

VILNIUS GEDIMINAS TECHNICAL UNIVERSITY

Rūta IVANEC-GORANINA

BIOCATALYTIC OXIDATION OF AROMATIC HYDROXY DERIVATIVES

SUMMARY OF DOCTORAL DISSERTATION

TECHNOLOGICAL SCIENCES,
CHEMICAL ENGINEERING (05T),
BIOTECHNOLOGY (T490)



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VILNIAUS GEDIMINO TECHNIKOS UNIVERSITETAS

Rūta IVANEC-GORANINA

BIOKATALIZINĖ AROMATINIŲ HIDROKSI DARINIŲ OKSIDACIJA

DAKTARO DISERTACIJOS SANTRAUKA

TECHNOLOGIJOS MOKSLAI,
CHEMIJOS INŽINERIJA (05T),
BIOTECHNOLOGIJA (T490)



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Introduction

Formulation of the problem

Phenol derivatives form one of the most important groups of chemicals that are widely used in chemical industry, scientific research centers and are found in nature. In nature phenol derivatives are widespread among secondary metabolites of plants that perform various functions. On the other hand, compounds based on phenol derivatives synthesized by chemical industry, are treated as environment pollutants. From the point of view of environment protection in many cases they are classified as toxic and should be decontaminated in a secure manner. One of the decontamination methods is biocatalytic oxidation of phenol derivatives. In order to insure effective biocatalytic oxidation of phenol derivatives it is necessary to select appropriate reagents, including newly discovered, and optimize the scheme of their application. The task of reagents selection and application scheme optimization is important from the scientific and practical point of view since on one hand it allows application of the most effective enzyme (in aspect of bioengineering, economics, etc.), and on the other hand defines an optimal enzyme application scheme. In this work oxidation of phenol derivatives in presence of *Coprinus cinereus* and horseradish peroxidases and *Coriolopsis byrsina* laccase is being analyzed. Phenol derivatives being analyzed: 4-hydroxyphenolacetic acid, 1-hydroxypyrene, phenol, 1-naphthol, 2-naphthol, 1-hydroxypyrene, 2-hydroxyanthracene, 9-phenanthrol. The phenol derivatives were selected based on their structure and environmental effect.

While selecting the conditions for biocatalytic oxidation reactions it is very important not limit the investigation only to the selection of enzymes. One of the most perspective research areas is application of mediators in oxidation processes of phenol derivatives. Mediator application tasks face with issues of mediator selection and application cost effectiveness. The recently started investigations of phenoxazine derivatives have shown that in biocatalytic processes they can be classified as high reactivity substrates. They have shown good mediator properties in oxidation of aromatic N-hydroxy derivatives. In this work while investigating the biocatalytic oxidation of aromatic hydroxy derivatives the representative of phenoxazines 3-(4a,10a-dihydro-phenoxazin-10-yl)-propane-1-sulfonic acid (PPSA) was used.

Topicality of the thesis

Aromatic hydroxy derivatives are treated as environment pollutants and effective methods of their decontamination are very important. Bioutilization methods are usually not very expensive and environmentally friendly.

Pollutants can be degraded with the help of enzymes synthesized by bacteria, fungi or plants. Progress of genetic engineering also provides possibilities for application of recombinant enzymes. It is important to create a model scheme that identifies the biocatalytic oxidation processes of these compounds.

Biocatalytic oxidation of phenol derivatives has several limitations: inactivation of enzymes during the reaction and slow oxidation of hardly degrading compounds. That is why it is important to investigate mechanisms and regularities of these reactions.

The object of research

The object of present work is enzyme-catalyzed oxidation of aromatic hydroxy derivatives.

The goal of the thesis – to investigate and optimize biocatalytic oxidation of aromatic hydroxy derivatives.

The tasks of the thesis

1. To investigate the kinetics of 4-hydroxyphenylacetic acid and 1-hydroxypyrene oxidation catalyzed with recombinant *Coprinus cinereus* (rCiP) and horseradish (HRP) peroxidases.
2. To investigate the kinetics of rCiP catalyzed oxidation of hydroxy aromatic derivatives phenol, 1-naphtol, 2-naphtol, 1-hydroxypyrene, 2-hydroxyanthracene, 9-phenanthrol in presence of surfactants.
3. To investigate oxidation of hardly degrading compound bisphenol A, catalyzed by newly discovered *Coriopsis byrsina* laccase and mediator PPSA.
4. To apply peroxidase-catalyzed 1-hydroxypyrene oxidation reaction for determination of hydrogen peroxide concentrations and perform mathematical modeling and optimization of this reaction.

Methodology of research

In this work kinetic fluorimetric, spectrophotometric, light scattering and dynamic light scattering methods and mathematical modeling were used.

Scientific novelty of the thesis

The aspects of scientific novelty for chemistry engineering (biotechnology) are as follows:

1. The scheme, used for the optimization of the biocatalytic oxidation of phenol derivatives in presence of monomer form of (bio)surfactants and micelles of (bio)surfactants, was proposed.

2. The investigation of hardly degrading compound bisphenol A oxidation catalyzed by newly discovered *Coriolopsis byrsina* laccase in presence of mediator PPSA was performed.
3. The new scheme for detection of nanomolar concentrations of hydrogen peroxide based on the peroxidase-catalyzed 1-hydroxypyrene oxidation reaction was proposed.

