was possible to place it in a packed bed bioreactor. What is more important,

continuous flow of dye solutions through the column with immobilized HRP allowed to remove of azo dyes C.I. Reactive Red 120, C.I. Reactive Orange 16 and anthraquinone dye C.I. Reactive Blue 4 with efficiencies equal to 72%, 80% and 87%, respectively. This work can be compared with other, presented by Gholami-Borujeni et al. (2011). They also encapsulated horseradish peroxidase in sodium alginate and applied this system in the decolorization process of azo dyes, C.I. Acid Orange 7 and C.I. Acid Blue 25 using a stirred batch reactor. It was shown that despite the fact that immobilized HRP possessed higher activity compared to the native counterpart, the relative activity was below 50% at acidic pH and temperatures above 70 °C. Moreover, the reusability drastically decreased after 4 consecutive catalytic cycles. However, the degradation efficiencies of AO7 and AB25 were relatively high, at 75% and 84%, respectively. Comparing these results with the work presented by Bilal et al. (2016), it can be seen that although different azo dyes were used, their degradation efficiencies were similar, which confirms effective decolorization of this type of dyes from aqueous solutions using encapsulated HRP. However, inaccurately selected supports can negatively affect the activity of produced biosystem at various process conditions. In the work presented by Zheng et al. (2016), laccase was entrapped into chitosan beads and used for decolorization of dyes, e.g. C.I. Basic Red 5, C.I. Direct Red 28 and C.I. Acid Green 1. The low decolorization efficiencies, which were below 40%, were probably caused by mass transport limitation in dye molecules diffusion to the active site of the immobilized enzymes. In another work, Tuncay and Yagar (2020) applied laccase from *Boletus edulis* immobilized onto the rice husk, and next, used it as a biocatalyst for removal of anthraquinone dye C.I. Reactive Blue 19 from aqueous solutions. It was shown that system with attached enzyme degraded over 90% of RB19 from the solution at pH 3.5, at 25 °C within 1 h. Despite the good efficiency of dye decolorization at specific conditions, the obtained biosystem possessed lower activity in the presence of $Fe^{2+}$ and Tween 80 in the reaction solution, which shows a decrease of enzyme stabilization in the presence of metal ions or surfactants in reaction solutions. In the work presented by Rani et al. (2017) laccase immobilized by adsorption onto ZnO and $MnO_2$ particles was used for decolorization of C.I. Mordant Red 3. The effect of various process parameters, such as time, pH, and amount of enzyme, was investigated. It was shown that the highest decolorization efficiency was obtained after the use of ZnO-laccase system for 120 min, at pH 7 and 50 mg of immobilized enzyme, and reached approx. 90%. However, it was also

confirmed that the dye degradation efficiency varied depending on process conditions; at acidic pH efficiencies decreased to 60%.

The published studies shown that the degradation of two groups of dyes, azo and anthraquinone, by immobilized oxidoreductases is most often investigated. This is caused by the fact that these groups of dyes are the most commonly used chemicals in textile industry and they are present in wastewaters (Costa et al., 2012). Therefore, besides the effect of type of support material, enzyme immobilization method and process conditions, it is important to consider the type of dye in its removal process. Moreover, it should be stated that the obtained decolorization efficiencies of dyes after application of systems with immobilized laccase or horseradish peroxidase usually differ from each other, which is mainly due to different mechanisms of dyes conversion by those biomolecules.

It should be noted that chemical and thermal stability of systems with immobilized enzymes are important for application in wastewater treatment. The qualitative composition and temperature of wastewaters may vary and depend on the type of processes in treatment plants. Morshed et al. (2016) investigated the physicochemical properties of textile wastewaters and it was shown that their pH was above 7, and even close to 12. In case of temperature, the optimal conditions of textile dyeing are between 50–90 °C (Zahid et al., 2017).

The presented Chapter highlights the negative impact of dyes on environment and human health with the special attention focused on azo, anthraquinone and triarylmethane dyes. Due to the fact that these dyes are widely used in textile industry, they are present in wastewaters and can even enter water reservoirs, which may result in the extinction of ecosystems and human health problems. Various methods of dyes degradation such as adsorption or ozonation are applied, however they possess many limitations, which affect final decolorization efficiencies of selected pollutants. Therefore, the application of biocatalytic systems with immobilized oxidoreductases, especially laccase and horseradish peroxidase, can be a future-proof way of dyes decolorization from aqueous solutions. As was presented in Table 2, many investigations of using immobilized oxidoreductases in the removal of dyes from aqueous solutions were conducted. However, in order to increase the degradation efficiencies of specific dyes by enzymes attached onto support materials, new biosystems characterized high catalytic activity and stability must be sought. Based on the

information provided above, it is crucial to produce systems made of immobilized oxidoreductases, characterized by high chemical and thermal stability. Moreover, these biocatalysts should be characterized by high efficiency in dyes decolorization process.

# 4. Motivation and aim of the work

The main research objective of the presented work was the development and production of new, effective environmental tools based on oxide materials or electrospun fibers and immobilized oxidoreductases, and their application for decolorization of dyes.

The main assumption of works within the framework of the PhD thesis was based on the hypothesis that oxidoreductases, such as laccase and horseradish peroxidase, immobilized onto oxide systems and/or electrospun fibers possess ability to remove environmental pollutants, such as dyes, from aqueous solutions. Moreover, it should be noted that the undertaken research topic is important in terms of the still increasing need to search for new, eco-friendly and effective systems that facilitate the removal of hazardous compounds, the presence of which in waters is one of the biggest problems and challenges for environmental protection. The main stages of the presented work are summarized in Figure 12.

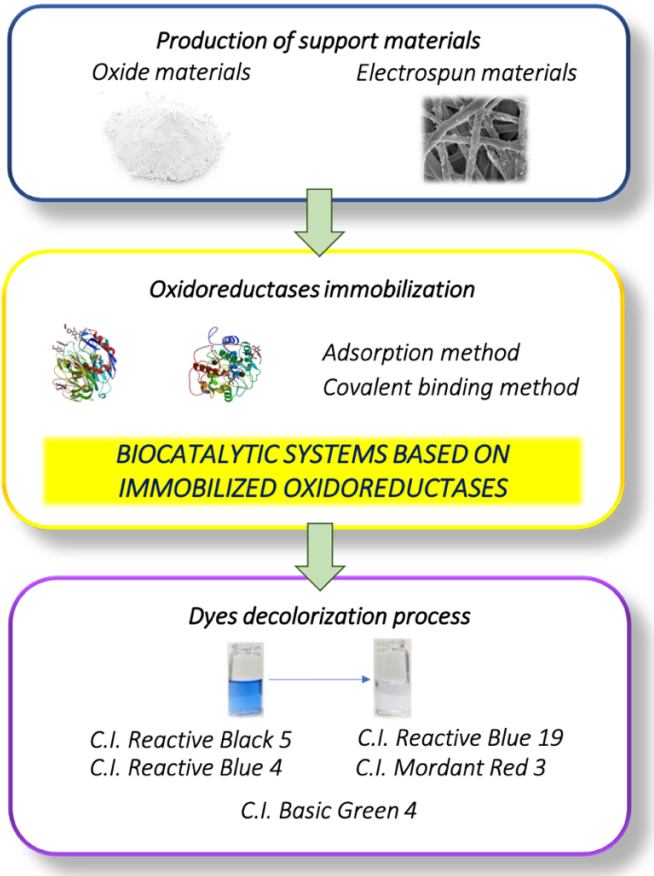


***Figure 12.*** *Schematic representation of the aim of the presented research and main research steps.*

To solve the above-mentioned problem and to achieve the assumed goal of the PhD study, numerous studies were carried out, which were divided into several more detailed stages, each containing detailed research tasks:

1. Synthesis of support materials made of hybrid oxide systems and/or electrospun materials;
2. Characterization of the obtained support materials and their functionalization to enhance enzyme-support affinity;
3. Immobilization of laccase and horseradish peroxidase on the obtained support using various immobilization approaches;
4. Characterization of the systems with immobilized oxidoreductases in terms of their catalytic activity and stability;
5. Decolorization of dyes from aqueous solutions using produced systems, under various process conditions;
6. Determination of dye decolorization efficiencies and analysis of the post-reaction mixture.

Additionally, in order to fulfil the main goal of the study as well as to better understand and explain the observed phenomena and dependencies, the following detailed research tasks have been formulated:

1. Advanced physicochemical characterization of the synthesized materials; before and after enzyme immobilization;
2. Selection of the immobilization conditions in order to obtain enzymatic systems characterized by the best catalytic properties;
3. Comparison of the immobilization effectiveness of laccase and horseradish peroxidase on the produced support materials;
4. Examination of the effect of surface modifiers on the immobilization efficiency and activity of immobilized biomolecules;
5. Detailed investigation of the activity, stability and reusability of the enzymes immobilized on various supports using different immobilization approaches under various reaction conditions;
6. Conducting dye decolorization experiments at various process conditions from solutions at different composition of solutions using various biocatalytic systems; comparison of the obtained decolorization efficiencies;
7. Study of the dye decolorization mechanism;

8. Determination and comparison of chemical oxygen demand and toxicity of the dye solutions before and after decolorization process.

It should be clearly stated that the above-presented detailed goals should be considered globally as the main assumptions of the study. However, during the undertaken research, other objectives have also been raised. The important issue was the optimalization of enzyme immobilization by selection of process conditions to obtain a stable connection between enzymes and support materials. Moreover, the determination of mechanisms of immobilization was the next objective, that allowed better understanding of the occurring enzyme-support interactions. Furthermore, optimalization of decolorization process was carried out and the mechanisms of dye degradation were proposed. The interesting point of the study was the decolorization of dyes from model aqueous solutions imitating sea waters. Moreover, the toxicity of the solutions after degradation process was investigated.

It could be concluded that the main goals of research, which included the production of novel supports and oxidoreductases immobilization, and the application of these biosystems for dye decolorization process, were crucial for preparation of effective tools for removal of these hazardous compounds from aqueous solutions. Moreover, due to the utility of the produced biocatalysts in dye degradation process, it can be assumed that the proposed systems may also find application in removal of other organic compounds in industrial processes. Moreover, the research objectives such as broadening of the knowledge on the application of immobilized enzymes in environmental protection and increasing awareness in this field cannot be omitted.

# 5. Description of the content of research achievements

Referring to the assumption that immobilized oxidoreductases can effectively remove dyes from aqueous solutions, it was decided to carry out studies, which confirm this hypothesis. Therefore, **Publications no. 1–6** are focused on the design, synthesis, and application of new biosystems made of oxide materials (**Publications no. 1** and **no. 2**) or electrospun fibers (**Publications no. 3–6**) and immobilized oxidoreductases for removal of selected dyes from water solutions.

The crucial step for each study was the design and synthesis of novel and effective support materials for enzyme immobilization. The selection of proper support for oxidoreductase attachment was dictated by the specific properties of the matrix, such as sufficient porosity or the presence of functional groups, compatible with enzymes, which increase the efficiency of biomolecules attachment and stabilization of proteins. Moreover, a suitability of immobilization technique as well as process conditions were investigated and selected mechanisms of enzyme attachment to the supports were determined in order to produce biocatalytic systems characterized by high catalytic properties. To confirm the application properties and versatility of the produced biosystems, they were tested in terms of decolorization of dyes, which was also a scientific novelty of the presented work. The colour compounds from various groups, such as azo, anthraquinone and triarylmethane dyes, were decolorized at various process conditions from aqueous solutions to find the optimal process parameters for the highest removal efficiency. The obtained data were presented and discussed in detail which allowed to draw conclusions on the use of biosystems with immobilized oxidoreductases in the decolorization of dyes.

## 5.1. Results for systems made of oxide materials and oxidoreductases

Currently oxide materials find many applications in various sectors of the industry and scientific areas, such as electrochemistry, sensors production, water purification and photocatalysis (Gao et al., 2011; Sun et al., 2017; Zhang et al., 2017; Kubiak et al., 2020). Their wide use is associated with the possibility of design of their properties, such as size of particles, porosity or surface area, due to the use of various synthesis methods including

co-precipitation, thermal decomposition, microemulsion, hydrothermal and sonochemical synthesis (Ganahari et al. 2019). To improve the properties of oxide materials, they can be combined into hybrid systems. Such systems are characterized by features of each of used components, therefore the obtained hybrids could be more suitable for enzyme immobilization, compared to the single oxide-based materials. Taking the above-mentioned facts into account, it was decided to investigate the application properties of designed hybrid oxide materials in oxidoreductase immobilization and to apply the produced biocatalytic systems for removal of dyes from aqueous solution, by synergistic enzymatic conversion and adsorption of dye molecules on the synthesized oxide systems.

In **Publication no. 1**, the hybrid oxide materials made of $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$, and in the **Publication no. 2** $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$ modified by $Cu^{2+}$ were synthesized, characterized and applied as supports for laccase immobilization. After careful characterization of the catalytic properties of produced systems, they were applied for removal of azo and anthraquinone dyes from model aqueous solutions. The selection of these hybrid oxide materials in enzyme immobilization was dictated by their specific properties, which include e.g. high porosity, well developed surface area, the presence of numerous of surface hydroxyl groups and stability under the reaction conditions. The synthesis and characterization of $TiO_2$-$ZrO_2$, $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ oxide systems were the first stages of this investigation.

After synthesis, the oxide materials were characterized using X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier transfer infrared spectroscopy (FTIR) and zeta potential analyses, which allowed to confirm the effective synthesis of hybrid oxide systems. Specifically, XRD, SEM and TEM results enabled the description of the morphology of the hybrid materials. It was shown that oxide materials are characterized by irregular particles at size around 10 µm and 5 µm in diameter for $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$, respectively, and 5 µm in diameter for both $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$. FTIR spectra confirmed the presence of specific functional groups on the surface of $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$ based on their characteristic signals. These bands are assigned to: υ –OH (3480 $cm^{-1}$), δ –ZrO, υ O–Ti–O, δ ≡Ti–O (broad peak approx. 600 $cm^{-1}$), υ ≡Ti–O–Si≡ (1540 $cm^{-1}$) and υ ≡Si–O–Si≡ (1110 $cm^{-1}$). In case of $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ oxide materials, the specific functional moieties and bonds were assigned

to the following signals: υ –OH (wide band 3650–3350 $cm^{-1}$), δ Zr–OH (1330 $cm^{-1}$), υ Si–O–Si and υ Zr–O–Zr (1200–950 $cm^{-1}$), δ Si–O (675 $cm^{-1}$) and δ Zr–O (600 $cm^{-1}$).

Moreover, the porous structure parameters were calculated using low-temperature $N_2$ adsorption/desorption analysis (Table 3).

