IOSUD - Universitatea Politehnica Timişoara
Şcoala Doctorală de Studii Inginereşti

BIOCATALYTIC SYSTEMS FOR SYNTHESIS AND CHARACTERIZATION OF SUSTAINABLE BIODERIVATIVES

Teză de doctorat – Rezumat

for obtaining the scientific title of doctor at
Polytechnic University of Timisoara
in the field of Chemical Engineering

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month September year 2023

The use of enzymes to obtain bioderivatives is currently an important direction of green chemistry, due to the high potential of using these compounds in various fields such as pharmaceutical, medical, cosmetic or food [1].

In recent years, interest in biocatalysis has expanded throughout the field of green chemistry, with the aim of achieving industrial processes with low environmental impact.

The main objective of the doctoral thesis was the development of sustainable methods for the synthesis of new polyesters, but also of possible monomers, using renewable raw materials in different combinations.

The doctoral thesis is structured in four parts:

1. Literature review detailing the current state of research in biopolymer synthesis using enzymes as catalysts.

2. Original contributions, in which the results obtained during the research are presented, begin with the presentation of the objectives of the doctoral thesis. The doctoral thesis is focused on the development and testing of biocatalytic systems for obtaining monomeric or polyester bioderivatives using renewable raw materials. Starting from the principles of green chemistry and the known advantages of enzyme catalysts to obtain new bioderivatives, compounds with multiple uses in various fields, the thesis had as its general objective the investigation of synthesis routes to obtain these products, in close connection with sustainability criteria what governs the current development of the bioeconomy.

Computational studies on lipases from different microbial sources.

In this chapter, different computational methods were used to evaluate: the effect of physical reaction parameters (temperature and pressure) on the three-dimensional structure and on the surfaces of two commercially available microbial lipases; and the interactions of these lipases with different ecological, aliphatic and aromatic oligoester substrates.

The purpose of these molecular modeling and docking methods was to provide a prediction of the changes in protein conformation and implicitly the active site that enzymes undergo when used under different reaction conditions.

Biotransformation of castor oil into unsaturated oligomers with furan units by the one-pot method using native and immobilized lipases

Within this subchapter, the optimization of the enzymatic synthesis of new oligomeric compounds using castor oil and 5-hydroxymethyl-2-furancarboxylic acid was achieved. Lipase from *Pseudomonas stutzeri* was used as biocatalysts. It should be highlighted that the raw materials used were of vegetable origin, their use meeting all the requirements of green chemistry. The studied reaction system was of the "one-pot" cascade type, in which it started from castor oil and using a single lipase both the hydrolysis of this oil and the transformation of the obtained RCA into oligoesters were carried out.

The structural analysis, the demonstration of the copolymer formation and its composition were carried out using demanding instrumental techniques, such as 2D-NMR and MALDI-TOF MS but also FT-IR.

NMR analysis confirmed the structure of the reaction products and provided relevant information on the reactivity and regioselectivity of the reactions, and thermal analysis confirmed the high stability of the terpolymer, which makes it available for various applications.

After the advanced characterization of the reaction products, their ability to form organogels in different organic solvents was tested. The characterization of the organogels was carried out by determining the viscosity but also by morphological analysis through scanning electron microscopy.

Enzymatic synthesis and characterization of adipic acid oligoesters with aliphatic, cyclic, or aromatic diols

Although CaLB has been shown to be an efficient catalyst for the conversion of a wide range of substrates with aliphatic functional groups into polyesters (e.g. polyesters with vinyl or hydroxy functions), for the lipase from *Ps. stutzeri* there are no systematic studies to evaluate such an activity. In the case of CalB, the studies were mainly oriented towards obtaining polyesters derived from renewable monomers, for example 2,5-furandicarboxylic, adipic or succinic acids and 1,4-butanediol [2].

In this chapter, the selectivity of CalB lipases and from *Ps. stutzeri* for 15 commercially available diols in the polycondensation reaction with adipic acid, using a green (eco) organic solvent as the reaction medium or in a solvent-free system. The reactions were performed at 2 temperatures 50 and 75/80°C and the reactions were monitored by gel permeation chromatography.

Characterization of the reaction products was carried out by different chromatographic techniques, mass spectrometry, NMR spectroscopy and TG and DSC thermal analysis.

These results were published in the journal Sustainable Chemistry and Pharmacy [3].

Evaluation of the ecological impact of some enzymatically obtained oligoesters: biodegradation studies in freshwater and saltwater liquid media

Polymer degradation is a process that brings about changes in properties (eg, shape, color, tensile strength) by favoring biological, chemical, or physical processes that result in bond breaking and subsequent chemical transformations. These transformations mainly include chemical changes, bond breaking and formation of new functional groups. Material properties such as mechanical, electrical or optical characteristics of polymers are influenced by degradation. Polymers do not corrode like metals, but undergo degradation processes through several means that could be achieved by the environment or by enzymes through biodegradation.