Practical value of the results

The performed investigations of biocatalytic oxidation of phenol derivatives can be used for intensification of peroxidase-catalysed biotechnological processes which can be applied while developing environmentally friendly decontamination methods of phenol compounds in different types of treatment plants. Environmentally friendly methods have an advantage over traditional decontamination methods due to their less environmental impact.

Biocatalytic 1-hydroxypyrene oxidation reaction was applied for detection of nanomolar concentrations of hydrogen peroxide. The proposed scheme can be used in environmental monitoring of hydrogen peroxide concentrations.

Defended propositions

1. Surfactants and biosurfactants, used with concentrations lower than the critical micelle concentration (CMC), decrease or completely stop the enzyme inactivation and increase the conversion of phenol derivatives. Surfactants and biosurfactants used over the CMC concentration again cause the enzyme inactivation.
2. Oxidation of hardly degrading compound bisphenol A can be increased by using newly discovered *Coriolopsis byrsina* laccase and PPSA mediator.
3. High 1-hydroxypyrene oxidation constant and fluorescence quantum yield allows using this reaction for determination of nanomolar hydrogen peroxide concentrations.

Approval of the results

4 scientific articles were published on the topic of thesis: 3 in scientific journals indexed in Thomson Reuters Web of Science and 1 in other reviewed publication.

The results of the dissertation were discussed at 4 international and national scientific conferences:

- The 10th International Symposium on Peroxidases „OxiZymes 2012 in Marseille“, 2012, Marseille, France;

- The 12th Lithuanian Biochemical Society Conference „50 years for Biochemistry studies in Lithuania“, 2012, Tolieja, Lithuanian;
- The 11th Lithuanian Conference of Young Scientists „Science – Future of Lithuania“, 2008, Vilnius, Lithuanian;
- The Conference of Young Scientists „Biofuture: Perspectives of Nature and Life Sciences“, 2008, Vilnius, Lithuanian.

The scope of the scientific work

The scientific work consists of introduction, 3 chapters, conclusions, references, list of the author's scientific publications on the dissertation topic. The total scope of the dissertation is presented in 123 pages, 30 pictures and 12 tables. 187 sources of literature have been used in the dissertation.

1. Literature overview on the topic of biocatalytic oxidation of aromatic hydroxy derivatives

The first chapter of dissertation is dedicated to literature overview. The phenol derivatives and their environmental impact are described. The peroxidase and laccase enzymes, catalyzing the phenol derivatives oxidation and oxidation mechanisms are described. Overview of research performed by other authors on the biocatalytic oxidation of phenol derivatives is also presented. The review covers the limitations of peroxidase and laccase catalysis: peroxidase inactivation dependence on hydrogen concentration; inactivation of peroxidases and laccases during phenol derivatives oxidation; slow oxidation of hardly degrading phenol derivatives; measures for eliminating the described limitations.

2. Investigation methods and materials

In the second chapter the materials and methods are presented: fluorimetric, spectrophotometric, light scattering and dynamic light scattering. Mathematical calculations and modeling schemes are presented: modeling of steady state kinetics based on classic peroxidase action scheme; modeling of phenol derivatives initial oxidation rates dependence on pH; modeling of non-steady state kinetics with added peroxidase inactivation steps to the peroxidase action scheme; modeling of monomer of surfactants interaction with oligomers produced during phenol derivatives oxidation; modeling of steady state kinetics based on laccase action scheme with mediator. Steady state conditions processes were modeled using the symbolic processor of the MathCAD 2001 Prof program. Non-steady state conditions processes were modeled using the

KinFitSim 2.0 program. KinFitSim solve ordinary differential equations corresponding to the analyzed schemes. The system of differential equations is omitted due to standard mathematical formulation. The program uses non-linear least squares regression to find the best fit between experimental and calculated curves. The search technique was used for parameters optimization, or for finding the minimum of the sum of squared differences function.

3. Results and discussion of the investigation of biocatalytic oxidation of aromatic hydroxy derivatives

3.1. Peroxidase catalyzed oxidation of phenol derivatives

The oxidation of 4-hydroxyphenylacetic acid and of 1-hydroxypyrene catalyzed with recombinant *Coprinus Cinereus* (rCiP) and horseradish peroxidases (HRP) was investigated at pH 8.0. The bimolecular constants of 4-hydroxyphenylacetic acid oxidation with the ferryl compounds of rCiP and HRP are equal to $(2.4 \pm 0.6) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $(1.4 \pm 0.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively. At pH 8.0 the bimolecular constants of 1-hydroxypyrene oxidation with ferryl compounds of rCiP and HRP are equal to $(1.0 \pm 0.3) \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and $(0.6 \pm 0.2) \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively.

The research showed that 4-hydroxyphenylacetic acid and of 1-hydroxypyrene oxidation with peroxidases initial reaction rates depend on pH. pKa values of rCiP and HRP active forms were estimated.

The fluorescence quantum yield of 1-hydroxypyrene is equal to 0.66 (pH 8.0, 25 °C).

3.2. Peroxidase catalyzed oxidation of phenol derivatives in presence of surfactants

It is known that addition of (bio)surfactants can decrease or stop peroxidase inactivation during the oxidation of phenol derivatives. Still the exact mechanism of (bio)surfactant action during the biocatalytic oxidation of phenol derivatives was not known and systemized. Due to that reason the further investigation covered the peroxidase catalyzed oxidation of phenol derivatives in presence of (bio)surfactants.