***Table 3.*** *Parameters of the porous structure of the hybrid support materials, made of $TiO_2$-$ZrO_2$, $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$.*

| Publication no. | System | Parameter | | |
|---|---|---|---|---|
| | | $A_{BET}$ ($m^2$/g) | $V_p$ ($cm^3$/g) | $S_p$ (nm) |
| 1 | $TiO_2$-$ZrO_2$ | 19.3 | 0.121 | 22.8 |
| | $TiO_2$-$ZrO_2$-$SiO_2$ | 47.1 | 0.082 | 5.1 |
| 2 | $ZrO_2$-$SiO_2$ | 440.2 | 0.369 | 3.4 |
| | $ZrO_2$-$SiO_2$/$Cu^{2+}$ | 445.7 | 0.344 | 3.0 |

The obtained results allowed to conclude that the produced $TiO_2$-$ZrO_2$ material was characterized by pore diameters ($S_p$) of 22.8 nm, whereas $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ possessed $S_p$ of approx. 5 nm. Moreover, the $TiO_2$-$ZrO_2$ system was characterized by the lowest surface area ($A_{BET}$) equal to 19.3 $m^2$/g, whereas after adding a new component, $SiO_2$, which was used to increase the inner surface area, the $A_{BET}$ increased to 47.1 $m^2$/g. Despite the results of porous structure parameters making $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$ materials suitable for enzyme immobilization, it was decided to produce oxide systems characterized by an even better developed porous structure. Therefore, the next oxide system was formed by $ZrO_2$ and $SiO_2$. The selection of these components was dictated by their features: $ZrO_2$ and $SiO_2$ are characterized by good mechanical properties and separately possess high surface area. As an effect of this study, for $ZrO_2$-$SiO_2$ synthesized system, $A_{BET}$ equal to over 440 $m^2$/g was reached. Moreover, the $ZrO_2$-$SiO_2$ hybrid was further modified by $Cu^{2+}$ in order to investagate the effect of copper ions on the activity of immobilized laccase. It was shown that the porous structure parameters did not vary significantly between $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ systems, indicating that modification of oxide material by metal ions does not affect its porosity and does not limit the surface area capable for enzyme binding.

The synthesized oxide materials, presented in **Publication no. 1** and **no. 2**, were used as supports for laccase immobilization by the adsorption method. This method of enzyme immobilization was chosen mainly due to its universality and retention of high catalytic activity

by the immobilized enzymes (Jesionowski et al., 2014). The kinetic parameters of the laccase immobilized using the obtained oxide systems as well as data on immobilization efficiency are shown in Table 4.

***Table 4.*** *Kinetic parameters of the free laccase and laccase immobilized onto $TiO_2$-$ZrO_2$, $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ hybrid oxide materials as well as amount of attached enzyme and immobilization yield.*

| Publication no. | Biocatalyst | Parameter | | | |
|---|---|---|---|---|---|
| | | $K_m$ (mM) | $V_{max}$ (U/mg) | Amount of enzyme (mg/g) | Immobilization yield (%) |
| | Free laccase | 0.057±0.004 | 0.046±0.006 | - | - |
| 1 | $TiO_2$-$ZrO_2$-laccase | 0.108±0.016 | 0.036±0.002 | 83±3.2 | 83±3.4 |
| | $TiO_2$-$ZrO_2$-$SiO_2$-laccase | 0.124±0.019 | 0.029±0.003 | 96±2.7 | 96±2.2 |
| 2 | $ZrO_2$-$SiO_2$-laccase | 0.132±0.009 | 0.029±0.007 | 86±3.8 | 86±3.9 |
| | $ZrO_2$-$SiO_2$/$Cu^{2+}$-laccase | 0.098±0.008 | 0.037±0.009 | 94±3.2 | 94±3.1 |

The determination of kinetic parameters, such as Michaelis-Menten constant ($K_m$) and maximal reaction rate ($V_{max}$), of free laccase and its immobilized forms are important in terms of enzyme affinity for a given substrate and maximum reaction rate. The higher value of $K_m$ means lower affinity of the enzyme to substrate, which usually leads to a decrease of $V_{max}$ of catalytic reaction (Cornish-Bowden, 2014). Based on the obtained data, it can be seen that free biomolecule possessed lower value of $K_m$ (0.057±0.004 mM) and higher value of $V_{max}$ (0.046±0.006 U/mg), compared to the laccase immobilized onto oxide materials, indicating higher affinity of the free enzyme to the substrate and limited diffusional limitations. The higher values of $K_m$ and lower values of $V_{max}$ of immobilized laccase may be caused by blocking of enzyme active sites upon immobilization and formation of diffusional limitations in the transport of the substrate and products. Nevertheless, high amount of immobilized laccase and immobilization yield, which was over 80% for each of biocatalytic system, confirmed effective laccase deposition, that is directly related to high porosity of the produced oxide materials and the presence of numerous of –OH groups onto their surface, which facilitates the formation of hydrogen bonds and ionic interactions between the supports and laccase (Zdarta et al., 2018b). Moreover, it should be highlighted that by using $TiO_2$-$ZrO_2$-$SiO_2$ hybrid oxide system, higher immobilization yield (96±2.2%) and amount of immobilized enzyme (96±27 mg/g) was observed, as compared to the binary $TiO_2$-$ZrO_2$

system, which were 83±3.4% and 83±3.2 mg/g, respectively. It also can be seen that biocatalytic system dopped with $Cu^{2+}$ ions is characterized by values of kinetic parameters indicating higher affinity of the immobilized enzyme to the substrate than the copper-free system. It indirectly confirms that copper ions positively affect laccase activity by supporting its oxidation properties (Morozova et al., 2007).

The investigation of storage stability of the produced systems with immobilized laccase was also an important aspect of the presented work. This parameter is one of the most important in terms of practical application of the biocatalysts at an industrial scale (Talebi et al., 2016). Therefore, it was decided to measure relative activity of free laccase and enzyme immobilized using $TiO_2$-$ZrO_2$, $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ materials over storage time (Figure 13).

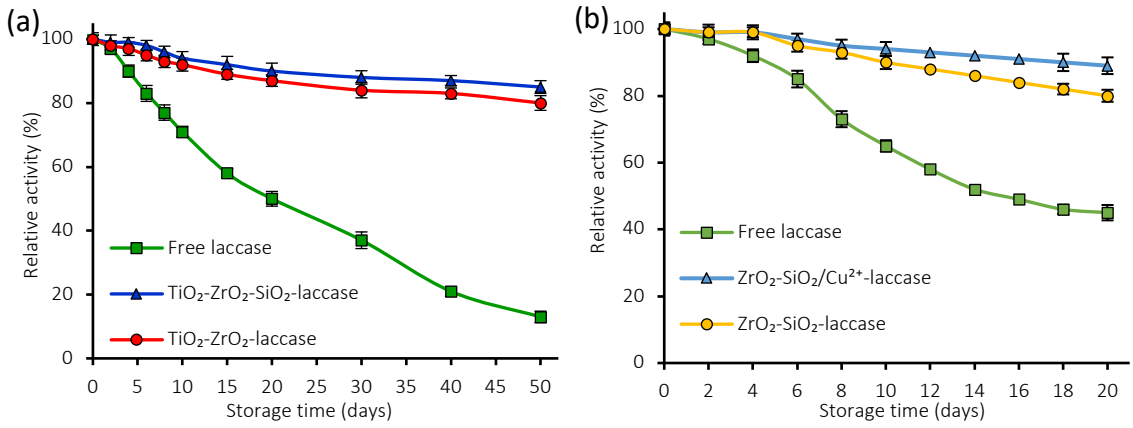


***Figure 13.*** *Storage stability of: (a) $TiO_2$-$ZrO_2$-$SiO_2$-laccase and $TiO_2$-$ZrO_2$-laccase and (b) $ZrO_2$-$SiO_2$/$Cu^{2+}$-laccase and $ZrO_2$-$SiO_2$-laccase, as well as storage stability of free enzyme.*

It can be seen that $TiO_2$-$ZrO_2$-laccase and $TiO_2$-$ZrO_2$-$SiO_2$-laccase systems exhibited approx. 90% of their initial catalytic activity after 50 days of storage. Furthermore, the $TiO_2$-$ZrO_2$-$SiO_2$-laccase biosystem possessed higher activity in the whole storage time, compared to the system without $SiO_2$. In case of $ZrO_2$-$SiO_2$-laccase and $ZrO_2$-$SiO_2$/$Cu^{2+}$-laccase systems, after 20 days of storage they retained approx. 80% and 90% of enzyme initial activity, respectively. The slight differences in catalytic activity of $ZrO_2$-$SiO_2$-laccase and system doped by copper ions may be explained by laccase hyperactivation by the $Cu^{2+}$ ions (Batule et al., 2015). It should be noted that a sharp decrease of catalytic activity of free laccase was observed over storage time, as compared to its immobilized forms. It was caused

by the low stability of free enzyme over time and lack of protective effect of support material on enzyme structure, which leads to its denaturation and inactivation.

To summarize the research presented so far, it can be concluded that use of synthesized hybrid oxide materials $TiO_2$-$ZrO_2$, $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$, facilitated the immobilization of high amounts of laccase biomolecules on them. Moreover, compared to the free oxidoreductase, these systems are characterized by high stability over storage time.

The essential part of **Publication no. 1** was the application of $TiO_2$-$ZrO_2$-laccase and $TiO_2$-$ZrO_2$-$SiO_2$-laccase biosystems as tools for removal of three dyes: azo dye–C.I. Reactive Black 5 (RB5), anthraquinone dyes–C.I. Reactive Blue 19 (RB19) and C.I. Mordant Red 3 (MR3). First of all, the sorption capacities of the synthesized oxide materials towards dyes molecules were investigated. It was important for determination of degradation mechanism of selected dyes. In this case, systems with thermally inactivated laccase were applied for dye removal from solutions at concentrations: 1, 5 and 10 mg/L. The application of the ternary system allowed to adsorb more dye molecules, compared to the binary oxide material, due to higher porosity of $TiO_2$-$ZrO_2$-$SiO_2$ and the presence of silica in its structure. For example, the highest sorption efficiency (100%) was obtained for adsorption of the MR3 dye molecules onto $TiO_2$-$ZrO_2$-$SiO_2$ from solution at concentration 1 mg/L, whereas the lower value (74%) was noted for adsorption of the same dye solution using $TiO_2$-$ZrO_2$ as sorbent material. Moreover, it could be concluded that the removal efficiency of dyes by adsorption onto oxide systems with inactive enzyme decreased with incresing concentration of dye solution. For instance, the sorption efficiency of RB19 dye solution at concentration 1 mg/L adsorbed onto the $TiO_2$-$ZrO_2$ material was equal to 76%, whereas after adsorption from dye solution at concentration 10 mg/L was at 46%. In order to increase the decolorization efficiencies of selected dyes, it was decided to use these oxide systems with active immobilized laccase. The removal of dyes using the obtained biosystems were carried out at various process conditions such as varying concentration, temperature and pH in order to clearly determine reaction parameters that facilitate high dye removal (Table 5).

***Table 5.*** *Effect of various dye concentration, temperature and pH on dye removal efficiency. The processes were carried out using laccase immobilized onto $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$, hybrid oxide systems. The errors values of measurements do not exceed 5%.*

| Parameter | Removal efficiency (%) | | | | | |
|---|---|---|---|---|---|---|
| | C.I. Mordant Red 3 | | C.I. Reactive Blue 19 | | C.I. Reactive Black 5 | |
| | $TiO_2$-$ZrO_2$-laccase | $TiO_2$-$ZrO_2$-$SiO_2$-laccase | $TiO_2$-$ZrO_2$-laccase | $TiO_2$-$ZrO_2$-$SiO_2$-laccase | $TiO_2$-$ZrO_2$-laccase | $TiO_2$-$ZrO_2$-$SiO_2$-laccase |
| **Concentration (mg/L)** | | | | | | |
| 1 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5 | 87 | 100 | 91 | 78 | 16 | 77 |
| 10 | 62 | 100 | 70 | 67 | 6 | 47 |
| **Temperature (°C)** | | | | | | |
| 5 | 74 | 87 | 70 | 62 | 7 | 42 |
| 25 | 87 | 100 | 91 | 78 | 16 | 77 |
| 50 | 83 | 91 | 82 | 66 | 7 | 49 |
| **pH** | | | | | | |
| 4 | 90 | 100 | 87 | 75 | 11 | 76 |
| 5 | 87 | 100 | 91 | 78 | 16 | 77 |
| 6 | 86 | 100 | 90 | 76 | 6 | 75 |

It can be seen that the removal efficiency of each of the tested dye decreased when the concentration of its solution increased. It is also correlated with the data obtained for sorption study. Moreover, it was observed that changing of temperature and pH only slightly affected the removal efficiency of each of dye. The highest removal efficiencies, among tested dyes, were obtained for the MR3 solution at each examined temperature. It was shown that at 5 °C and 50 °C, the removal of dyes was approx. 30% less effective than after the process conducted at 25 °C (up to 100%). Similar correlations were noted for RB19 and RB5: the obtained decolorization efficiencies of both these dyes were the highest at 25 °C, whereas after increase or decrease of temperature to 5 °C and 50 °C, respectively, the removal rates decreased by approx. 30%, irrespectively to the used biosystem. In case of pH, the differences between removal efficiencies of dyes at various pH, for each of the used system with immobilized laccase, did not exceed 5%, which was equal to error values.

The high removal efficiencies of dyes using the produced biosystem at various temperatures and pH can confirm that the proposed $TiO_2$-$ZrO_2$-laccase and $TiO_2$-$ZrO_2$-$SiO_2$-laccase systems are characterized by high enzyme activity retention at various process conditions. It is probably caused by the protective effect of support on the laccase structure, which provided better stabilization of protein and its high activity under changing reaction conditions. The effect of type of applied hybrid oxide system as support for laccase immobilization on the removal efficiency of each dye cannot be ommited. It can be seen that for MR3 and RB5, the highest removal efficiencies were obtained after application

of biosystem based on $TiO_2$-$ZrO_2$-$SiO_2$, which can be explained by the presence of $SiO_2$ in this material and, in consequence, a more developed inner surface area compared to the binary system. It allowed to immobilize higher amount of laccase and adsorb more dye molecules compared to the $TiO_2$-$ZrO_2$-laccase system. In contrast, in case of RB19 removal, the highest decolorization efficiencies were noted after application of binary system with immobilized laccase. It was caused by higher sorption efficiency of RB19 onto $TiO_2$-$ZrO_2$, compared to the $TiO_2$-$ZrO_2$-$SiO_2$. This can be explained by the fact that binary system possessed two times higher content of $ZrO_2$ in its structure and zirconia could more effectively bind RB19 by interactions with single N–H bond in the dye molecule (Kamaz et al., 2018). It should be also noted that the type of dye affects its removal. The highest efficiencies of removal were observed for MR3, compared to the RB19 and RB5. This is caused by more complicated structures of RB19 and RB5, which limit their efficient adsorption and/or enzymatic conversion. Moreover, it should be highlighted that in the study, the synergistic mechanism of dye degradation by sorption and enzymatic conversion was confirmed. Solutions of MR3, RB19 and RB5 dyes at concentration of 5 mg/L possessed sorption efficiency equal to 51%, 48% and 23%, respectively, after using $TiO_2$-$ZrO_2$-$SiO_2$ with inactivated enzyme as a sorbent. However, after application of the hybrid oxide material with immobilized oxidoreductases, the removal efficiencies increased up to 100%, 78% and 77%, respectively. The promising results obtained for single dye removal by proposed binary and ternary biosystems encouraged to investigate the removal efficiency of mixture of studied dyes. It was shown that after 24 h of biodegradation at pH 5 and 25 °C, the removal efficiencies of dyes form their mixture at total concentration of 5 mg/L, using both $TiO_2$-$ZrO_2$-laccase and $TiO_2$-$ZrO_2$-$SiO_2$-laccase biosystems, were equal to approx. 90%, indicating the potential of the produced materials for removal of mixture of dyes. Finally, the reusability study was also carried-out, which showed the possiblity to reuse the systems with attached oxidoreductase for removal of azo and anthraquinone dyes with promising efficiencies.