The necessary conditions for a polyester to be used in cosmetic applications are biocompatibility, biodegradability and non-toxicity. The biodegradability of plastics in a liquid environment refers to the degradation in fresh water (lakes, rivers), in salt water (seas, oceans) or in aerobic and anaerobic sludges (wastewater treatment) [4].

In this subchapter, the biodegradability of some oligoesters synthesized in the doctoral thesis was studied, these being: oligoesters derived from furan, aliphatic oligoesters but also aliphatic or aromatic monomers. To evaluate the biodegradability, the studies were carried out using specific OxiTop devices that are equipped with sensors that measure the biochemical oxygen consumption (CBO) necessary for aerobic microorganisms to degrade the organic matter in a certain environment. The study was carried out in liquid culture media, in which water collected from the Bega River in Timișoara, Romania and water collected from the Adriatic Sea, Italy were used as inoculum.

Enzymatic oxidation of glycerol using covalently immobilized laccases.

In this chapter, the enzymatic oxidation of glycerol was studied. In this regard, four commercially available native laccases from *Aspergillus sp*., *Trametes versicolor*, *Rhus vernicifera* and *Agaricus bisporus* were selected, using five different mediators. The most active laccases were covalently immobilized on six functionalized solid supports: three magnetic matrices and three based on methacrylic polymers with epoxy or amine active groups (Lifetech™). Compared to the well-known Fe3O4 magnetic particles, spinel ferrite Ni-Zn or Ni-Zn-Co magnetic particles (MFe2O4) with different metal cations (M: Zn, Co, Ni) were used in this chapter. The activity of the 18 resulting enzyme preparations was evaluated and the most active biocatalysts were characterized in detail in terms of stability and reusability, demonstrating improved storage stability, pH and thermal stability compared to native enzymes. Biocatalysts were successfully used for glycerol oxidation at 50°C, demonstrating high selectivity for glyceric acid synthesis. These results were published in the journal Enzyme and Microbial Technology [5].

Evaluation of the enantioselectivity of lipase from *Ps. stutzeri* for the kinetic resolution of a heterocyclic secondary ketoalcohol

The biological activity and important role of enantiopure secondary alcohols containing heterocyclic systems as intermediates for drug synthesis have attracted much interest in recent years. Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) have proven to be suitable biocatalysts for obtaining optically pure stereoisomers, since these enzymes show high stability and enantioselectivity in organic solvents [5].

In this subsection, 2,2'-furoin was tested as a substrate for enantioselective kinetic resolution catalyzed by native and immobilized lipases. All reactions were performed at 50°C using 2-methyltetrahydrofuran as the reaction medium and vinyl acetate as the acyl donor. Enantiomeric excesses of both substrate and product were determined by chiral chromatography using Phenomenex Lux® 5µm i-Amylose-3 chiral stationary phase. Among the lipases tested, lipase from *Pseudomonas stutzeri* immobilized by sol-gel entrapment using a binary mixture of precursor silanes (3GOPrTMOS:TMOS=1:1) was the most effective biocatalyst in terms of stereoselectivity for the enantioseparation of 2,2' -furoin. The efficiency of the immobilized lipase was proven by the high values of substrate conversion, enantiomeric excess (ee), after five reaction cycles.

3. The experimental part includes the materials and experimental procedures used in the synthesis of derivatives but also for the characterization of immobilized enzymes or in other procedures, as well as the analysis methods used (MALDI TOF-MS, NMR, ATR FT-IR, TG,

DSC, HPLC, XPS as well as the software package Gromacs, Grid și Autodock).

4. Final conclusions and original contributions, resulting from experimental and theoretical studies, in accordance with the proposed objectives.

Achieving the objectives of the doctoral thesis through the experimental research carried out led to the following conclusions:

1. The literature study, presented in the first chapter of the thesis, emphasized the following aspects regarding the development of biocatalytic systems for obtaining sustainable derivatives:

- Biopolymers represent a sustainable solution for replacing polymers obtained from raw materials of petroleum origin.
- Currently there are effective computational methods, applicable in the field of biocatalysis.
- There are a multitude of renewable raw materials that have been successfully used in the enzymatic synthesis of polyesters.
- Biodegradability assessment methods must be carefully selected.
- Glycerol-type polyols represent an important source for the enzymatic synthesis of some compounds of interest in cosmetics and the pharmaceutical industry.

2. Computational molecular dynamics and docking methods allowed to evaluate the stability of two lipases under various temperature and pressure conditions.