The kinetics of fungal peroxidase-catalyzed phenol derivatives oxidation was investigated in presence of synthetic surfactant Dynol 604 at pH 5.5 and 25 °C. It was shown that the presence of micromole concentrations of surfactant didn't influence initial rate of phenol derivatives oxidation. The calculated apparent bimolecular rate constants are $(1.8 \pm 0.2) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$,

$(1.4 \pm 0.4) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $(1.30 \pm 0.06) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $1.1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for phenol, 1-naphthol, 2-naphthol and 1-hydroxypyrene, respectively.

Table 1. Kinetic parameters of peroxidase-catalyzed oxidation of phenol, 1-naphthol, 2-naphthol and 1-hydroxypyrene in the presence of Dynol 604 in 50 mM acetate buffer pH 5.5, 25 °C

Phenol derivatives	Dynol 604, μM	k_2 , $\text{M}^{-1}\text{s}^{-1}$	k_3 , $\text{M}^{-1}\text{s}^{-1}$	k_{in} , $\text{M}^{-1}\text{s}^{-1}$
Phenol	0	1.5×10^5	1.2×10^5	6.0×10^2
Phenol	16	1.9×10^5	1.1×10^5	6.0×10^2
Phenol	77	1.9×10^5	1.1×10^5	6.0×10^2
1-naphthol	0	1.0×10^7	4.0×10^6	2.2×10^4
1-naphthol	0.8	1.3×10^7	3.4×10^6	1.2×10^4
1-naphthol	1.6	1.0×10^7	3.4×10^6	7.0×10^3
1-naphthol	4	1.2×10^7	3.8×10^6	3.5×10^3
1-naphthol	8	1.8×10^7	3.9×10^6	1.4×10^3
1-naphthol	16	2.1×10^7	4.3×10^6	5.0×10^2
2-naphthol	0	1.3×10^7	1.1×10^7	1.1×10^4
2-naphthol	1.6	1.3×10^7	1.1×10^7	5.6×10^3
2-naphthol	4	1.2×10^7	1.0×10^7	2.9×10^3
2-naphthol	8	1.4×10^7	1.0×10^7	2.6×10^3
2-naphthol	16	1.3×10^7	1.0×10^7	1.7×10^3
2-naphthol	48	1.3×10^7	1.2×10^7	6.0×10^2
1-hydroxypyrene	0	1.1×10^8	1.0×10^8	3.7×10^3
1-hydroxypyrene	32	1.1×10^8	1.0×10^8	1.8×10^3
1-hydroxypyrene	117	1.1×10^8	1.0×10^8	2.0×10^2

During an extensive substrates conversion Dynol 604 showed diverse action for different phenol derivatives. The oxidation of phenol practically didn't change, whereas the surfactant enhanced the conversion of 1-naphthol, 2-naphthol and 1-hydroxypyrene in dose response manner. The results accounted by a scheme, which contains a stadium of enzyme inhibition by phenol derivatives intermediates. The fitting of data gave constants of phenol derivatives reactivity with the ferryl compounds of rCiP (with compound I (k_2) and with compound II (k_3)), which were similar at different surfactant concentrations (Table 1). The calculated k_{in} was low for phenol, and did not change at different surfactant concentration correlating with the surfactant influence to the reaction. For 1-naphthol, 2-naphthol and 1-hydroxypyrene the k_{in} value decreased if surfactant concentration has increased (Table 1). The results have shown that k_2 and k_3 values did not change at different surfactant concentration for all substrates. This means that surfactant Dynol 604 does not interact with peroxidase. The decrease of the inhibition constant (k_{in}) value

when surfactant concentration has increased could be explained by reversible oligomeric phenol derivatives oxidation products interaction with surfactant.

It can be seen from the investigation of peroxidase catalyzed oxidation of phenol derivatives in presence of Dynol 604 that the highest enzyme inactivation and the best biosurfactants application results were achieved in case of naphthol oxidation. Due to that reason the 2-naphthol substrate was selected for further investigation and more detailed analysis of peroxidase catalyzed 2-naphthol oxidation in presence of different (bio)surfactants was performed. The critical micelle concentrations (CMC) of (bio)surfactants were determined using the dynamic light scattering technique.

The kinetics of the rCiP-catalyzed 2-naphthol oxidation was investigated in presence of synthetic surfactants Surfydol 465, Triton X-100 and biosurfactants JBR 425 and Escin at pH 5.5 and 25 °C, with concentrations of (bio)surfactants both less than critical micelle concentrations (CMC) and larger than CMC.