The important part of **Publication no. 1** was also the determination of chemical oxygen demand (COD) value of untreated and treated dye solutions, which is an important parameter in controlling of wastewater quality (Alves et al. 2020). Based on COD reduction, it was also possible to confirm removal of dyes from model aqueous solutions after enzymatic treatment (Table 6).

***Table 6.*** *Chemical oxygen demand determination after dyes solutions treatment by $TiO_2$-$ZrO_2$-laccase and $TiO_2$-$ZrO_2$-$SiO_2$-laccase systems. The errors of measurements do not exceed 5%.*

| Solution | Biocatalytic system | COD reduction efficiency (%) |
|---|---|---|
| C.I. Mordant Red 3 | $TiO_2$-$ZrO_2$-laccase | 78.58 |
| | $TiO_2$-$ZrO_2$-$SiO_2$-laccase | 84.66 |
| C.I. Reactive Blue 19 | $TiO_2$-$ZrO_2$-laccase | 71.52 |
| | $TiO_2$-$ZrO_2$-$SiO_2$-laccase | 64.34 |
| C.I. Reactive Black 5 | $TiO_2$-$ZrO_2$-laccase | 20.35 |
| | $TiO_2$-$ZrO_2$-$SiO_2$-laccase | 87.65 |
| Dyes' mixture | $TiO_2$-$ZrO_2$-laccase | 89.40 |
| | $TiO_2$-$ZrO_2$-$SiO_2$-laccase | 93.84 |

The COD values were decreased significantly after the process with binary and ternary oxide systems with immobilized laccase, irrespectively of the treated solution. The reduction of COD of selected dyes and their mixture was probably caused by the synergistic sorption of dyes molecules onto oxide materials, enzymatic conversion and sorption of bioconversion products. The obtained results allow to state that application of produced systems made of oxide materials and immobilized laccase may be a prospective tools for dye removal from aqueous solutions and reduction of organic matter. Moreover, it suggests mineralization of the dye solutions after treatment by the produced biosystems and reduction of toxicity that is extremely desirable in treatment plants (Alinsafi et al., 2006).

The promising results obtained for removal of dyes by $TiO_2$-based hybrid oxide systems with immobilized oxidoreductase encouraged to extend the studies using dye solutions at higher concentrations. In this case, hybrid oxide system $ZrO_2$-$SiO_2$, characterized by higher porosity, as compared to the $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$, was synthesized and used for decolorization of C.I. Reactive Blue 19 from solution at a concentration of 50 mg/L under various process conditions (Figure 14). Moreover, due to use of high concentration of RB19 for decolorization study, it was decided to modify $ZrO_2$-$SiO_2$ by copper ions. The presence of $Cu^{2+}$ in the support material could increase the activity of immobilized laccase, therefore the comparison of dye removal by $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ with immobilized laccase was made. The obtained results are presented in **Publication no. 2**.

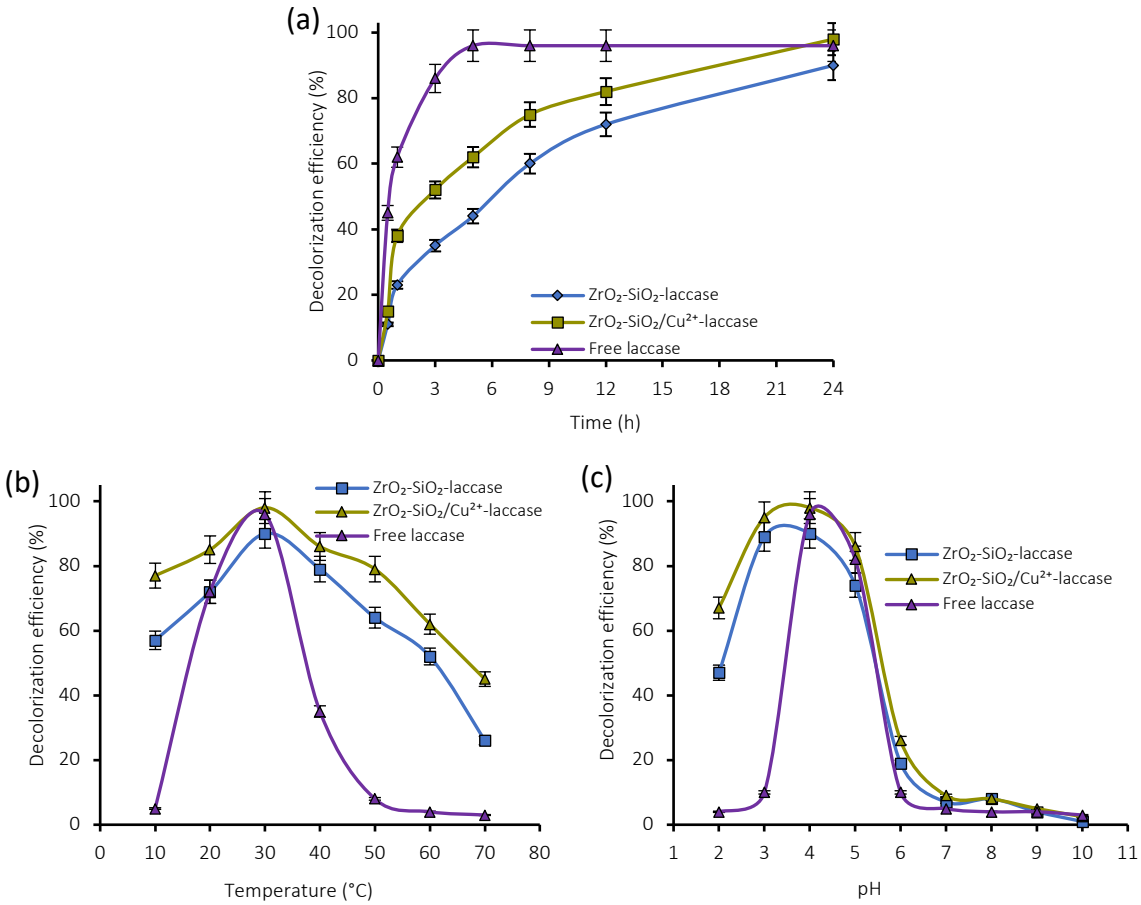


***Figure 14.*** *Effect of: (a) time of the process, (b) temperature and (c) pH on C.I. Reactive Blue 19 decolorization efficiency. The processes were carried out using $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ systems with immobilized laccase. The initial dye solution concentration was 50 mg/L.*

The effect of process time, temperature and pH on enzymatic removal of RB19 was followed in detail. The highest decolorization efficiency of the dye was obtained after 24 h of the process at temperature 30 °C and pH around 4. These results are directly related to the fact that fungal laccases show the highest activity at mild temperatures and slightly acidic pH (Kurniawati and Nicell, 2008). It should be noted that in case of free laccase, the decolorization efficiency of the dye sharply decreased under process conditions different than optimal (30 °C and pH 4). Moreover, it was observed that higher decolorization efficiency over the whole analyzed pH and temperature range was obtained using the system doped by copper ions, indicating positive effect of these ions on activity of laccase immobilized onto the $ZrO_2$-$SiO_2$ system. Surprisingly, despite high BET surface area, the sorption of dye molecules onto oxide materials with inactivated enzyme was lower than 7%, making catalytic conversion

a dominant process in dye removal. It could be explained by saturation of inorganic $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2/Cu^{2+}$ adsorbents active sites by the biomolecules. Therefore, the amount of active centers on the sorbent surface was not sufficient for dye adsorption from solution at such a high concentration (50 mg/L).

The determination of toxicity of solutions with pollutants is important in terms of their effect on environment. Moreover, its should be highlighed that the study on toxicity of solutions after treatment needs to be conducted due to the fact that such solutions could include products that are even more harmful for aquatic ecosystems than parent compounds. Therefore, study based on *Artemia salina* larvae as a model microorganism allowed to determine the toxicity of RB19 dye solution before and after enzymatic treatment (Figure 15). It was shown that the mortality of larvae decreased after incubation in solutions of C.I. Reactive Blue 19 after the decolorization process using systems with immobilized laccase. The mortality of solutions after the process using $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2/Cu^{2+}$ with oxidoreductase decreased by approx. 20% and 30%, respectively, compared to the dye solution before treatment. This decline is due to removal of the initial dye as well as formation of less toxic products obtained after enzymatic conversion of RB19 (Osma et al., 2010a).

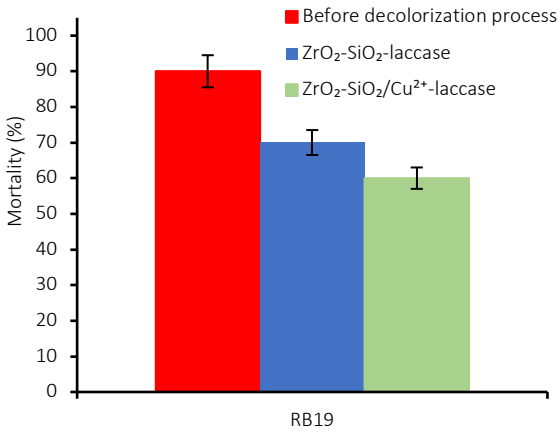


***Figure 15.*** *Mortality of Artemia salina larvae in the RB19 solution before and after treatment by $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2/Cu^{2+}$ systems with immobilized laccase.*

## 5.2. Results for systems made of electrospun fibers and oxidoreductases

Aside from materials of inorganic origin, hybrid materials composed of organic precursors may also be used as supports for enzyme immobilization. Thus, the next materials, which were investigated in this study as supports for oxidoreductases immobilization and removal of dyes from aqueous solutions, were fibers produced by electrospinning. The decision to use this type of support materials was made in order to enhance the possibility to obtain more effective tools for removal of pollutants from aqueous solutions. Electrospinning allows to produce materials with different shapes, such as membranes, foams and single fibers of various shape and structure, from various polymers and biopolymers. Therefore it is possible to choose the most suitable material in terms of the presence of specific functional groups, compatible with moieties in the structure of enzyme, and characterized by sufficient porosity. However the main advantage of this technique is the possibility to control the synthesis process by manipulation of various conditions among others humidity, temperature, voltage, flow rate or polymer concentration. These changes affect thickness of fibers, their texture and porosity of a whole material (Kijeńska and Swieszkowski, 2017; Haider et al., 2018; Xue et al., 2019), that strongly affect its final properties, stability and applicability as well as might influence properties of the immobilized enzymes. Moreover, such electrospun materials could be tailor-made by designing of their properties to be suitable for specific process and systems. Therefore **Publications no. 3**–**6** present the production of electrospun fibers at specific conditions and their application properties in oxidoreductase immobilization. To attach oxidoreductases onto the produced electrospun fibers, two immobilization methods, adsorption and covalent binding, were applied and compared in terms of activity and stability of immobilized enzymes. Moreover, various surface modifiers were used to create the most stable connections between the electrospun materials and immobilized biomolecules. Such biosystems were characterized in detail and applied in dye removal processes at various conditions.

In **Publication no. 3** the production of electrospun fibers from nylon 6 and their use as supports for laccase immobilization by adsorption and covalent binding method, using *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide chloride (EDC) coupling with

*N*-hydroxysuccinimide (NHS) approach, was presented. The prepared biocatalytic systems were applied for decolorization process of two dyes: azo dye–C.I. Reactive Black 5 and anthraquinone dye–C.I. Reactive Blue 4 (Figure 16). In this work, it was crucial to compare two types of enzyme immobilization methods and their effect on dye decolorization efficiencies. Moreover, it was decided to investigate the effect of various process parameters, such as dye concentration, pH, temperature and reaction time, on the degradation of RB5 and RB4.

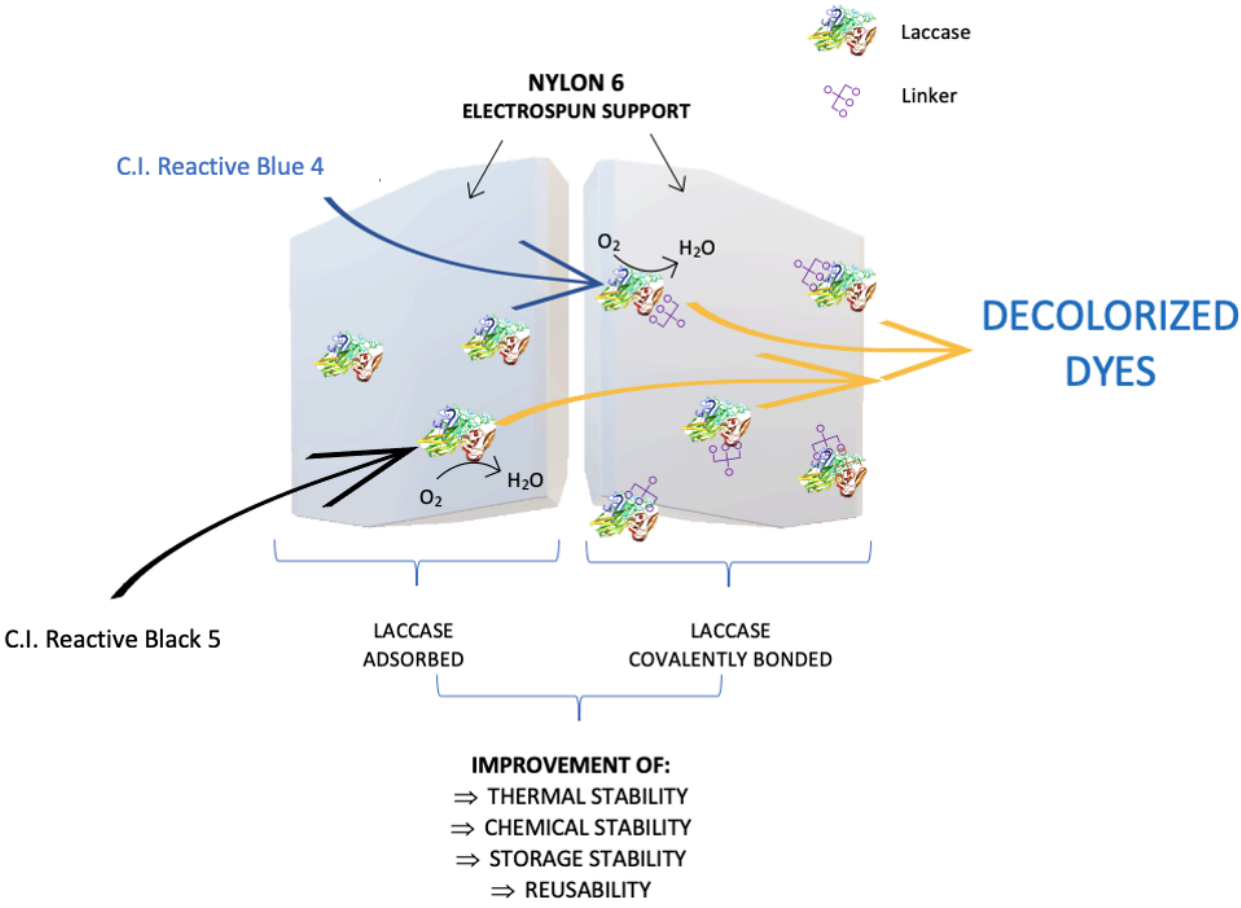


***Figure 16.*** *The schematic presentation of RB4 and RB5 removal by laccase immobilized by adsorption and covalent binding methods onto nylon 6 electrospun support.*

Results obtained by FTIR, EDS and SEM analyses confirmed effective fabrication of electrospun fibers and enzyme immobilization. It was shown that specific signals characteristic for stretching vibrations of –NH and –C=O groups occured in the FTIR spectrum of nylon 6. These data stays in with agreement with the results of EDS microanalysis of surface composition, which showed the presence of elements such as N, C and O on the surface of nylon 6 electrospun fibers. Moreover, after analysis of SEM photos, the average fiber diameter was calculated, which was equal to 784 ± 215 nm. The analysis of FTIR spectra of

systems after immobilization allowed to state that the signals characterized for protein structure corresponding to amide I, II and III bonds occurred after enzyme attachment by both methods. Moreover, after enzyme attachment, an increase of fiber diameter to even 1599 ± 850 nm for laccase covalently bonded was observed, which is related to coverage of fibers by enzyme particles. The next important part of this work was the investigation of effect of various process conditions on decolorization of two selected dyes: azo dye RB5 (Figure 17) and anthraquinone dye RB4 (Figure 18) by both immobilized enzymes.

