University of Belgrade
Faculty of Technology and Metallurgy

POROUS AMINO MODIFIED LIGNIN MATERIALS FOR ENZYME IMMOBILIZATION

Dr Jelena Bebić

jelenabebic@dmdm.rs

Ministry of Economy
Republic of Serbia

Directorate of Measures and Precious Metals (DMDM)

The main goal of the study

So far, it has been demonstrated that bio-waste based amino-modified microspheres have excellent adsorption properties and have good potential use in bioremediation, thus our intention was to expand their application potential, mainly:

- to investigate the possibility of use of the A-LMS microspheres as a novel matrix for enzyme immobilization
- To investigate the possibilities of use of such obtained and optimized immobilized preparations, and evaluate the results.

Introduction

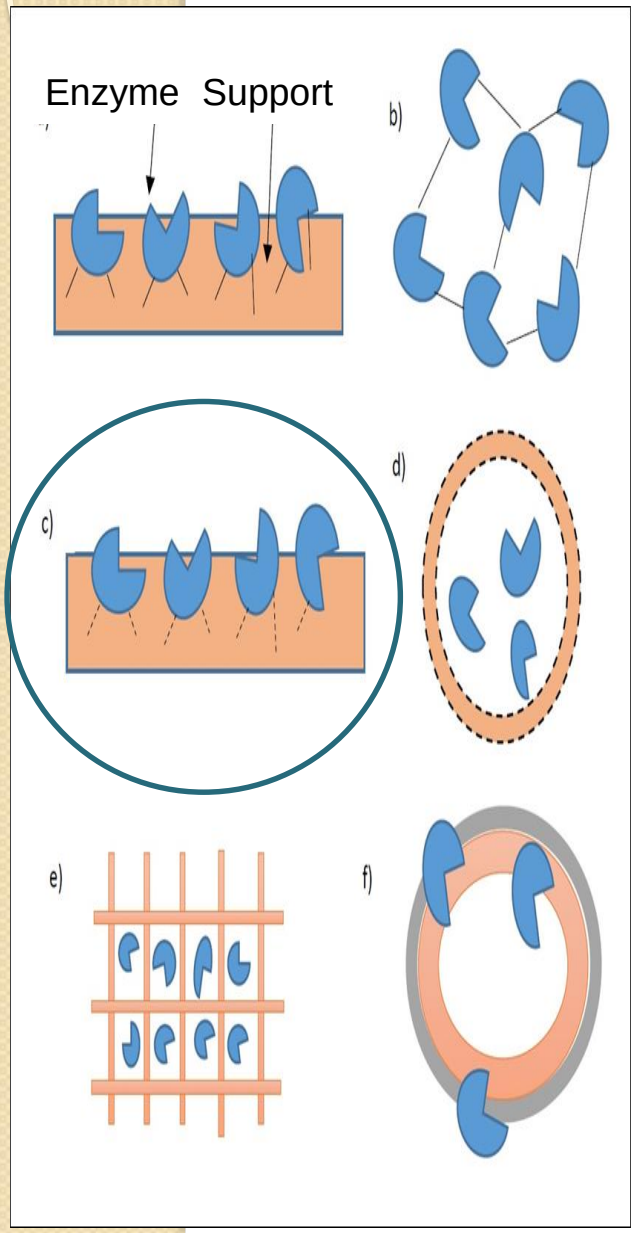
- Selected enzymes
- Enzyme immobilization – advantages for industrial use
- Amino-modified lignin microspheres (A-LMS) as a carrier for enzyme immobilization
- A-LMS_5 and A-LMS_10 types
- A-LMS synthesis and
- A-LMS characterisation

Selected enyzmes

- Two selected enzymes: ***β*-galactosidase** from *Aspergillus oryzae* and **laccase** from *Myceliophthora thermophila* expressed in *Aspergillus oryzae* (Novozym 51003® laccase)
- **β-galactosidase**, is a family of glycoside hydrolase enzymes that catalyzes the hydrolysis of β-galactosides into monosaccharides through the breaking of a glycosidic bond.
- Generally, **laccases** (EC 1.10.3.2) belong to a family of multicopper containing oxidoreductases, also known as blue copper oxidases, that catalyze the oxidation of variety of organic substrates, including POPs. Laccases have vast possible industrial applications and biotechnological potential in bioremediation of waste waters and contaminated soil.

- Characteristics and structure are origin-related, and the biggest laccase producers are white rot fungi
- Both of these enzymes have exhibited affinity toward supports with free amino groups on their surfaces.
- Both enyzmes have been successfully immobilized on different carriers, including glass beads, nanoparticles, molecular sieves, silica gel, microspheres, carbon nano-tubes, carbon paste, chitosan, graphite powder, membrane (magnetic carbon paste-chitosan/silica), nanofibers, glutaraldehyde and nanoflowers etc.