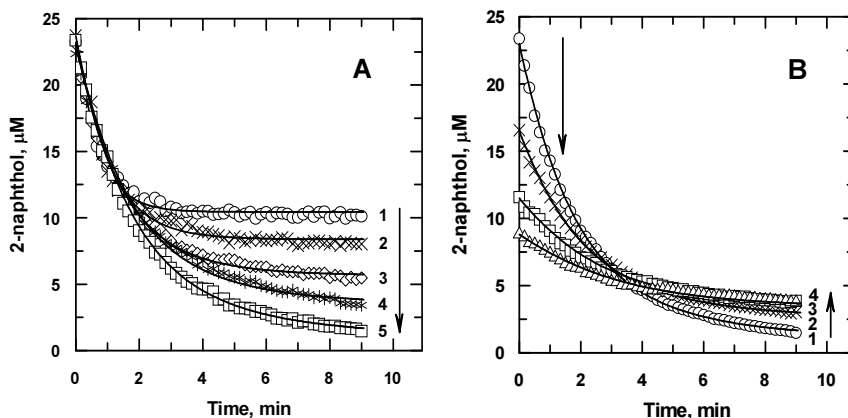


Fig. 1. Kinetics of peroxidase-catalyzed 2-naphthol oxidation in presence rhamnolipid biosurfactant JBR 425 when concentrations of biosurfactant are less than CMC (A) and when concentrations of biosurfactant are larger than CMC (B). The reaction mixture contained 25 μM 2-naphthol, 1 nM rCiP, 100 μM H_2O_2 , JBR 425 (A): 0 μM (1), 0.25 μM (2), 1 μM (3), 3 μM (4), 6 μM –0.1 mM (5); JBR 425 (B): 0.1 mM (1), 1 mM (2), 2 mM (3), 3 mM (4); in 50 mM acetate buffer pH 5.5, 25 °C. Curves were drawn through the points are the approximations of the experimental data according to the proposed model

It was shown that monomers of synthetic surfactants as well as monomers of biosurfactants had an enhancing effect on the conversion of 2-naphthol in

dose response manner and did not influence the initial rate of 2-naphthol oxidation (Fig. 1A). When (bio)surfactants' concentrations are larger than CMC (bio)surfactants have an opposite effect on the oxidation of 2-naphthol by peroxidase (Fig. 1B).

The results were explained by a scheme, which contains a stadium of enzyme inhibition by oligomeric 2-naphthol oxidation products. The action of the (bio)surfactant's monomers was explained by avoidance of the enzyme active center clothing with oligomers (Fig. 2B). When (bio)surfactants concentrations are larger than CMC (bio)surfactants the 2-naphthol distributes between water and the micellar phase, and rCiP catalyzes the oxidation of the 2-naphthol that is in water solution. In this case (Fig. 2C) the enzyme inhibition during the reaction was shown again.

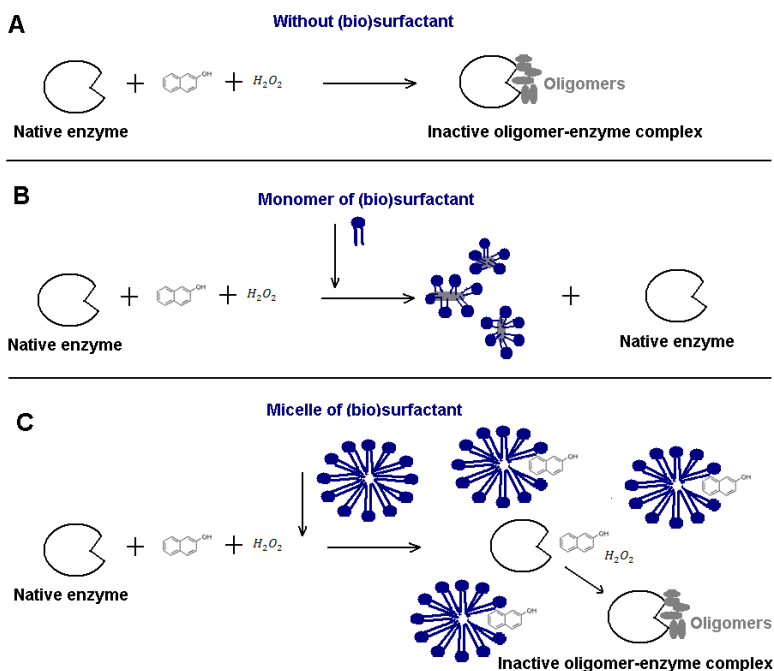


Fig. 2. Schematic representation of the peroxidase-catalyzed oxidation of 2-naphthol without (bio)surfactant (A), with addition of (bio)surfactant in a monomer form (B), with addition of (bio)surfactant in a micelle form (C)

It was shown that both synthetic surfactants and biosurfactants had same effect on the oxidation of 2-naphthol by peroxidase. Similar results have demonstrated the potential of biosurfactants due to their biodegradability. Because of that reason it was decided to use only biosurfactants (JBR 425 and Escin) in further investigations of biocatalytic oxidation of phenol derivatives.

The biocatalytic oxidation of 2-hydroxyanthracene and 9-phenanthrol in presence of JBR 425 and Escin was investigated. It was shown that monomers of biosurfactants had an enhancing effect on the conversion of both investigated phenol derivatives in dose response manner and did not influence the initial rate of these substrates oxidation. The obtained results are similar to those in 2-naphthol case.

3.3. Laccase catalyzed oxidation of hardly degrading compound bisphenol A in presence of mediator

Another limitation of phenol derivatives oxidation – oxidation of hardly degrading derivatives. The newly isolated from *Corioloropsis byrsina* laccase (CbL) slowly oxidizes bisphenol A. The addition of 3-(4a,10a-dihydro-phenoxazin-10-yl)-propane-1-sulfonic acid (PPSA) acting as a mediator increased laccases-catalyzed BisA oxidation rate. Kinetic parameters were calculated (Table 2).