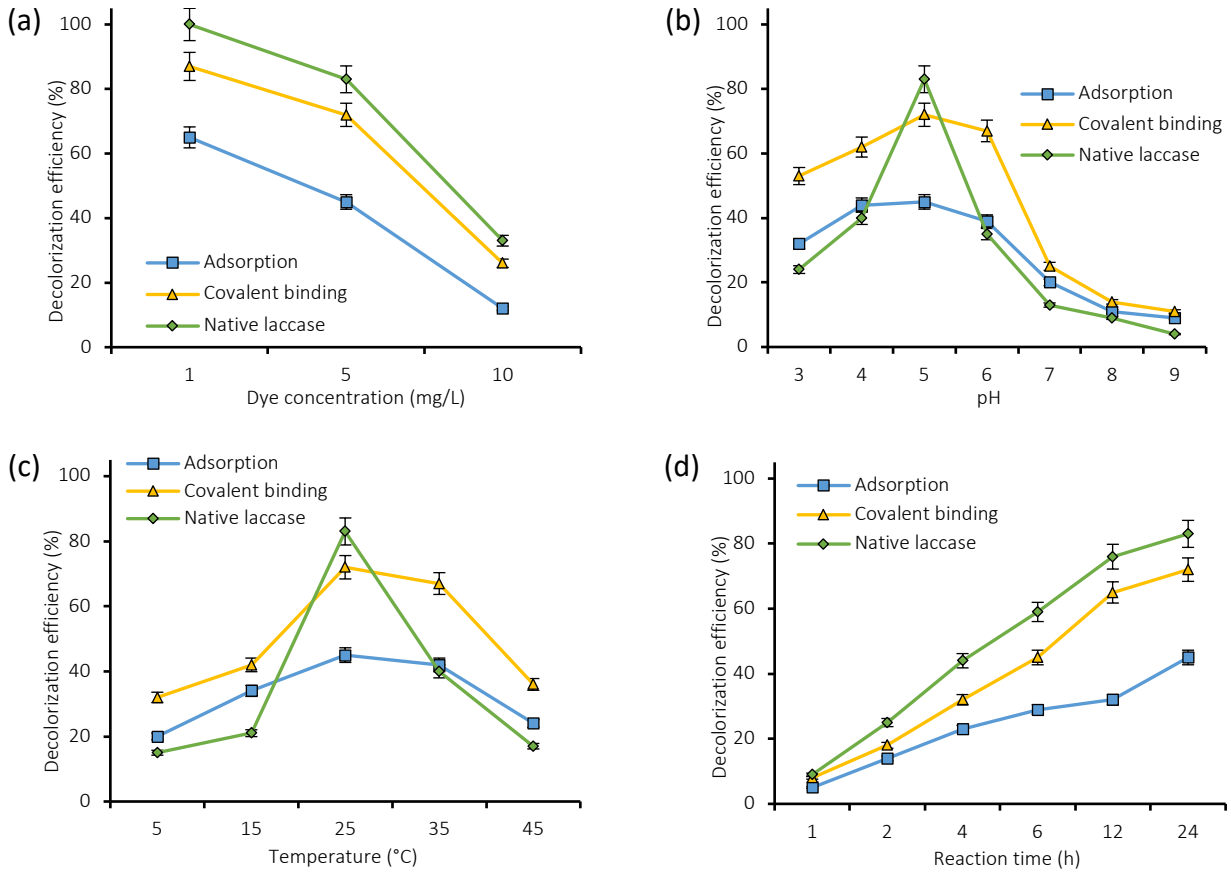


***Figure 17.*** *Effect of: (a) dye concentration, (b) pH, (c) temperature and (d) reaction time on decolorization efficiency of RB5 using laccase adsorbed and covalently bonded onto nylon 6 electrospun fibers.*

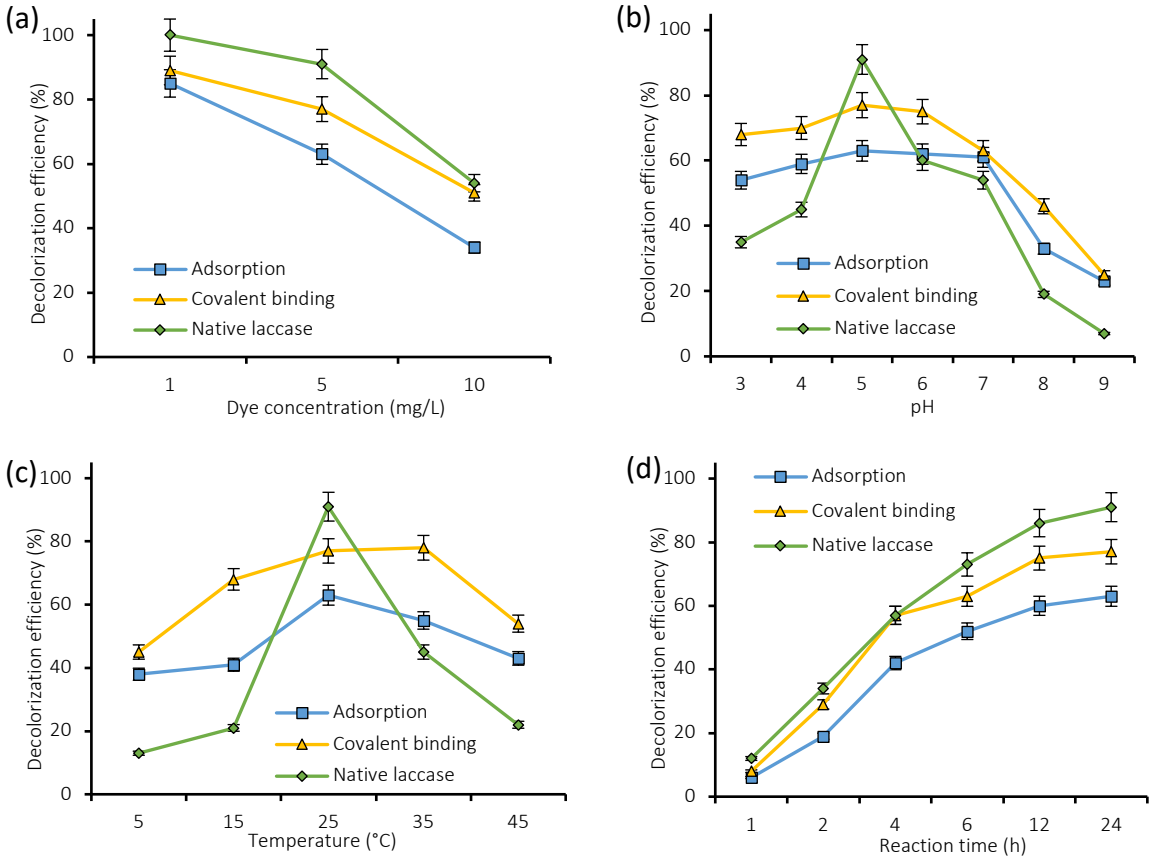


***Figure 18.*** *Effect of: (a) dye concentration, (b) pH, (c) temperature and (d) reaction time on decolorization efficiency of RB4 using laccase adsorbed and covalently bonded onto nylon 6 electrospun fibers.*

The selection of dye decolorization parameters is important to achieve high efficiency of pollutant removal. It can be seen that for each investigated parameter, similar trends in decolorization efficiencies by both immobilized enzymes were observed. The highest decolorization efficiency for each applied laccase was obtained after processes from the lowest dye concentration, which was 1 mg/L. Moreover, it was shown that increasing the dye concentration decreased the decolorization efficiency. In case of effect of pH and temperature on decolorization efficiency of analyzed compounds, it was confirmed that the highest decolorization efficiencies for both removed dyes were noted after the process at pH 5 and temperature of 25 °C, which corresponds with the optimal operation parameters for laccase from *Trametes versicolor* (Kurniawati and Nicell, 2008). However, oxidoreductase covalently bonded onto nylon 6 fibers decolorized both dyes with higher efficiency, compared to the laccase adsorbed onto electrospun material. This fact is caused by better stabilization of enzyme structure by covalent binding, compared to the laccase adsorption, and retention

of higher activity by the covalently bonded enzyme. Furthermore, enzyme attached *via* the EDC/NHS approach is less prone to leaching from prepared support material. Moreover, the differences between decolorization efficiencies of RB5 and RB4 under the same reaction conditions by the same biocatalytic system were noted. The higher efficiencies of removal of anthraquinone dye, compared to the azo compound, may be explained by different oxidation mechanism of dyes. In case of RB5, in the possible pathway of its degradation, an oxidation step occurs with the presence of a mediator, such as ABTS which was added to the reaction mixture, whereas in case of RB4 laccase is able to convert this dye molecule directly, without mediator addition, only in the presence of oxygen in reaction environment (Legerska et al., 2016).

After determination of suitable process conditions which allowed to decolorize dyes solutions with high efficiencies, further research was undertaken in terms of storage stability and reusability of the obtained systems made of laccase adsorbed or covalently bonded onto nylon 6 electrospun fibers (Figure 19). These parameters play an important role in practical application of the produced biosystems in the industry.

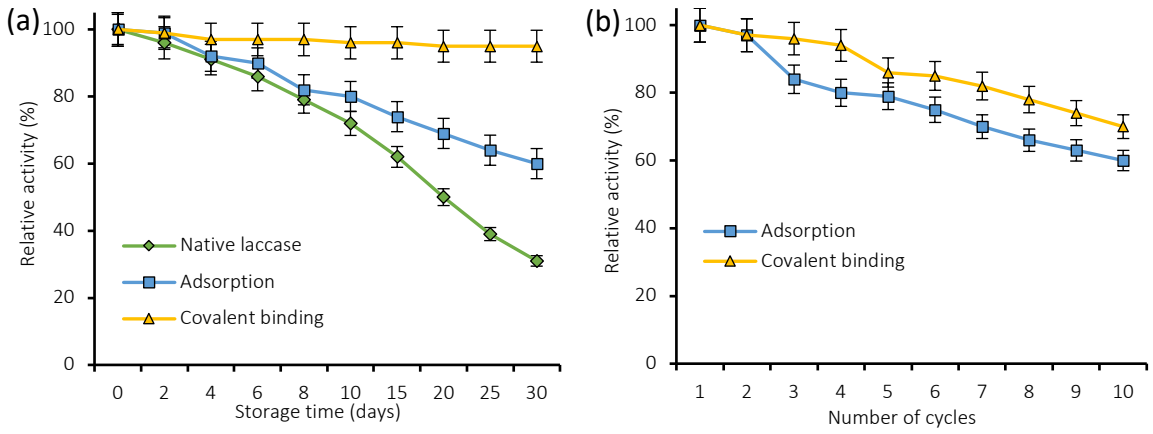


***Figure 19.*** *(a) Storage stability and (b) reusability of laccase adsorbed and covalently bonded onto nylon 6 fibers.*

After 30 days of storage laccase adsorbed and covalently bonded retained 60% and 95% of its initial activity, respectively. Moreover, the activities of laccase adsorbed and covalently bonded onto nylon 6 fibers were characterized by similar values of approx. 70–80% after 10 consecutive uses. It shows that laccase attached onto electrospun materials retains its activity regardless of the immobilization method, which is caused by the formation

of stable interactions between enzyme structure and nylon 6 that stabilize the enzyme and limit its elution.

The future-proof results in application of laccase immobilized on electrospun fibers encouraged to conduct a study with a different oxidoreductase, to determine differences between them in terms of catalytic activity and application properties in dye removal. Therefore, in **Publication no. 4** another oxidoreductase, horseradish peroxidase (HRP), was immobilized onto nylon 6 fibers by both adsorption and covalent binding methods. However, compared to **Publication no. 3**, glutaraldehyde was used as a linker between the biomolecule and the support to simplify the immobilization methodology. Moreover, it should be highlighted that HRP differs from laccase by the type of reaction mechanism and co-substrate, which is $H_2O_2$ (Grönqvist et al., 2005), which can also affect the final removal efficiencies of selected dyes. Finally, it should be emphasized that the crucial stage of this work was the selection of immobilization conditions to obtain final biocatalysts, characterized the highest catalytic activities, which were then used for dye decolorization from model aqueous solutions imitating sea waters.

The first step of this work was the selection of HRP immobilization conditions. In this case parameters such as glutaraldehyde concentration, time of process, temperature and pH were considered (Figure 20). This research stage was planned and carried out in order to obtain biocatalysts characterized by high activity and stability.

The investigation of the effect of glutaraldehyde concentration on HRP activity was performed using 5 various concentrations of linker in the range from 1% to 5%. The highest amount of HRP was attached onto fibers modified by 5% solution of glutaraldehyde and reached 730 μg/mg. However, the highest catalytic activity of enzyme was noted for system modified by 3% of GA solution, which possessed a lower amount of HRP (678 μg per 1 mg of support). Therefore, in subsequent studies regarding the selection of immobilization process conditions, nylon 6 fibers modified by 3% glutaraldehyde were used to covalently immobilized laccase.