2.1. Molecular dynamics studies confirmed the stability of lipases from *Candida antarctica* and *Ps. stutzeri* at the temperature and pressure conditions tested.

2.2. The results obtained in the docking studies confirmed the selectivity of the lipases for substrates with primary and secondary OH groups and indicated a higher selectivity of the lipase from *Ps. stutzeri* for the furanic substrate.

3. Oligoesters with different reactive functions were synthesized, using castor oil and a furanic derivative in a "one-pot" enzymatic reaction system, using a single lipase.

3.1. The possibility of using castor oil as a substrate for lipase, in order to synthesize unsaturated oligoesters with heteroaromatic functions, was demonstrated.

3.2. The sustainability of the process was improved demonstrated by performing the reactions in a solvent-free environment at temperatures up to 80°C.

3.3. In the "one-pot" system studied, the formation of terpolymers with units of ricinoleic acid, furan derivative and glycerol represents a possibility to increase the hydrophilicity of the products.

Terpolymers with units of ricinoleic acid, furan derivative and glycerol obtained in one-pot system represent an opportunity to increase the hydrophilicity of the products.

3.4. The proposed reaction system has high biotransformation efficiency and may be feasible for the recovery of triricinolein from vegetable oils.

3.5. The one-pot synthesis of poly(5-hydroxymethyl-2-furancarboxylate-co-ricinoleate)

was demonstrated and optimized using a factorial experimental program using ricin acid as a starting material and lipase from *Pseudomonas stutzeri* as a biocatalyst. The optimal reaction conditions were temperature 63°C, molar ratio castor oil:HFA 3.3:1, amount of enzyme 33 U/mmol (total substrate).

3.6. The introduction of heterocyclic furan units provides access to a wide range of new oligomers with original properties through simple and efficient processes. Structural analysis, copolymer formation and its composition were performed using modern instrumental techniques such as 2D-NMR and MALDI-TOF MS.

3.7. The oligoesters obtained under the optimal reaction conditions were tested to obtain organogels in different organic solvents. The organogels obtained in cyclohexane and heptane showed uninterrupted morphology with porosities of approximately 50 μm.

4. The selectivity of two immobilized lipases against 15 diols in polyester synthesis reactions with adipic acid units was evaluated. The scientific importance of these results is in demonstrating the activity of a less studied lipase in polyesterification reactions, as well as in the possibility of directing the synthesis of products with well-defined properties.

4.1. The selectivity of lipase from *Ps. stutzeri* was systematically evaluated for the first time for the synthesis of some oligoesters, under different temperature conditions and reaction media.

4.3. The average molecular weights of the products were determined by steric exclusion chromatography, and the structure of the reaction products was demonstrated by 1H-NMR spectroscopy.

4.4. The use of a green organic solvent as a reaction medium had no significant effect on increasing the degree of polymerization of the products.

4.5. Among the enzymes tested, CalB proved its efficiency in this reaction, at the selected temperature of 85°C.

4.6. The thermal stability of oligoesters with the highest average molecular masses was studied by thermogravimetry. Among the 8 oligoesters evaluated, the most stable one proved to be the derivative with cyclic units obtained on the basis of 1,4-cyclohexanedimethanol.

5. The biodegradability of 6 synthesized and characterized oligoesters and 3 raw materials was evaluated in freshwater and saltwater liquid media by monitoring dissolved oxygen, using OxyTop monitoring systems.

5.1. For all tested compounds the degradability (Dt) expressed as a percentage after 21 days was higher in the liquid medium inoculated with salt water.

5.2. Among the 3 oligoesters with furanic units, the highest Dt value was obtained for the poly(2,5-bis-hydroxymethyl furan adipate) derivative, followed by poly(5-hydroxymethyl-2-furancarboxylate-co-ricinoleate).

5.3. For aliphatic polyesters, the highest Dt values >70% were obtained for poly(1,8-octanediol adipate).

5.4. Furanic monomers demonstrated slow biodegradability, and the Dt value obtained for the derivative with a hydroxyl group was superior to 2,5-furandicarboxylic acid.

5.5. The preliminary results obtained in the biodegradability evaluation study are promising and frame the products obtained as potential candidates for applications in the food or cosmetic field.

6. The utilization of glycerol to obtain glyceric acid was successfully carried out enzymatically, using laccases immobilized by covalent binding on magnetic supports.

6.1. 4 commercial laccases from different sources were tested in the glycerol oxidation reaction, the most efficient was the laccase from *Trametes versicolor*.

6.2. 5 initiators were tested and TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) proved to be the most effective for the glycerin oxidation reaction.

6.3. The covalent immobilization of laccases from *Trametes versicolor* and *Aspergillus sp.* obtaining 24 enzyme preparations whose activity was evaluated in the oxidation reaction of 2,6-dimethoxyphenol.