Table. 2. Kinetic characteristics of laccase-catalyzed oxidation of bisphenol A in the presence of PPSA in 50 mM acetate buffer pH 5.5, 25 °C

Calculation conditions	k_2' , $M^{-1}s^{-1}$	k_3' , $M^{-1}s^{-1}$	k_4' , $M^{-1}s^{-1}$
Apparent bimolecular constants calculated from direct experimental data	$(3.3 \pm 0.2) \times 10^5$	$(5.2 \pm 0.6) \times 10^3$	-
Steady-state kinetics bimolecular constants calculated by scheme modeling	$(3.9 \pm 0.2) \times 10^5$	$(5.8 \pm 0.3) \times 10^3$	$> 1.6 \times 10^3$

As it can be seen from Table 2, k_2' and k_3' values, obtained both from modelling and experimental data, are equal in error limits. Approximated constant k_4' shows the lowest value, at which chemical reaction can limit the process.

Coincidence of constants show that steady– state kinetics model conform to real processes and characterize them correctly. The calculated value of k_2' constant characterize the reactivity of PPSA with laccase, which is almost 100 times higher than bimolecular constant k_3' , characterizing reactivity of bisphenol A with laccase. The high PPSA reactivity determines the proportional increase of mediator process rate.

3.4. Application of peroxidase catalyzed 1-hydroxypyrene oxidation for hydrogen peroxide determination

Practical application of the investigated 1-hydroxypyrene oxidation processes is its use for determination of hydrogen peroxide concentration, which can be also important in some tasks of environment monitoring. High bimolecular constants and fluorescence quantum yield of 1-hydroxypyrene permitted detection as low as 21 nM of hydrogen peroxide. To optimize the detection system 1-hydroxypyrene oxidation was modeled at steady-state conditions in the range of pH from pH 5.0 to pH 8.0 (Fig. 3A).

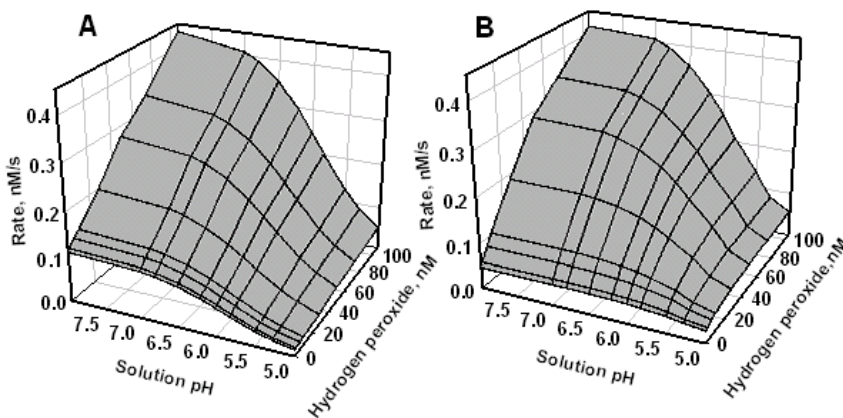


Fig. 3. The dependence of initial 1-hydroxypyrene (A) and Amplex Red (B) oxidation rate on hydrogen peroxide concentration and solution pH. For calculations 150 nM 1-hydroxypyrene (A) / Amplex Red (B), 0.4 nM rCiP, 0–107 nM hydrogen peroxide, (A): $pK_1' = 5.9$, $k_1^* = 3.9 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $pK_1 = 5.4$, $pK_2 = 8.4$ and $pK_3 = 10.4$, $k_{\text{lim}}^* = 1.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, (B): $pK_1' = 3.9$, $k_1^* = 1.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, $pK_1 = 6.2$ and $pK_2 = 9.0$, $k_{\text{lim}}^* = 1.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ was used

The 1-hydroxypyrene based detection system was compared with Amplex Red system (Fig. 3B). Peroxidase-catalyzed 1-hydroxypyrene oxidation system was used for determination of hydrogen peroxide in the buffer solution.

General conclusions

1. At pH 8.0 the bimolecular constants of 4-hydroxyphenylacetic acid oxidation with the ferryl compounds of recombinant *Coprinus Cinereus* (rCiP) and horseradish peroxidases (HRP) are equal to $(2.4 \pm 0.6) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $(1.4 \pm 0.1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively. The bimolecular constants of 1-hydroxypyrene oxidation with the ferryl compounds of rCiP and HRP are equal to $(1.0 \pm 0.3) \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and $(0.6 \pm 0.2) \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively. Fluorescence quantum yield of 1-hydroxypyrene is equal to 0.66 (pH 8.0, 25 °C).
2. Surfactants and biosurfactants, used with concentrations lower than the critical micelle concentration (CMC), decrease or fully stop the enzyme inactivation and increase the conversion of phenol derivatives. With surfactants and biosurfactants used over the CMC concentration, enzyme inactivation was detected during the peroxidase catalyzed phenol derivatives oxidation.
3. Addition of PPSA mediator increases the laccase catalyzed initial oxidation rate of bisphenol A up to 3 times. The calculated constant, characterizing reactivity of PPSA with laccase, is almost 100 times higher than bimolecular constant, characterizing reactivity of bisphenol A with laccase.
4. Peroxidase-catalyzed 1-hydroxypyrene oxidation reaction was applied for detection of nanomolar concentrations of hydrogen peroxide, mathematical modeling and optimization of the proposed scheme was performed (the highest reaction rate was achieved at pH 8.0). The determined detection limit of hydrogen peroxide is equal to 21 nM (pH 8.0, 25 °C).