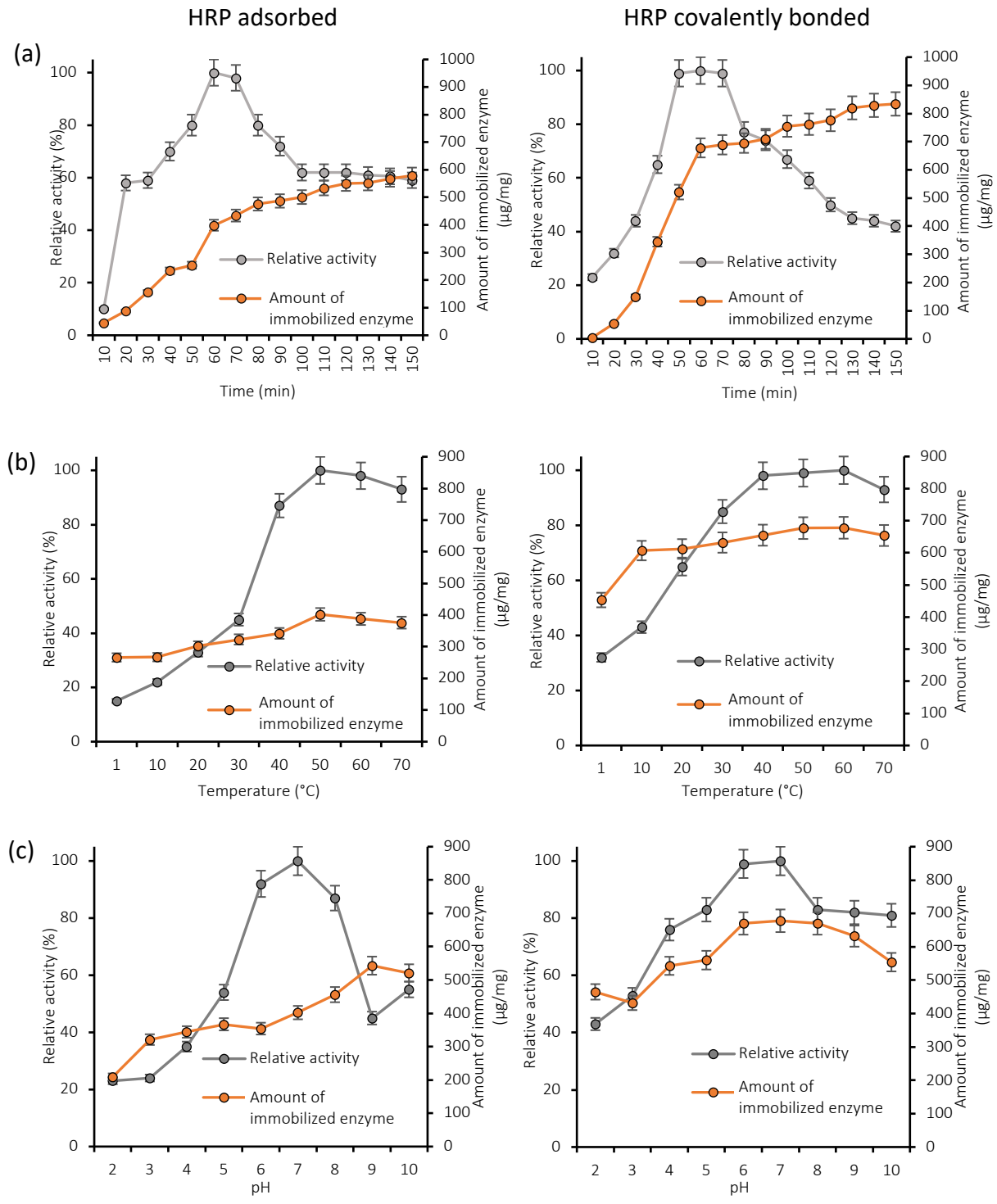


***Figure 20**. Effect of: (a) time of process, (b) temperature and (c) pH of immobilization on relative activity and amount of immobilized horseradish peroxidase onto nylon 6 fibers using adsorption and covalent binding methods.*

The parameter which affects the amount of immobilized biomolecule and enzymatic activity, is immobilization time. It was shown that the highest catalytic activities of enzyme

adsorbed and covalently bonded were noted after 60 min of immobilization process, whereas the highest amount of HRP was immobilized after 150 min of the process for both immobilization approaches. This is probably caused by the fact that with increasing time of immobilization the more biomolecules are attached to the support, which can block the active centers of already deposited enzymes, therefore the activity of such systems may decrease with immobilization time. These observations allowed to state that the activity of the biosystem is not directly related to the amount of immobilized enzyme. The next important parameters for the immobilization process were temperature and pH. The highest activity of adsorbed and covalently bonded HRP was observed after immobilization process at 50 °C and 60 °C, respectively. This difference in temperature conditions for HRP adsorbed and covalently bonded may be explained by changes in amino acids residues in active center of this enzyme by connection to glutaraldehyde which, in consequence, caused an increase of HRP activity at the temperature of 60 °C. Moreover, pH 7 was specified as the optimal immobilization pH, that corresponds with previously published study (Monier et al., 2010).

To summarize, the optimal conditions for immobilization were selected and the biocatalytic systems made of nylon 6 and enzyme were produced at following conditions: 60 min, 60 °C, pH 7 and 3% of glutaraldehyde concentration (covalent binding method), 60 min, 50 °C and pH 7 (adsorption method). After that, storage stability and reusability of HRP immobilized onto nylon 6 fibers were investigated (Figure 21).

Storage stability results showed that regardless of the form of immobilized HRP, it possessed higher relative activity after 30 days of storage compared to the free counterpart of this enzyme. The catalytic activities of HRP adsorbed and covalently bonded after 30 days of storage were 36% and 74%, respectively, whereas the native enzyme retained 23% of its initial activity. Furthermore, HRP covalently bonded, due to connection with nylon 6 by glutaraldehyde, is more rigid and stable and therefore it could retain more activity, compared to the horseradish peroxidase adsorbed onto electrospun fibers. Moreover, it should be noted that after 20 consecutive catalytic cycles adsorbed and covalently bonded oxidoreductase retained 63% and 70% of its relative activity, respectively. The decrease of activity for both adsorbed and covalently bonded horseradish peroxidase may be caused by inhibition of its active sites by reaction products, however, also minor enzyme elution cannot be excluded.

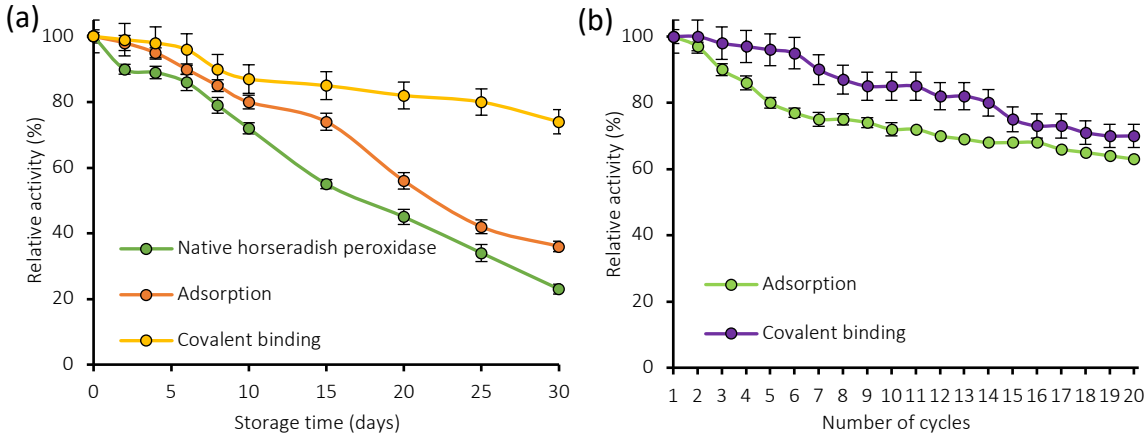


***Figure 21.*** *(a) Storage stability and (b) reusability of horseradish peroxidase adsorbed and covalently bonded onto nylon 6 electrospun material.*

The crucial part of this work was the application of systems with immobilized HRP, characterized by the highest activity, in removal of two selected dyes from aqueous solutions. However, compared with the previous works (**Publications no. 1–3**), the dyes solutions were prepared to imitate model sea waters. In this case, the concentrations of salts, such as sodium chloride and sodium sulphate were adjusted to be similar with those occurring in real sea water. Azo dye–C.I. Reactive Black 5 and triarylmethane dye–C.I. Basic Green 4 were used as model dyes commonly used in textile industry (Przystaś et al., 2012). The preparation of such composed solutions was motivated by increasing amount of pollutants from textile industry in sea waters (Banks, 2017). The reactions of dye decolorization were carried out with and without the presence of $H_2O_2$. It was shown that higher amount of hydrogen peroxide in reaction solution also increased the decolorization efficiency, which is related to the fact that HRP requires $H_2O_2$ to catalyze the oxidation reaction (Veitch, 2004). Figure 22 shows the decolorization efficiencies of dye solutions after application of biosystems with HRP adsorbed and covalently bonded onto nylon 6 fibers with the presence of 1 mM of hydrogen peroxide.

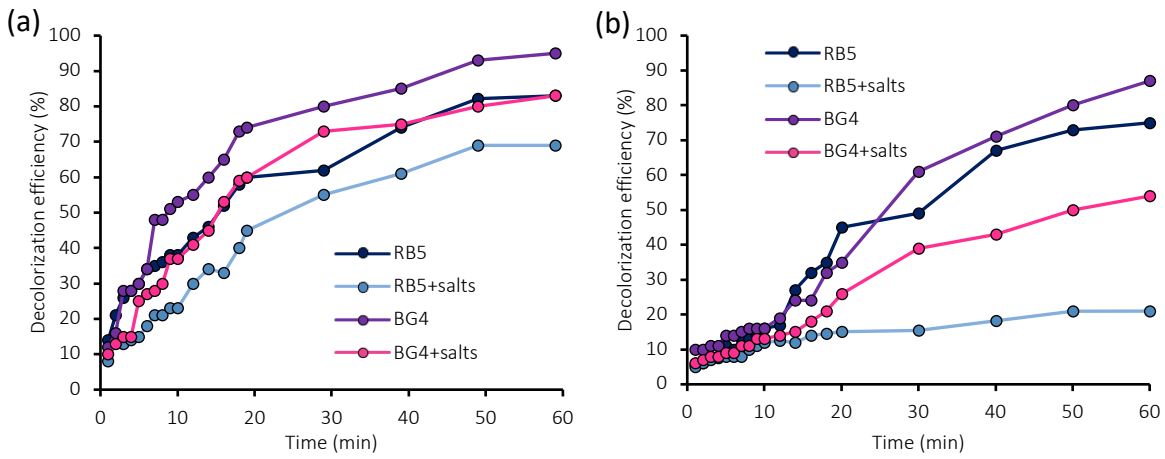


***Figure 22.*** *Decolorization efficiency of dyes from model aqueous solutions obtained after a process catalyzed by: (a) HRP adsorbed and (b) covalently bonded onto nylon 6. Reactions were carried out with the presence 1 mM of $H_2O_2$. Error values do not exceed 8%.*

It can be seen that lower decolorization efficiencies were noticed after the use of biosystem with covalently bonded HRP, compared to the enzyme adsorbed onto electrospun fibers. It may be explained by the presence of ions of salts in solutions, which affect the interactions between HRP and glutaraldehyde and, in consequence, decrease the activity of biomolecules or increase elution of this oxidoreductase from the support. The more perspective results were obtained after application of HRP adsorbed onto electrospun support, which is probably caused by lack of interference on the enzyme structure, compared to the covalent binding method, and its stabilization as well as providing a protective effect by the support material. Therefore HRP adsorbed onto nylon 6 fibers decolorized dye solutions with higher efficiencies compared to the covalently bonded enzyme. It should be highlighted that the decolorization efficiencies, regardless of the used form of immobilized HRP, are lower for dye solutions with the presence of salts, which suggests an inhibition effect of salts ions on HRP activity. It was shown that the highest decolorization efficiencies of RB5 and BG4 with the presence of salts, were equal to 69% and 83%, respectively, whereas for pure dyes it was 83% and 95%, respectively, using HRP adsorbed onto nylon 6.

The promising results in terms of application of electrospun fibers made of nylon 6 for oxidoreductases immobilization and removal of dyes from aqueous solutions were an inspiration for synthesis of new electrospun hybrid material characterized by more developed structure. In **Publication no. 5** electrospun fibers were synthesized from poly(methyl methacrylate) (PMMA) and polyaniline (PANI). The selection of these precursors

for production of novel polymer blend was dictated by the presence of carbonyl and amide groups in the structure of monomers, which are compatible with biomolecules and facilitate surface modification and enzyme binding. The effective fabrication of PMMA/PANI material was confirmed based on the FTIR results. It was shown that FTIR spectrum for electrospun material included signals characteristic for PMMA, at 1770 $cm^{-1}$ assigned to C=O bonds and at 1150 $cm^{-1}$ assigned to C–O–C bonds. Moreover, the presence of $C_{Ar}$–$C_{Ar}$ bonds at 1550–1450 $cm^{-1}$, characteristic for PANI, was also demonstrated.

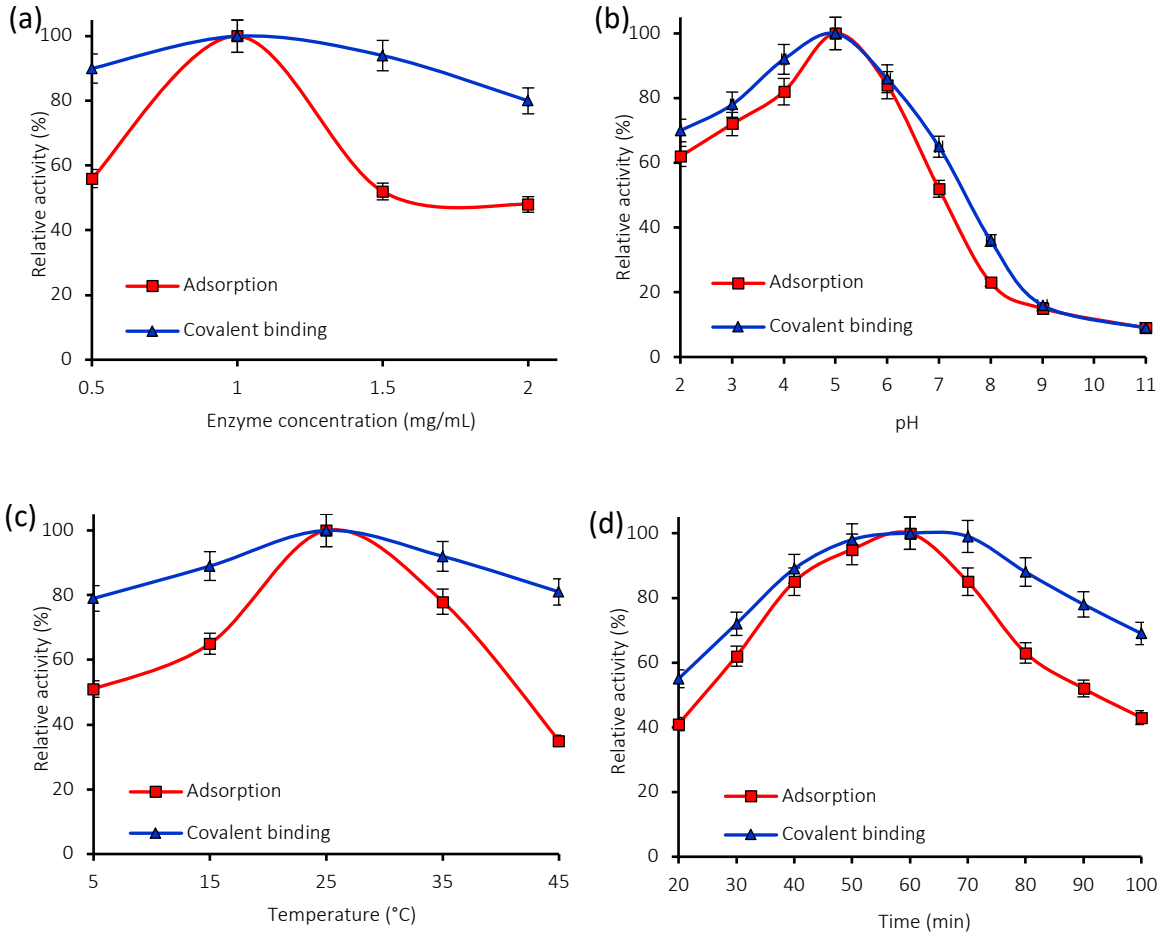


***Figure 23.*** *Effect of immobilization conditions on relative activity of laccase immobilized onto PMMA/PANI hybrid electrospun fibers by adsorption and covalent binding: (a) enzyme concentration, (b) pH, (c) temperature and (d) time of process.*

The crucial part of this work was the selection of the optimum conditions for laccase immobilization onto electrospun fibers. In this case two types of enzyme immobilization methods were applied: adsorption and covalent binding via *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide chloride coupling with *N*-hydroxysuccinimide (EDC/NHS).