6.4. The highest high values of the enzyme activity for the immobilized laccases from *Trametes versicolor* - 760 μmol/min/g preparation, were obtained when a polymethacrylic resin functionalized with butyl-epoxy groups was used as a support. The highest enzyme activity using magnetic nanoparticles as a support was obtained for the immobilized laccase from *Trametes versicolor*, 360 μmol/min/g prepared on NiZn mixed ferrite support.

6.5. The immobilized biocatalyst maintained its activity in the temperature range 30-50°C, even an increase in thermal stability was observed compared to the native enzyme.

6.6. Reduction of the imine bond with $NaBH_4$ resulted in increased operational stability by approximately 20%.

6.7. Both native and immobilized *Trametes versicolor* laccase were successfully used for the first time as biocatalysts in the glycerol oxidation reaction.

7. The lipase capacity of native and immobilized *Ps. stutzeri* for the enantioselective kinetic resolution of 2,2'-furoin.

7.1. Enantioselective acylation of 2,2'-furoin catalyzed by immobilized lipases was demonstrated for the first time.

7.2. Entrapment of lipases in sol-gel matrices proved effective for the conversion of heteroaromatic substrates such as 2,2'-furoin.

7.3. Among the 9 lipases tested, the highest conversion and enantiomeric excess values were obtained using the lipase from *Ps. stutzeri* immobilized by inclusion in sol-gel matrices using the mixture of precursor silanes 3GOPrTMOS:TMOS in a 1:1 molar ratio.

7.4. Among the solvents tested as a reaction medium, 2-methyl tetrahydrofuran, a green solvent, proved to be the most effective.

7.5. It was possible to reuse the biocatalyst, maintaining 21.7% of its activity after 5 cycles of use.

ORIGINAL CONTRIBUTIONS (NEW SCIENTIFIC RESULTS)

I. Computational studies were carried out under different temperature and pressure conditions for the lipase from *Ps. stutzeri* that has been less studied previously.

1. Molecular dynamics studies demonstrated the high stability of lipase from *Ps stutzeri* at temperatures of 50 and 75°C.

2. Evaluation of the catalytic sites of lipases from *Candida antarctica* and *Ps stutzeri* indicated greater flexibility and ability of the lipase from *Ps. stutzeri* to establish interactions with H-bond donors in a wider area of the active site.

3. The docking studies performed represent the preliminary results for evaluating the selectivity and substrate specificity of the lipase from *Ps. stutzeri*

II. Development of a one-pot enzymatic process to synthesize ricinoleic acid oligoesters with furan units, using castor oil and 5-hydroxymethyl-2-furoic acid as substrates.

4. The first transformation of castor oil into aromatic oligoesters in a solvent-free system.

5. Establishing the optimal reaction conditions through a factorial experimental program.

6. Demonstration by 2D NMR spectroscopy of the structure of poly(5-hydroxymethyl-2-furancarboxylate-co-ricinoleate).

7. The first use of oligoesters with unsaturated functions and furanic units to obtain organogels.

III. Biocatalytic synthesis and characterization of oligoesters, using natural and synthetic diols and immobilized lipases from different sources.

8. Establishing the influence of the position of the OH group, the degree of unsaturation of the substrate, the source of the enzyme, the temperature and the reaction medium on the formation of oligomerization products.

9. Demonstration of the superiority of the lipase from *Candida antarctica* for the synthesis of aliphatic oligoesters, respectively the lipase from *Ps. stutzeri* catalysts for aromatic or cyclic oligoesters.

IV. Ecological Impact Assessment of Enzymatically Synthesized Oligoesters in Freshwater and Saltwater Liquid Media

10. Evaluation of biodegradability in liquid media of 6 oligoesters with furanic and aliphatic units.

11. Using the thermal properties of oligoesters for a possible correlation with biodegradability properties.

V. Enzymatic valorization of glycerol using native and immobilized oxidative enzymes.

12. The influence of laccase source, initiators and immobilization support on the preferential synthesis of glyceric acid using glycerol as a substrate was determined.

13. The effectiveness of laccase from *Trametes versicolor* immobilized by covalent binding on magnetic supports for this reaction was demonstrated for the first time.

VI. Comparative evaluation of the capacity of CalB lipases and from *Ps. stutzeri* immobilized for the enantioselective acylation of 2,2'-furoin.

14. 9 commercial enzyme preparations or those obtained within the biocatalysis group were evaluated in order to establish the catalytic efficiency in the enantioselective acylation reaction of 2,2'-furoin and the superiority of lipase from *Ps stutzeri* immobilized by entrapment in matrixes was demonstrated for the first time sol-gel for this reaction.

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