List of published works on the topic of the dissertation in the reviewed scientific periodical publications

Ivanec-Goranina, R.; Kulys, J. 2013. Effects of rhamnolipid biosurfactant JBR 425 and synthetic surfactant Surfynol 465 on the peroxidase-catalyzed oxidation of 2-naphthol, *Journal of Environmental Sciences* 25(7): 1431–1440. ISSN 1001-0742. (Thomson Reuters Web of Knowledge) IF = 1,773 (2012)

Kulys, J.; Ivanec-Goranina, R. 2009. Peroxidase catalyzed phenolic compounds oxidation in presence of surfactant Dynol 604: A kinetic investigation, *Enzyme and Microbial Technology* 44: 368–372. ISSN 0141-0229. (Thomson Reuters Web of Knowledge) IF = 2,592 (2012)

Ivanec-Goranina, R.; Kulys, J. 2008. Kinetic study of peroxidase-catalyzed oxidation of 1-hydroxypyrene. Development of a nanomolar hydrogen peroxide detection system, *Central European Journal of Biology* 3(3): 224–232. ISSN 1895-104X. (Thomson Reuters Web of Knowledge) IF = 0,818 (2012)

In the other editions

Dorožko, D; Ivanec-Goranina, R.; Kulys, J. 2008. The analysis of peroxidase-catalyzed p-hydroxyphenylacetic acid oxidation (P-hidroksifenilacto rūgšties peroksidazinės oksidacijos tyrimas), in *Proc. of the 11th Lithuanian Conference of Young Scientists „Science – Future of Lithuania“*. Vilnius: Technika, 25–34 (in Lithuanian). ISBN 9789955283010.

About the author

Rūta Ivanec-Goranina was born in Vilnius, on 28 of January 1983.

Bachelor degree in Bioengineering, from Faculty of Fundamental Sciences, Vilnius Gediminas Technical University (VGTU), 2005. Master of Science in Bioengineering, from Faculty of Fundamental Sciences, VGTU, 2007. In 2005–2007 worked as a technician, later as research engineer at the Department of Enzyme Chemistry at Institute of Biochemistry. In 2007–2013 – PhD student at VGTU. In 2007–2008 was granted by State Studies Foundation and by the Research Council of Lithuania in 2012 for the investigations performed. Since 2012 till present – junior research associate at the Department of Chemistry and Bioengineering at VGTU.

BIOKATALIZINĖ AROMATINIŲ HIDROKSI DARINIŲ OKSIDACIJA

Problemos formulavimas

Fenolio dariniai (FD) sudaro vieną svarbiausių cheminių medžiagų grupių, sutinkamų gamtoje ir naudojamų chemijos pramonėje bei moksliniuose centruose. FD gamtoje yra plačiai paplitę tarp augalų antrinių metabolitų, kurie atlieka įvairias funkcijas, tačiau chemijos pramonėje FD pagrindu susintetinti junginiai, patekę į aplinką, tampa teršalais. Aplinkos apsaugos požiūriu – tai dažnai pavojingos medžiagos, kurios turi būti saugiai šalinamos. Vienas iš FD šalinimo būdų yra biokatalizinė FD oksidacija. Norint užtikrinti efektyvią biokatalizinę FD oksidaciją, reikia parinkti tinkamas biokatalizines priemones, įskaitant naujai atrandamas, ir optimizuoti jų taikymo schemą. Biokatalizinių priemonių ir taikymo schemas optimizavimo problema yra svarbi moksliniu ir praktiniu požiūriu, kadangi leidžia iš vienos pusės naudoti efektyviausią biokatalizatorių (bioinžineriniu, ekonominiu ir kitais aspektais), o iš kitos pusės sudaryti optimalią jo panaudojimo schemą. Šiame darbe analizuojama FD oksidacija, naudojant rekombinantinę *Coprinus cinereus* ir krienų peroksidazes bei *Corioloropsis byrsina* lakazę. Tiriami FD: 4-hidroksifenilacto rūgštis, 1-hidroksipirenas, fenolis, 1-naftolis, 2-naftolis, 2-hidroksiantracenas, 9-fenantrolis, bisfenolis A. Tyrimams FD buvo parinkti atsižvelgiant į jų struktūrą ir kenksmingumą.

Parentant biokatalizinės oksidacijos sąlygas, yra labai svarbu neapsiriboti vien tik fermentų parinkimu. Viena perspektyvių tyrimo krypčių yra mediatorių panaudojimas FD oksidacijos procese. Taikant mediatorius susiduriama su tokiomis problemomis kaip efektyviausio mediatoriaus parinkimas ir panaudojimo ekonominis naudingumas. Neseniai pradėti tyrinėti fenoksazino dariniai biokataliziniuose procesuose priskiriami prie aukšto reaktingumo substratų. Jie pasižymėjo labai geromis mediatorinėmis savybėmis oksiduojant aromatinius N-hidroksi darinius. Todėl šiame darbe, tyrinėjant aromatinių hidroksi darinių biokatalizinę oksidaciją, buvo pasirinktas fenoksazino atstovas 3-(4a,10a-dihidro-fenoksazin-10-il)-propan-1-sulfoninė rūgštis (PPSA).

Darbo aktualumas

Aromatinių hidroksi darinių pagrindu susintetinti junginiai, patekę į aplinką, tampa teršalais ir yra svarbu juos efektyviai šalinti. Bioutilizaciniai metodai – tai ne itin brangūs ir aplinkai nekenksmingi būdai. Teršalai yra skaidomi bakterijų, grybų, augalų sintetiniais fermentais, o vystantis genų inžinerijai, taip pat ir rekombinantiniais fermentais. Svarbu sukurti modelinę sistemą, identifikuojančią šių junginių biokatalizinės oksidacijos procesus.