Figure 23 presents the effect of various immobilization conditions on the relative activity of immobilized laccase.

It was shown that the highest relative activity of both immobilized laccases was attained after immobilization at following conditions: enzyme concentration at 1 mg/mL, pH 5, temperature 25 °C, process duration 60 min, indicating these conditions as optimal for laccase immobilization using PMMA/PANI support. It should be also clearly stated that higher relative activities were obtained for systems with laccase covalently bonded onto PMMA/PANI fibers over whole range of investigated process parameters. Finally, it was also calculated that the amount of attached enzyme was equal to 110 mg/g and 185 mg/g for systems with adsorbed and covalently bonded laccase, respectively.

Moreover, the storage stability and reusability of PMMA/PANI fibers with immobilized laccase were determined (Figure 24).

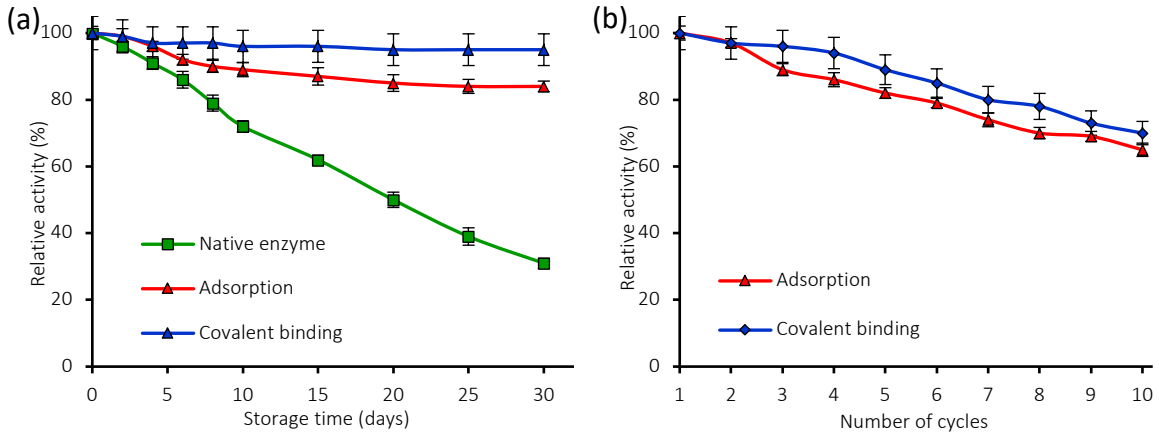


***Figure 24.*** *(a) Storage stability over 30 days of storage and (b) reusability over 10 consecutive catalytic cycles of laccase adsorbed and covalently bonded onto PMMA/PANI fibers.*

The obtained results showed that even after 30 days of storage and 10 consecutive catalytic cycles laccase covalently bonded onto the produced electrospun fibers possessed higher activity than enzymes adsorbed onto PMMA/PANI. However, both biocatalytic systems were characterized by improved storage stability compared to the free laccase, which retained less than 30% of its catalytic activity after 30 days of storage. These data are strictly related with stabilization of enzyme molecules upon their attachment to electrospun fibers and the protective effect of used support material on immobilized laccase (Bilal et al., 2019).

Biosystems characterized by the highest catalytic activities were then applied in decolorization of anthraquinone dye C.I. Reactive Blue 19 from model aqueous solutions (Figure 25).

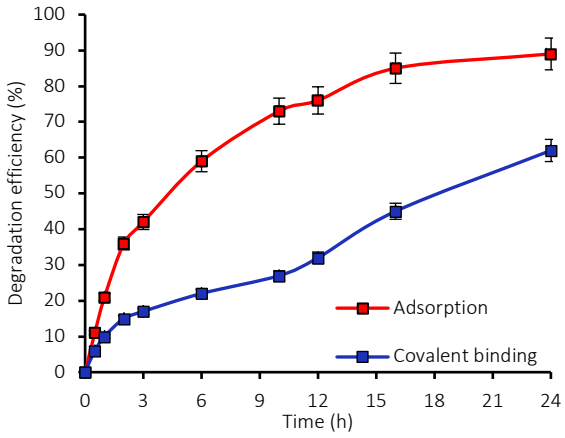


***Figure 25.*** *Decolorization efficiency of RB19 dye after process using systems made of PMMA/PANI fibers and adsorbed and covalently bonded laccase.*

The decolorization process was carried out under mild conditions, which were pH 5 and 30 °C. The most surprising observation was that the highest decolorization efficiency, equal to 87%, was obtained after 24 h of the process using a system with laccase immobilized by the adsorption method. The explanation of this phenomenon could be the fact that in systems with adsorbed laccase, there is no modifying agent on the PMMA/PANI surface, which facilitates the diffusion of dye molecules to the active sites of biomolecules. Furthermore, the active sites of laccase are more accessible to the substrate molecules due to higher flexibility of the absorbed laccase, as compared to covalently bounded enzyme.

The results presented above for application of electrospun fibers for effective oxidoreductases immobilization and application of the produced systems for dye decolorization, allow to state that materials produced by the electrospinning technique may be a universal base for enzyme immobilization and may act as a base to create effective tools for removal of pollutants from aqueous solutions. However, in order to produce active biocatalytic systems, it is essential to determine the mechanisms of oxidoreductase attachment to the electrospun support. Therefore, in **Publication no. 6** electrospun fibers made of polystyrene/poly(D,L-lactide-*co*-glycolide) were fabricated and applied for laccase

immobilization by two covalent binding approaches using (3-aminopropyl)triethoxysilane (APTES) with glutaraldehyde (GA) and polydopamine (PDA) as surface modifying agents.

The important step before enzyme immobilization onto fabricated support was its characterization in terms of porosity and the presence of specific functional groups, which strictly affect the immobilization process onto such material. Based on results of FTIR analysis, effective production of PS/PDLG fibers was confirmed. This is mainly due to the presence of signals characteristic for –OH and C–O groups and C–H bonds in the analyzed spectra. The porosity parameters of fabricated fibers were determined and it was shown that the PS/PDLG material possessed surface area equal to 217 $m^2/g$ while pore volume and pore size were at 5.806 $cm^3/g$ and 7.946 μm, respectively. Due to the fact that the produced material possessed relatively big pores, and to prevent leakage of enzyme from the support, it was decided to modify fibers using APTES+GA and PDA, separately.

Figure 26 presents mechanisms of laccase immobilization *via* APTES+GA and PDA approaches onto PS/PDLG fibers. It can be seen that, depending on the applied surface modifier/linker, the immobilization mechanisms vary from each other. Especially, the differences occur in the type of connections between support material and modifier. In case of the APTES and GA approach, the covalent binding occurs between APTES and electrospun fibers, whereas in system where electrospun material is modified by PDA, the connections between support and modifier are based mainly on hydrogen bonds. In order to immobilize enzymes using the first approach, the support was subjected to further crosslinking by GA, which allowed to form an α,β-unsaturated aldehyde product as a spacer for enzyme attachment. However, in the PDA approach it is possible to form covalent bonds between PDA and enzyme's amine groups directly due to the presence of carbonyl groups in the structure of polydopamine. Moreover, it should be emphasized that the type of enzyme can also affect the mechanism of immobilization. In case of laccase, a monomeric enzyme, the molecule was attached to both modifiers, separately, by a single specific group, such as $–NH_2$.

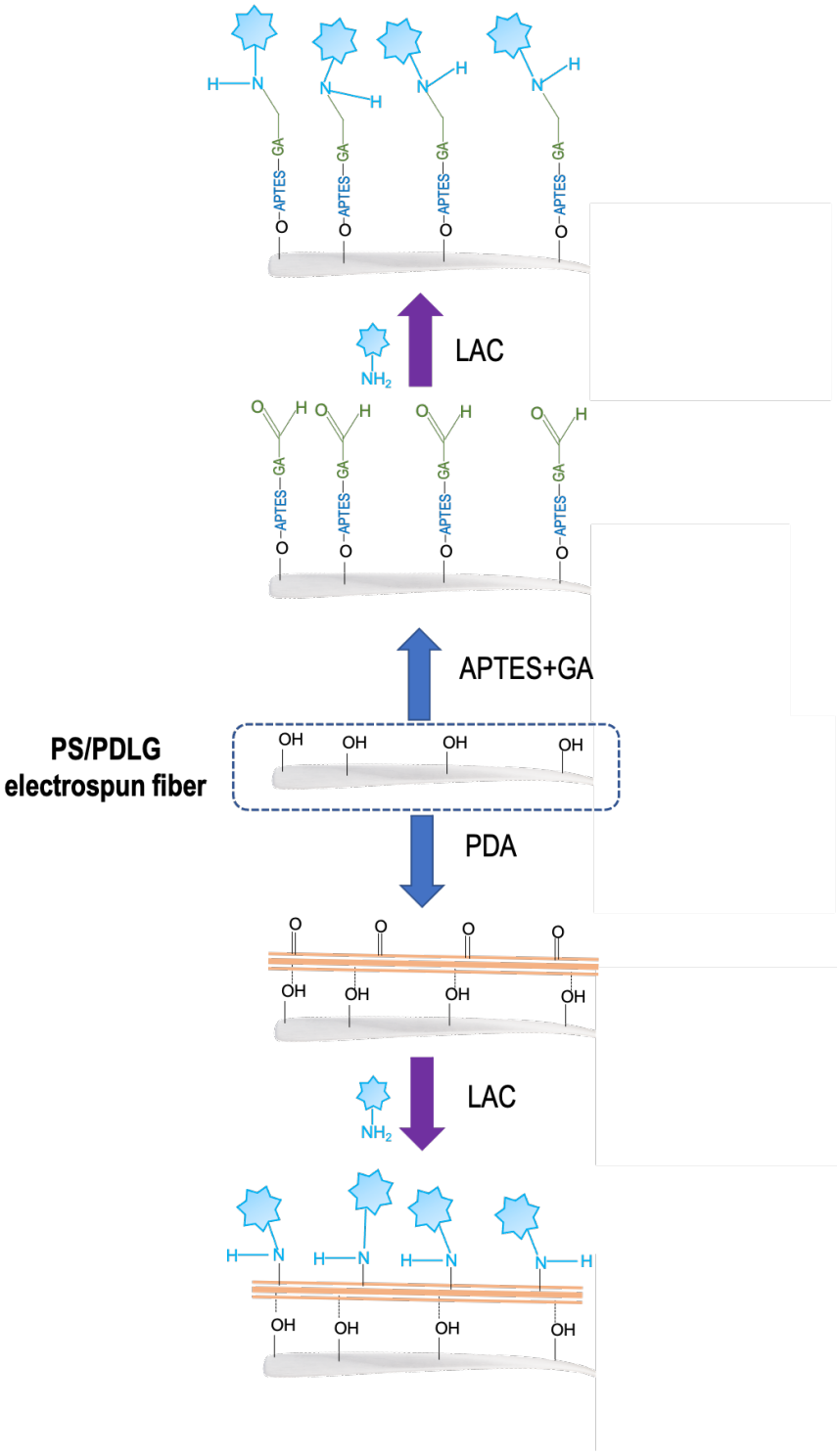


***Figure 26.*** *Mechanisms of laccase (LAC) immobilization via APTES+GA and PDA approaches onto PS/PDLG electrospun fibers.*

After examination of enzyme immobilization mechanisms, which are governed mainly by type of the applied linkers, the effect of pH and temperature on conversion efficiency of ABTS, as a model substrate, by immobilized oxidoreductase was investigated (Figure 27).

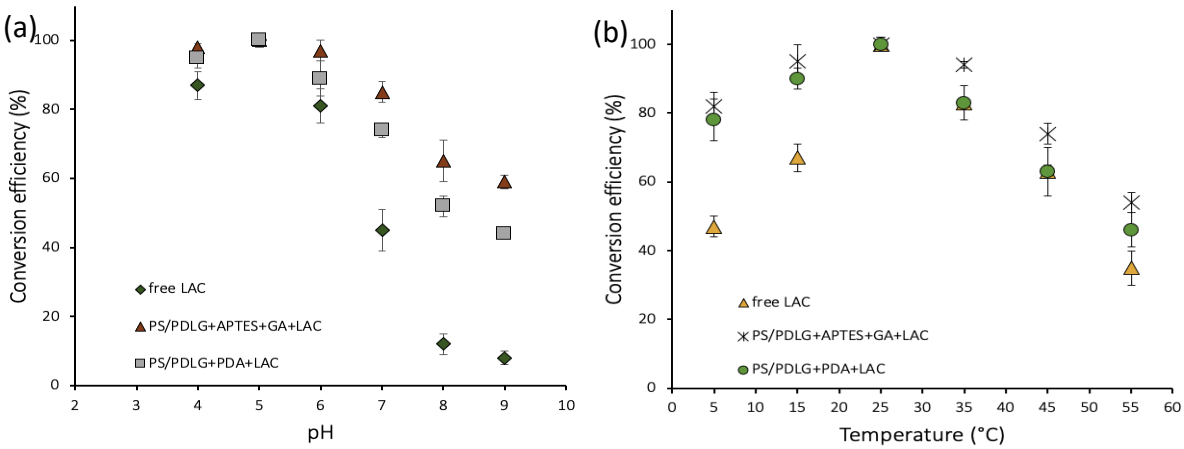


***Figure 27.*** *Effect of: (a) pH and (b) temperature on ABTS conversion efficiency catalyzed by free laccase and laccase attached onto PS/PDLG fibers via APTES+GA and PDA approaches, separately.*

It was confirmed that the highest conversion efficiencies of ABTS by free and immobilized laccase were noted for reactions carried out at pH 5 and 25 °C, which stays in agreement with the results obtained in **Publications no. 1**, **no. 2** and **no. 4** that laccase converts specific substrates at mild reaction conditions. Moreover, both immobilized oxidoreductases converted the substrate with higher efficiency over wider pH and temperature range compared to the free enzyme, which suggests stabilization of the biomolecule structures upon immobilization. The important observations, related directly to the mechanisms of oxidoreductase immobilization, were made after a comparison of conversion efficiencies obtained after reactions using enzymes immobilized on PS/PDLG+APTES+GA and PS/PDLG+PDA systems. It was shown that oxidoreductase immobilized onto PS/PDLG+APTES+GA converted the substrate with higher efficiency compared to the enzymes attached by PDA approach. This difference is strictly related to different immobilization mechanisms (see Fig. 26). In case of laccase immobilized by the APTES+GA approach, the enzyme structure is stabilized by pointwise connection to the previously mentioned α,β-unsaturated aldehyde product. Therefore, in this form of biocatalyst, the substrate possesses better access to active sites of immobilized laccase. In contrast to this system, laccase attached to PS/PDLGA+PDA is characterized by lower activity. Enzyme immobilized onto PS/PDLG using PDA approach was probably densely attached to the electrospun fibers modified by PDA, causing partial blocking of active sites and

limitation of substrate and product diffusion between reaction environment and active centers of laccase.

The fabricated biocatalytic systems have also been tested in terms of their storage stability and reusability (Figure 28).