Biokatalizinė fenolio darinių oksidacija turi keletą apribojimų: tai reakcijos metu vykstanti fermentų inaktyvacija, taip pat sunkiai degraduojamų junginių lėta oksidacija. Todėl svarbu, tinkamai parinkus priemones, ištirti šių reakcijų dėsningumus ir mechanizmus.

Tyrimų objektas

Disertacijos tyrimo objektas yra fermentais katalizuojama aromatinių hidroksi darinių oksidacija.

Darbo tikslas – ištirti ir optimizuoti biokatalizinę aromatinių hidroksi darinių oksidaciją.

Darbo uždaviniai

Darbo tikslui įgyvendinti, iškeliama šie uždaviniai:

1. Ištirti rekombinantine *Coprinus cinereus* (rCiP) ir krienų peroksidazėmis katalizuojamą aromatinių hidroksi darinių atstovų 4-hidroksifenilacto rūgšties ir 1-hidroksipireno oksidacijos kinetiką.
2. Ištirti rCiP katalizuojamą aromatinių hidroksi darinių atstovų fenolio, 1-naftolio, 2-naftolio, 1-hidroksipireno, 2-hidroksiantraceno, 9-fenantrolio oksidaciją, naudojant paviršinio aktyvumo medžiagas (PAM).
3. Ištirti sunkiai degraduojamo junginio bisfenolio A oksidaciją, katalizuojamą neseniai atrasta *Coriolopsis byrsina* lakaze (CbL), naudojant mediatorių 3-(4a,10a-dihidro-fenoksazin-10-il)-propan-1-sulfoninę rūgštį (PPSA).
4. Atlikti ištirtų aromatinių hidroksi darinių biokatalizinės oksidacijos mechanizmų matematinį modeliavimą ir optimizavimą.
5. Pritaikyti 1-hidroksipireno peroksidazinės oksidacijos reakciją praktiniam panaudojimui – žemų vandenilio peroksido koncentracijų nustatyme bei atlikti matematinį šios reakcijos pritaikymo nanomolinių vandenilio peroksido koncentracijų nustatyme modeliavimą ir optimizavimą.

Tyrimų metodika apima kinetinius fluorimetrinius, spektrofotometrinius, šviesos išbarstymo ir dinaminio šviesos išbarstymo metodus bei matematinį modeliavimą.

Darbo mokslinis naujumas

Rengiant disertaciją buvo gauti šie chemijos inžinerijos (biotechnologijos) mokslui nauji ir svarbūs rezultatai:

1. Pasiūlyta schema, kuri yra pritaikoma biokatalizinės fenolio darinių oksidacijos, naudojant monomerinės ir micelinės formos paviršinio aktyvumo medžiagas, optimizavimui.
2. Atlikti sunkiai degraduojamo junginio bisfenolio A oksidacijos tyrimai su neseniai atrasta *Coriolopsis byrsina* lakaze, naudojant mediatorių PPSA.
3. Pasiūlyta nauja nanomolinių vandenilio peroksido koncentracijų nustatymo sistema, paremta 1-hidroksipireno peroksidazinės oksidacijos reakcija.

Darbo rezultatų praktinė reikšmė

Atlikti biokatalizinės fenolio darinių (FD) oksidacijos tyrimai suteikia galimybę intensyvinti peroksidaze ir lakaze katalizuojamus biotechnologinius procesus, kurie gali būti panaudoti kuriant „draugiškus aplinkai“ FD nukenksminimo būdus, turinčius pritaikymą įvairaus tipo valymo įrenginiuose. „Draugiški aplinkai“ būdai yra geresni lyginant su šiuo metu naudojamais metodais dėl mažesnio šalutinio poveikio aplinkai.

Biokatalizinės 1-hidroksipireno oksidacijos reakcija pritaikyta nanomolinių vandenilio peroksido koncentracijų nustatymui. Pasiūlyta sistema gali būti taikoma vandenilio peroksido aplinkos monitoringo tyrimuose.

Ginamieji teiginiai

1. Sintetinės ir biologinės kilmės paviršinio aktyvumo medžiagos (PAM), naudojant koncentracijas iki kritinių micelių koncentracijų (CMC), mažina arba visiškai stabdo FD peroksidazinės oksidacijos metu vykstančią fermento inaktyvaciją bei didina FD konversiją. Naudojant PAM koncentracijas didesnes nei CMC, peroksidazinės FD oksidacijos metu fermentas vėl inaktyvuojasi.
2. Pridėjus mediatoriaus PPSA, *Coriolopsis byrsina* lakaze katalizuojama sunkiai degraduojamo junginio bisfenolio A oksidacija greitėja.
3. Aukšta 1-hidroksipireno oksidacijos konstanta ir didelė kvantinė šio junginio fluorescencijos išeiga leidžia 1-hidroksipireno peroksidazinę oksidacijos reakciją taikyti nanomolinėms vandenilio peroksido koncentracijoms matuoti.

Darbo rezultatų apibūdinimas

Disertacijos tema yra atspausdinti 4 moksliniai straipsniai: trys – mokslo žurnaluose, referuojamuose Thomson Reuters Web of Science duomenų bazėje ir vienas – respublikinėje konferencijos medžiagoje.