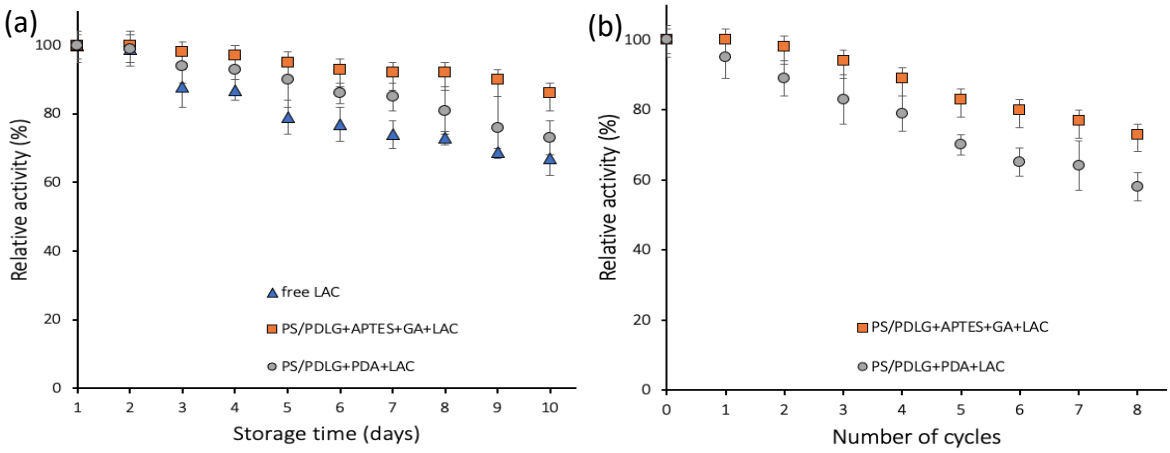


***Figure 28.*** *Effect of: (a) storage time and (b) number of consecutive reaction cycles on activity of laccase immobilized onto PS/PDLG+APTES+GA and PS/PDLG+PDA systems.*

It can be seen that free laccase and its immobilized form lost its activity within consecutive days of storage. However, both immobilized laccases were characterized by improved catalytic activity compared to the native counterpart over whole tested storage time. The highest activity of the enzyme was retained for systems based on PS/PDLG+APTES+GA, which confirmed the stabilization of the LAC structure by attachment to support material by APTES and GA approach. Moreover, the protection effect of support onto laccase cannot be omitted. The porous structure of PS/PDLG was probably responsible for creating a suitable microenvironment for catalytic reaction, allowing easy transport of substrates and products between the laccase's active centre and reaction solution. It should be noted that the same conclusions were drawn after determining the reusability of immobilized enzymes. In case of laccase, after 8 catalytic cycles, its immobilized forms possessed 73% and 58% of its initial activity for system with APTES+GA and PDA, respectively. The obtained results indicate that immobilized laccase was characterized by stability in terms of storage and catalyzing of consecutive catalytic cycles. Therefore, it can be concluded that this form of oxidoreductase, due to its catalytic properties, can be used in removal of hazardous compounds, e.g. dyes from aqueous solutions.

# 6. Summary and results discussion

The studies conducted within the frame of the presented PhD thesis were intended to confirm the assumed scientific hypothesis that oxidoreductases immobilized onto selected supports can be used as effective tools for removal of hazardous pollutants, such as dyes, from aqueous solutions. Therefore, the first stage the performed studies were focused on the design and fabrication of novel support materials for enzyme immobilization. In this case two types of support materials were synthesized: (i) oxide materials and (ii) electrospun fibers. The next step of the investigation was immobilization of oxidodoreductases, including laccase and horseradish peroxidase, onto the obtained materials by adsoption and covalent binding methods and insightful characterization of the produced systems. Such systems with immobilized enzymes were further applied in decolorization of dyes from model aqueous solutions at various process conditions. The detailed characterization of developed biosystems, investigation of procedures regarding attachment of biomolecules and dye removal as well as discussion and interpretation of the obtained data were presented in **Publications no. 1–6**, which constitute a series of articles which are the basis for the presented doctoral dissertation. The assumed research objectives have been achieved and discussed in detail.

**Publication no. 1** shows production of novel $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$ hybrid oxide materials, which were characterized in terms of their morphology and porosity. However, to obtain the hybrid oxide materials with higher BET surface area, that affects efficient enzyme immobilization, it was decided to produce systems made of $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ (**Publication no. 2**). These systems were characterized by more a developed surface area, equal to over 400 $m^2/g$, as compared to the $A_{BET}$ values of $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$ equal to 19.3 $m^2/g$ and 47.1 $m^2/g$, respectively. However, it was shown that the amounts of immobilized laccase onto supports, presented in both **Publications no. 1** and **no. 2**, did not differ much from each other, and ranged between 83 mg/g and 96 mg/g. This fact may probably be caused by the presence of spatial hindances in $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ materials, which prevented more efficient enzyme attachment onto these supports. The obtained results could be compared with work, which presented the synthesis of support for laccase immobilization from $Al_2O_3$. It was shown that BET surface area of aluminium oxide material was 159 $m^2/g$, which allowed to attach approx. 500 mg/g of enzyme to this support

(Kołodziejczak-Radzimska et al., 2020). Although higher amount of laccase was immobilized compared to **Publication no. 1** and **no. 2**, it was shown that $Al_2O_3$-laccase system was characterized by lower activity, approx. 70%, after 20 days of storage, compared to the $TiO_2$-$ZrO_2$, $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ with immobilized laccase, in case of which the activity exceed even 80% after the same time of storage. This shows that the obtained biocatalytic systems are characterized by high storage stability, probably caused by the stabilization of enzyme structure by immobilization and protective effect of support materials. Moreover, the kinetic parameters of free and immobilized enzymes were determined. It was presented that the $K_m$ value of a native laccase was lower, compared to $K_m$ of the oxidoreductase deposited onto the surface of produced oxide materials, which indicated higher affinity of the free laccase to the substrate. These results are also connected with $V_{max}$ values, which decidedly confirmed faster reaction rate in case of the process using free laccase, compared to its immobilized forms. The possible explanation of these observations was the formation of enzyme-support interactions and the occurrence of diffusional limitations. Although the produced biosystems possessed lower kinetic parameters, they retained high activity and exhibit improved stability compared to free proteins, which was confirmed in storage stability study. It was shown that after 20 days of storage, the produced biosystems made of $TiO_2$-$ZrO_2$, $TiO_2$-$ZrO_2$-$SiO_2$, $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ with immobilized laccase possessed over 80% of their catalytic activities, indicating good storage stability of these biocatalysts after a relatively long storage time. Further, the obtained catalytic activity of biosystems after specific storage time was higher than in recently published studies. In the work presented by Amin et al. (2018), laccase immobilized onto $Fe_3O_4$@$SiO_2$@Kit-6 retained approx. 70% of its activity after 21 days of storage. In another work, laccase immobilized onto $SiO_2$ modified by cysteine possessed only 40% of its activity after 20 days of storage (Kołodziejczak-Radzimska and Jesionowski, 2020). Finally, it was emphasized that addition of copper ions to the support material $ZrO_2$-$SiO_2$, which play crucial role in redox reaction catalyzed by laccase, positively affects activity of immobilized enzyme, which is also beneficial in terms of practical use of this biosystem.

Nevertheless, the crucial part of the research presented in **Publication no. 1** and **no. 2**, was the application of biosystems produced in removal of dyes from various groups, such as azo and anthraquinone dyes. The mechanism of dye degradation was determined, and it was

confirmed that synergistic decolorization of dye solution by adsorption onto the produced hybrid oxide materials and bioconversions by laccase occurred. Moreover, it was shown that it was possible to remove 100% of MR3 from solution at the highest investigated concentration equal to 10 mg/L using $TiO_2$-$ZrO_2$-$SiO_2$-laccase system. Furthermore, part of the investigations presented in **Publications no. 1** and **no. 2** was a study of effect of various process conditions on decolorization of selected azo and anthraquinone dyes. The attention was paid to this study as proper selection of decolorization conditions could not only increase the removal efficiency of dyes, but also optimize the entire process, especially in terms of energy use. As was presented in **Publication no. 1**, the highest decolorization efficiencies for MR3, RB19 and RB5 were obtained after 24 h of reaction at pH 5 and 25 °C, and reached 100%, 91% and 77%, respectively. In case of studies presented in **Publication no. 2**, the removal efficiencies of RB19 decolorized by both $ZrO_2$-$SiO_2$ and $ZrO_2$-$SiO_2$/$Cu^{2+}$ systems with immobilized laccase were over 90% at pH 4, 30 °C and 24 h. However, it should be strongly emphasized that the obtained results of decolorization efficiencies were higher compared to the removal data after using free form of this enzyme under reaction conditions different than optimal. These results could be compared with the study presented by Osma et al. (2010b), who immobilized laccase onto $Al_2O_3$ and used it for removal of RB19 dye from aqueous solution. Only 44% of dye decolorization efficiency was reported after reaction at room temperature and during 42 h. In another study, presented by Rani et al. (2017), it was shown that laccase immobilized onto $MnO_2$ allowed to degrade 71% of MR3 from aqueous solutions, however there was no significant difference between the efficiencies of this dye degradation by a system with immobilized laccase and control $MnO_2$. Furthermore, the toxic effect of dye solutions before and after enzymatic decolorization was determined based on results of chemical oxygen demand and toxicity studies. Both $TiO_2$-$ZrO_2$ and $TiO_2$-$ZrO_2$-$SiO_2$ with adsorbed laccase allowed for a significant reduction in the COD values of the solutions after decolorization, whereas RB19 dye solution after treatment by $ZrO_2$-$SiO_2$-laccase and $ZrO_2$-$SiO_2$/$Cu^{2+}$-laccase was characterized by a significantly lower toxicity compared to the initial dye solution. This was probably caused by production of less toxic products of catalytic conversion and also their adsorption on the obtained systems.

It was demonstrated that the produced biosystems made of immobilized laccase and oxide materials are effective tools for dye removal from aqueous solutions under various

reaction conditions. Therefore, it can be concluded that the obtained biosystems not only decolorize dye solutions, but can also decrease their harmfulness and toxicity, which can be associated with simultaneous biocatalytic conversion and sorption of dye molecules and bioconversion products onto supports. The obtained results decidedly show that the undertaken studies are important in terms of production of new, efficient tools for removal of dyes from aqueous solutions, however to improve the applicability of the proposed biosystems, future studies regarding use of these biocatalysts in continuous flow reactors are needed.

The next group of materials, investigated in terms of application in oxidoreductases immobilization and dye decolorization, were electrospun fibers. **Publication no. 3** presents production of electrospun material from nylon 6 and immobilization of laccase onto these fibers by adsorption and covalent binding method. The results obtained by analyses such as FTIR, SEM and EDS, confirmed effective production of fibers and enzyme attachment. In the next step of this study, it was decided to investigate the removal of azo dye RB5 and anthraquinone dye RB4. This decision was dictated by the fact that RB5 is one of the most commonly used dyes in textile industry and it is known to be resistant to removal from aqueous solutions (Murugesan et al., 2007), whereas removal of RB4 by biological methods is poorly presented in recently published studies. Moreover, the investigation of the effect of the dye structure on the decolorization efficiency was also performed. The most important part of **Publication no. 3** concerns the application of the proposed biosystems in decolorization of RB5 and RB4 dyes from model aqueous solutions at various process conditions, in order to determine the most suitable removal parameters. It was shown that laccase immobilized onto nylon 6 by covalent binding method allowed to degrade selected dyes with 72% and 77% efficiencies for RB5 and RB4, respectively. These results were decidedly higher, compared to the decolorization efficiencies obtained after using of biosystems with adsorbed oxidoreductase, which were equal to 45% for RB5 and 63% for RB4. This shows that laccase immobilized by covalent binding onto nylon 6 using EDC/NHS approach is better stabilized than laccase adsorbed onto this support, mantaining higher catalytic activity under reaction environment. This, in consequence, allowed to degrade the selected dyes with higher efficiencies using oxidoreductases attached to the electrospun fibers by covalent bonds. What is worth emphasizing, the obtained decolorization efficiencies

for both dyes, irrespectively of the applied biosystems, were noted at the same conditions: pH 5, 25 °C and 24 h of process.

In **Publication no. 3** it was confirmed that the systems with laccase immobilized by adsorption and covalent binding methods onto nylon 6 electrospun fibers can be effectively used for dye decolorization. The crucial part of this work was the selection of dye removal conditions to obtain the highest decolorization efficiencies of these pollutants. The obtained results of dye decolorization efficiencies after a process using laccase immobilized by both methods and its native form allowed to state that immobilized laccase, irrespectively of applied immobilization methods, decolorizes dyes solutions with higher efficiencies at different reaction conditions than optimal compared to its free counterparts. These facts definitely confirmed the increase of enzyme's catalytic properties after the immobilization process.

Promising results obtained for dye decolorization using laccase immobilized onto nylon 6 electrospun fibers, were the basis for a decision to use the same material for adsorption and covalent binding attachment of another oxidoreductase, horseradish peroxidase, and to apply this biocatalyst for removal of dyes from aqueous solutions (**Publication no. 4**). In this investigation it was crucial to select the best conditions of HRP immobilization, such as glutaraldehyde concentration (covalent binding approach), pH, temperature or time of process, for high activity of final biosystems. It was shown that the highest activities of HRP adsorbed and covalently bonded onto nylon 6 were obtained after immobilization process carried out at different conditions. For example, HRP attached to the electrospun material by adsorption was characterized by the highest catalytic activity after immobilization conducted for 60 min, at 50 °C and pH 7, whereas the covalently bonded enzyme showed the highest activity after a process performed using 3% of glutaraldehyde solution, for 60 min, 60 °C at pH 7. The important step of this investigation concerned the application of horseradish peroxidase immobilized onto nylon 6 electrospun fibers for decolorization of two types of dyes, azo dye RB5 and triarylmethane dye BG4, from model solutions imitating sea waters which contained $Na^+$, $Cl^-$ and $SO_4^{2-}$ ions. It was shown that decolorization efficiencies of RB5 and BG4 equal to even 69% and 83% were obtained in these model solutions. What is more important, after comparison of dye decolorization efficiencies, it could be seen that irrespective of the applied system made of enzyme (laccase or horseradish

peroxidase) immobilized onto nylon 6 fibers, the efficiencies of dye removal were high, the despite the fact that these enzymes possess different catalytic reaction mechanisms and co-subtrates, which are $O_2$ and $H_2O_2$, respectively.

Although different types of enzymes were immobilized onto the produced electrospun fibers in **Publications no. 3** and **no. 4**, their comparison would be benefficial for better explanation of the possibility of future use of these biosystems for a wide range application in dye removal. In this term, parameters such as storage stability and reusability of the produced biosystems made of nylon 6 and oxidoreductases were determined and compared. In case of storage stability, laccase immobilized by both adsorption and covalent binding retained 60% and 95% of its activity, respectively, after 30 days of storage, whereas after the same time horseradish peroxidase attached by adsorption and covalent bindning to the nylon 6 fibers possessed 36% and 74% its initial activity, respectively. The differences in activity of enzymes may be caused not only by the properties of the oxidoreductases, but also the type of applied linkers, which were *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide chloride (EDC) and *N*-hydroxysuccinimide (NHS) for laccase immobilization, and glutaraldehyde for horseradish peroxidase attachment. In case of the EDC/NHS approach, the treatment of nylon 6 by these chemicals consisted of modification of carbonyl group on the surface of electrospun fibers, to allow connection of enzyme by amino group to EDC/NHS arm. The different mechanism of covalent binding was presented for use of glutaraldehyde as a linker for HRP immobilization, which consisted of creation of a spacer arm as an α,β-unsaturated aldehyde product in the glutaraldehyde molecule attached to the support, which is suitable for binding of the enzyme. Therefore, the distance between the support and immobilized biomolecule can be different for both covalent binding immobilization approaches, which can strictly affect the activity and stability of attached enzymes. Despite these differences, the reusability of systems with immobilized laccase and horseradish peroxidase was similar. After 10 catalytic cycles they possessed over 60% of their initial activities, irrespectively of the applied immobilization approach. The obtained results can be compared with the work presented by Fatarella et al. (2014), in which laccase was immobilized onto nylon 6 nanofibers modified by glutaraldehyde. It was shown that only 29% of residual activity of laccase was retained after 7 catalytic cycles, which is much worse result compared to data presented in **Publication no. 3**.

After a detailed investigation of applicability of nylon 6 fibers for oxidoreductases immobilization and dye decolorization, it was decided to continue studies using electrospun fibers. Therefore, the production of hybrid electrospun material poly(methyl methacrylate)/polyaniline (PMMA/PANI) was presented in **Publication no. 5**. Similarly to **Publication no. 4**, the crucial step in this investigation was the selection of laccase immobilization conditions onto fabricated fibers by adsorption and covalent binding, which is important in terms of final properties of the obtained biosystems. For both applied immobilization approaches it was shown that the best immobilization conditions were pH 5, 25 °C, enzyme concentration 1 mg/L and time of process equal to 1 h. It should also be noted that the selected conditions for laccase immobilization and horseradish peroxidase attachment were different from each other (especially pH and temperature values), which is related to different properties of these oxidoreductases. It was shown that the best conditions for HRP immobilization were pH 7 as well as temperatures equal to 50 °C and 60 °C for adsorption and covalent binding, respectively.

After immobilization, the systems characterized by the highest catalytic activity were applied for decolorization of dye RB19 from aqueous solutions. It was shown that laccase adsorbed onto PMMA/PANI fibers removed 87% of the dye, which is higher compared to RB19 solution treatment using enzyme covalently bonded onto produced fibers (62%). This can be compared with the removal of RB19 by $TiO_2$-$ZrO_2$-laccase (**Publication no. 1**), in case of which the decolorization efficiency of this dye was equal 91%. However, the synergistic removal of RB19 using laccase adsorbed onto $TiO_2$-$ZrO_2$ should be remembered, whereas in case of system with PMMA/PANI and immobilized oxidoreductase the sorption process of dye molecules onto fibers is negligible, which indicates the enzymatic conversion as the main driving force of the dye conversion. An interesting finding was made for decolorization efficiencies of dyes, the results of which were presented in **Publications no. 4** and **no. 5**. It was noted that dyes were decolorized with higher efficiencies using laccase adsorbed onto electrospun fibers, which were nylon 6 and PMMA/PANI, respectively, compared to the enzyme covalently bonded onto such fibers. The most probable reason for these observations was the fact that covalent bonds may disrupt the structure of the enzyme. Additionally, the presence of modifiers on the support surface disturbs the flow of the substrate and products between enzyme and reaction environment. In this work

the storage stability studies were also conducted, which showed that laccase immobilized by both adsorption and covalent binding methods possessed approx. 80% of its catalytic activity after 30 days of storage. This can be compared with other works, which present laccase immobilization onto different electrospun fibers. Xu et al. (2013) immobilized this oxidreductase onto modified fibers made of chitosan and poly(vinyl alcohol) by glutaraldehyde. Such biosystem possessed only 20% of initial activity of laccase after 14 days of storage. In another work Dai et al. (2010) immobilized laccase by encapsulation method into PDLLA/PEO-PPO-PEO fibers and it was shown that the produced biocatalyst possessed approx. 50% of activity after 2 weeks of storage.

The important conclusion, which can be drawn after analysis of the data presented in **Publications no. 3–5**, is that the electrospun fibers from nylon 6 and hybrid PMMA/PANI with immobilized oxidoreductases can be be effective, future-proof biotools for removal of dyes from aqueous solutions. However, to better understand the mechanism of laccase covalent immobilization using electrospun fibers as supports, it was decided to conduct a study presented in **Publication no. 6**. In this investigation, two approaches of laccase immobilization by covalent binding onto polystyrene/poly(D,L-lactide-*co*-glycolide) (PS/PDLG) fibers were presented and compared. It was shown that laccase immobilized onto electrospun fibers using both methods can convert substrates with 100% efficiency at 25 °C and pH 5. This can be compared with the study regarding laccase immobilization onto nylon 6 electrospun material (**Publication no. 3**) and PMMA/PANI (**Publication no. 5**). These works also showed that immobilized laccase is able to convert dye substrates at mild conditions with the highest efficiencies. Moreover, it should also be emphasized that activity of laccase immobilized onto PS/PDLG stored for 10 days and reused in 8 consecutive catalytic cycles was lower compared to the laccase immobilized by covalent binding onto nylon 6 (**Publication no. 3**) and PMMA/PANI (**Publication no. 5**). After this time of storage, the laccase immobilized onto PS/PDLG fibers by two proposed immobilization approaches (APTES+GA and PDA) retained approx. 80% of its activity, whereas after the same time of storage, laccase covalently bonded using the EDC/NHS approach onto nylon 6 and PMMA/PANI fibers exhibited over 90% of its relative activity. This shows that enzyme activity strongly depends on the applied modifier/linker, used for enzyme immobilization by covalent binding.

The proposed hybrid oxide and/or electrospun materials with attached oxidoreductase, presented in **Publications no. 1–6**, may effectively decolorize selected azo, anthraquinone and triarylmethane dyes from aqueous solutions. Therefore, to highlight the application potential of the presented solutions and produced systems for dye decolorization, they were compared with selected, previously published studies on dye removal (Table 7).

***Table 7**. Comparison of efficiencies of removal of hazardous compounds by biosystems made of hybrid oxide materials and/or electrospun fibers with immobilized oxidoreductases.*

| Type of support | Enzyme | Support | Pollutant | Process conditions | Process efficiency | Storage stability | Literature |
|---|---|---|---|---|---|---|---|
| Oxide materials | Laccase | $MnO_2$ | MR3 | pH 7, 31 °C, 60 min | 71% | na | (Rani et al., 2017) |
| | Laccase | $SiO_2$ | RB19 | pH 5, 23 °C, 6 h | Around 70% | 71% of dye decolorization after 4 months of biosystem storage | (Champagne and Ramsay 2007) |
| | Laccase | $Al_2O_3$ | RB19 | room temperature, 42 h | 44% | na | (Osma et al., 2010b) |
| | Laccase | $TiO_2$-$ZrO_2$<br>$TiO_2$-$ZrO_2$-$SiO_2$ | 1. MR3<br>2. RB19<br>3. RB5 | pH 5, 25 °C, 24 h | 1. 100%<br>2. 91%<br>3. 77% | Approx. 80% of laccase catalytic activity after 50 days of storage | **Publication no. 1** |
| | Laccase | 1. $ZrO_2$-$SiO_2$<br>2. $ZrO_2$-$SiO_2$/$Cu^{2+}$ | RB19 | pH 4, 30 °C, 24 h | 1. 90%<br>2. 98% | Approx.80% of laccase catalytic activity after 20 days of storage | **Publication no. 2** |
| Electrospun fibers | Laccase | PDLLA/PEO-PPO-PEO | BV3 | pH 6, 30 °C, 5 h | 88% | Approx.50% of laccase catalytic activity after 14 days of storage | (Dai et al., 2010) |
| | Laccase | HPEI/PES | BPA | pH 7, 25 °C, 4 h | 89% | na | (Koloti et al., 2018) |
| | Laccase | CS/PVA | 2,4-DCP | pH 6, 50 °C, 6 h | 87.6% | Approx.20% of laccase catalytic activity after 14 days of storage | (Xu et al., 2013) |
| | Laccase | Nylon 6 | 1. RB5<br>2. RB4 | pH 5, 25 °C, 24 h | 1. 72%<br>2. 77% | 95% of laccase catalytic activity after 30 days of storage | **Publication no. 3** |
| | Horseradish peroxidase | Nylon 6 | RB5<br>BG4 | pH 7, 25 °C, 60 min | Over 70% for both decolorized dyes | Approx. 80% of horseradish peroxidase catalytic activity after 30 days of storage | **Publication no. 4** |
| | Laccase | PMMA/PANI | RB19 | pH 5, 30 °C, 24 h | 87% | Approx. 80% of laccase catalytic activity after 30 days of storage | **Publication no. 5** |
| | Laccase | PS/PDLG | ABTS | pH 5, 25 °C, 1 h | 100% | Approx. 80% of laccase catalytic activity after 10 days of storage | **Publication no. 6** |

2,4-DCP – 2,4-dichlorophenol; ABTS – 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; BG4 – Basic Green 4; BPA – bisphenol A; BV3 – C.I. Basic Violet 3; CS – chitosan; HPEI – hyperbranched polyethyleneimine; MR3 – C.I. Mordant Red 3; PANI – polyaniline; PDLLA – poly(D,L-lactide); PEO – poly(ethylene oxide); PES – polyethersulfone; PMMA – poly(methyl methacrylate); PPO – poly(*p*-phenylene oxide); PVA – poly(vinyl alcohol); RB19 – C.I. Reactive Blue 19; RB5 – C.I. Reactive Black 5; na – not available

The most important conclusion is that in most cases the biosystems presented in this study are capable of more efficient removal of various dyes at mild conditions or during shorter process duration, as compared to previously published studies. It should be stated that the occurred differences between decolorization efficiencies presented in publications which are the basis of the doctoral thesis and other studies are probably due to more stable interactions between the immobilized oxidoreductases and used hybrid oxide materials and electrospun fibers, which possess properties that make them suitable supports for enzyme immobilization. Moreover, it should be emphasized that scientific reports on application of electrospun fibers with immobilized enzymes in dye decolorizations are very limited, making the novelty of the presented study undeniable. Therefore, the examples regarding electrospun materials with immobilized enzymes and their application in removal of other pollutants from waters are given. The presented comparison shows that the biosystems made of novel hybrid oxide materials and electrospun fibers and immobilized oxidoreductases possess great application potential in removal of dyes from aqueous solution and high stability during storage.

# 7. Conclusions and future outlooks

The presented doctoral thesis is focused on the production of a novel group of multifunctional materials made of hybrid oxides and/or electrospun fibers with immobilized oxidoreductases. The obtained biocatalytic systems were applied as tools for dye decolorization from model aqueous solutions. Results of characterization of the produced systems before and after enzyme immobilization confirm the effective production of oxide materials and electrospun fibers as well as the attachment of enzymes onto their surface using various approaches. The conducted tests regarding dye decolorization using the obtained biosystems allowed to confirm the applicability of the immobilized laccase and horseradish peroxidase for dye removal.

The first step of the study was the design and production of support materials at specific synthesis conditions, which was one of the research tasks. The well-developed porous structure of the produced supports and the presence of specific funtional groups on their surface, such as hydroxyl, carbonyl or amino moieties, were confirmed, making the oxide and electrospun materials susceptible for enzyme immobilization. Therefore, such supports were next used for immobilization of laccase and horseradish peroxidase by two types of enzyme attachment approaches: (i) adsorption and (ii) covalent binding. The produced systems with immobilized biomolecules were characterized in detail in order to determine changes in support morphology upon immobilization as well as to confirm effective immobilization of proteins onto the produced supports, to determine the amount of immobilized laccase and horseradish peroxidase and to calculate kinetic parameters of free and immobilized enzymes, which was one of the crucial objectives during the conducted research. The most suitable immobilization conditions of laccase and horseradish peroxidase were determined in order to obtain biosystems with the highest activity. It was shown that the best immobilization conditions for laccase were similar for each of the applied biosystems with this oxidoreductase, whereas in case of horseradish immobilization the optimal process conditions varied from the best attachment conditions of “blue oxidoreductase”, which was caused by different features of these two enzymes. After optimization of immobilization, highly active biocatalytic systems were obtained, which was one of the goals of the conducted research and a milestone for further studies. The systems with immobilized oxidoreductases, characterized by high catalytic properties, were applied for decolorization of dyes from three

groups of dyes: (i) azo, (ii) anthraquinone and (iii) triarylmethane. It should be noted that the dye decolorization process was investigated under various conditions, such as dye concentration, pH, temperature, and process duration, which is important in terms of optimization of decolorization reactions and future application of the obtained biosystems in technological processes. These studies demonstrated high decolorization efficiencies at mild reaction conditions, using free and immobilized enzymes. However, under different conditions that optimal the immobilized enzymes degraded selected pollutants with higher efficiencies than their free counterparts, which decidedly confirmed the application potential of the produced biosystems. The obtained data allow to state that biocatalytic systems capable of effective dye decolorization, based on hybrid oxide materials or electrospun fibers and oxidoreductases, were obtained within the frame of the study. Moreover, such biosystems were characterized by improved storage stability and reusability compared to other systems presented in recently published studies. Even after 10 consecutive catalytic cycles, all tested immobilized oxidoreductases retained over 60% of their initial activity. Mechanisms of enzyme immobilization by adsorption and covalent binding as well as dye degradation by adsorption and catalytic conversion were also determined and described. It was demonstrated that the mechanism of enzyme immobilization has a significant impact on the efficiency of dye removal. Furthermore, it was shown that the possibility of dye decolorization by synergistic biocatalytic conversion and adsorption using oxides-based biosystems is promising for further widespread application.

The obtained results were discussed in detail and presented in **Publications no. 1–6.** It should be strongly emphasized that the conducted investigations allowed to confirm the hypothesis that immobilized oxidoreductases, such as laccase and horseradish peroxidase, may act as efficient biocatalytic tools for dye conversion from aqueous solutions. The obtained results and determined dependencies undeniably confirmed the importance of the novel biocatalytic tools for decolorization of selected pollutants from waters. The satisfactory results of dye removal allow to conclude that the proposed systems made of hybrid oxides and/or electrospun materials and immobilized oxidoreductases are effective biocatalyst for dye decolorization and may be a promising alternative for other methods of pollutants removal. The conducted researches and drawn conclusions are sources of new knowledge about the mechanisms of enzyme immobilization and removal of dyes from model

solutions. However, the obtained biocatalytic systems possess some limitations in use, which should be undertaken and solved in future investigations. One of them is the relatively low decolorization efficiencies of dyes under extremely harsh reaction conditions. To obtain higher removal rates of such compounds, the activity of immobilized oxidoreductase should be improved. One of the ways can be controlled dosing of $O_2$ or $H_2O_2$ as co-substrates for laccase and horseradish peroxidase, respectively, to the reaction mixture with dyes. Moreover, in case of horseradish peroxidase, the effect of iron and calcium ions, the presence of which affects the activity and stability of this enzyme, should be examined. Moreover, the future investigation in terms of the use of these biosystems in continuous flow bioreactors is required. It might improve the applicability of the proposed biosystems for dye degradation from real wastewaters, which are usually delivered continously to the sewage treatment plant. Nevertheless, it should be emphasized that the biocatalytic systems presented in this study can also be used in bioremoval processes of other hazardous compounds such as pharmaceuticals or phenols. The advanced investigations in this area can confirm the usability of the produced systems for removal of various compounds from aqueous solutions. Finally, the application of oxidoreductases attached onto hybrid oxide materials and electrospun fibers in real textile wastewaters would help to determine dye removal efficiencies from solutions in the presence of other hazardous compounds and inhibitors. Despite the fact that the production and application of immobilized oxidoreductases require further research, solutions presented in this study should be considered as promising alternatives for real wastewater treatment. Therefore, the obtained results and dependencies as well as developed solutions can help in the wider application of immobilized enzymes for the removal of not only dyes, but also other hazardous compounds.

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