Disertacijoje atliktų tyrimų rezultatai buvo paskelbti keturiuose mokslinėse konferencijose Lietuvoje ir užsienyje:

- 10-ajame tarptautiniame peroksidazių simpoziume „OxiZymes 2012 in Marseille“, 2012 m. Marselyje, Prancūzijoje;
- 12-ojoje Lietuvos biochemikų draugijos konferencijoje „Biochemijos studijoms Lietuvoje – 50 metų“, 2012 m. Toliejoje;
- 11-ojoje Lietuvos jaunųjų mokslininkų konferencijoje „Mokslas – Lietuvos ateitis“, 2008 m. Vilniuje;
- Jaunųjų mokslininkų konferencijoje „Bioateitis: gamtos ir gyvybės mokslų perspektyvos“, 2008 m. Vilniuje.

Disertacijos struktūra

Darbą sudaro įvadas, 3 skyriai, bendrosios išvados, literatūros sąrašas, autorės mokslinių publikacijų disertacijos tema sąrašas. Įvadiniamе skyriuje aptariama tiriamoji problema, darbo aktualumas, formuluojamas darbo tikslas bei uždaviniai, darbo mokslinis naujumas ir praktinė reikšmė, pristatomos paskelbtos mokslinės publikacijos ir pranešimai konferencijose. Pirmasis disertacijos skyrius skirtas literatūros apžvalgai. Jame aprašomi fenolio dariniai (FD), jų poveikis aplinkai, aprašomi fermentai, oksiduojantys FD. Apžvelgiami peroksidazinės ir lakazinės katalizės apribojimai: nuo vandenilio peroksido koncentracijos priklausanti peroksidazės inaktyvacija; fermentų inaktyvacija, vykstanti FD biokatalizinės oksidacijos metu, bei lėta sunkiai degraduojamų FD oksidacija. Apžvelgiami panaudoti sprendimai, šalinantys išvardintus apribojimus. Antrajame skyriuje aprašomi disertaciniame darbe naudoti metodai: fluorimetriniai, spektrofotometriniai, šviesos išbarstymo ir dinaminio šviesos išbarstymo metodai bei darbe naudoti skaitiniai metodai ir modeliai. Trečiajame skyriuje pateikiami eksperimentiniai duomenys apie 4-hidroksifenilacto rūgšties ir 1-hidroksipireno peroksidazinę oksidaciją; apie fenolio, 1-naftolio, 2-naftolio, 1-hidroksipireno, 2-hidroksiantraceno, 9-fenantrolio peroksidazinę oksidaciją, naudojant paviršinio aktyvumo medžiagas; apie bisfenolio A lakazinę oksidaciją, naudojant mediatorių PPSA, bei atliekamas visų ištirtų FD biokatalizinės oksidacijos mechanizmų matematinis modeliavimas ir optimizavimas. Taip pat pateikiamas detaliam ištirtas 1-hidroksipireno peroksidazinės oksidacijos reakcijos praktinis pritaikymas žemų vandenilio peroksido koncentracijų nustatyme. Bendra disertacijos apimtis – 123 puslapiai, 30 paveikslų, 12 lentelių ir 187 literatūros šaltiniai.

Bendrosios išvados

1. Tyrimo metu nustatytos bimolekulinės 4-hidroksifenilacto rūgšties reakingumo su rekombinantine *Coprinus cinereus* (rCiP) ir krienų (HRP) peroksidazėmis konstantos atitinkamai lygios $(2,4 \pm 0,6) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ir $(1,4 \pm 0,1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. Bimolekulinės 1-hidroksipireno reakingumo su rCiP ir HRP peroksidazėmis konstantos atitinkamai lygios $(1,0 \pm 0,3) \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ ir $(0,6 \pm 0,2) \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. Kvantinė 1-hidroksipireno fluorescencijos išeiga lygi 0,66 (pH 8,0, 25 °C).
2. Sintetinės ir biologinės kilmės paviršinio aktyvumo medžiagos (PAM), naudojant koncentracijas iki kritinės micelių koncentracijos (CMC), mažina arba visiškai stabdo analizuotų fenolio darinių (FD) peroksidazinės oksidacijos metu vykstančią fermento inaktyvaciją bei didina FD konversiją. Naudojant PAM koncentracijas didesnes nei CMC, peroksidazinės FD oksidacijos metu fermentas vėl inaktyvuojasi.
3. Pridėjus mediatoriaus PPSA, *Coriolopsis byrsina* lakaze katalizuojamos bisfenolio A oksidacijos pradinis greitis padidėja iki 3 kartų. Nustatyta bimolekulinė mediatoriaus PPSA reakingumo su lakaze konstanta beveik 100 kartų didesnė už bimolekulinę konstantą, charakterizuojančią bisfenolio A reakingumą su lakaze.
4. 1-hidroksipireno peroksidazinės oksidacijos reakcija pritaikyta nanomolinėms vandenilio peroksido koncentracijoms matuoti – atliktas matematinis šios sistemos modeliavimas ir optimizavimas (reakcijos greitis didžiausias esant pH 8,0 reikšmei). Nustatyta peroksido žemutinė aptikimo riba lygi 21 nM (pH 8,0, 25 °C).

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BIOCATALYTIC OXIDATION OF AROMATIC HYDROXY DERIVATIVES

Summary of Doctoral Dissertation

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