Enzyme immobilization – advantages for industrial use

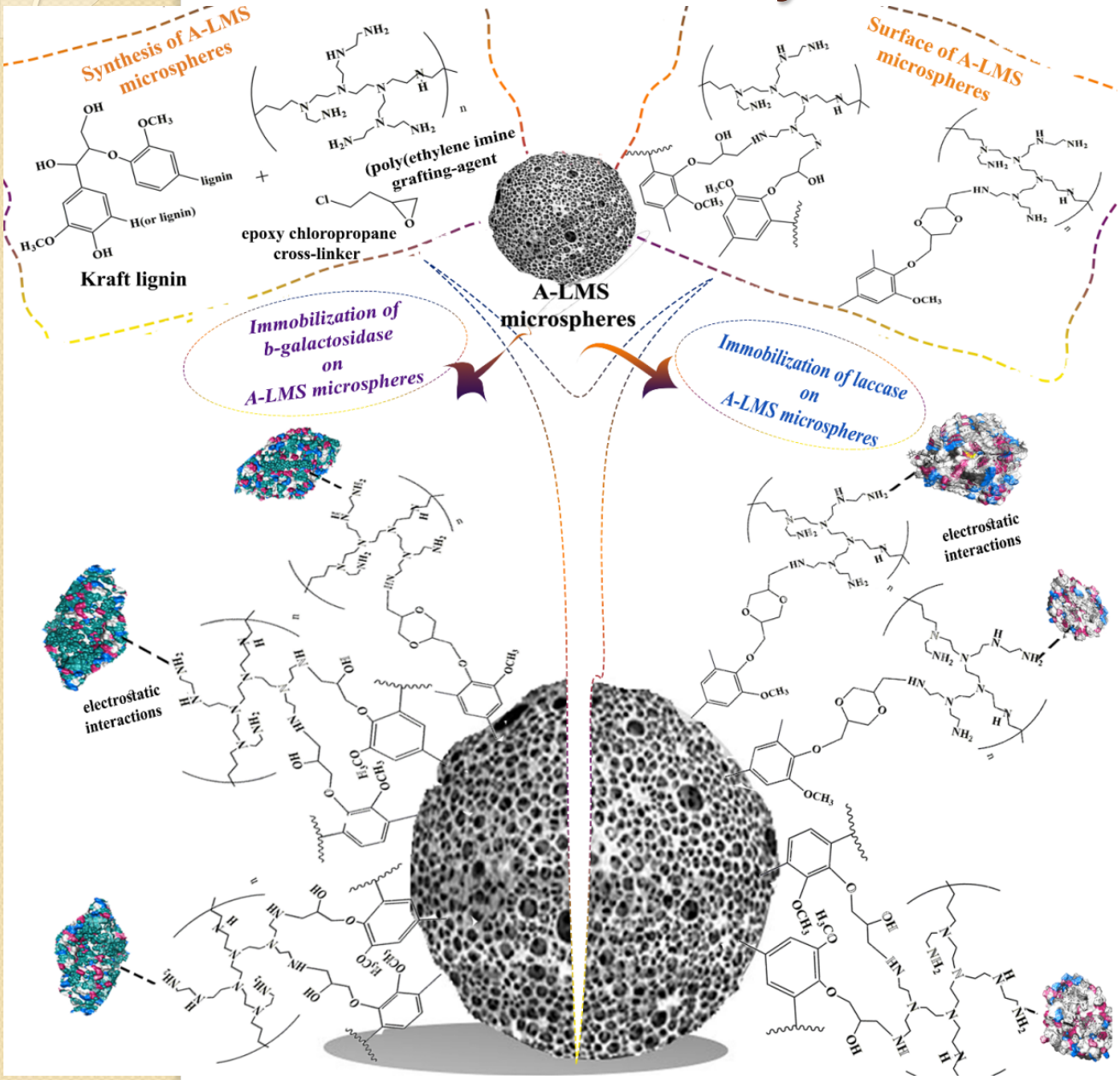


- Immobilization is a commonly used technique for improving the possible extensive industrial application of enzymes.
- Immobilized enzymes are more stable at usually non favourable process conditions, can be reused in continuous operations and more easily separated from the reaction media by non-chemical means.
- Two types of immobilisation: physical and chemical E-S interaction
- The selection of the carrier is based on the most important parameters, which are immobilization capacity, activity recovery and stability.
- Regardless of the support material type, for the formation of stable and efficient biocatalytic systems, the optimization of the attachment technique must be performed individually, for the specific enzyme, the specific material and the intended biocatalytic process.

Amino-modified lignin microspheres (A-LMS) as a carrier for enzyme immobilization

- As an immobilization support, lignin has been used so far in chitin-lignin novel matrix for immobilization of lipase by adsorption, and silica-lignin matrix as a stable and reusable biocatalytic system
- As a precursor for synthesis of A-LMS, kraft lignin is used, a type of industrial lignin which is obtained as residue from Kraft pulping process.
- Relatively cheap bio-waste derived material which has a positive impact with regard to the economic aspects of A-LMS synthesis
- In course of A-LMS microspheres formation, the process of copolymerization between kraft lignin, poly(ethylene imine) grafting-agent and epoxy chloropropane cross-linker was optimized and described in previous studies.
- The presence of amino functional groups in the surface of A-LMS increases their affinity to biomolecules.
- Two types of A-LMS were investigated, A-LMS_5 and A-LMS_10

A-LMS synthesis



Scheme : Immobilization of enzymes on lignin microspheres (A-LMS).

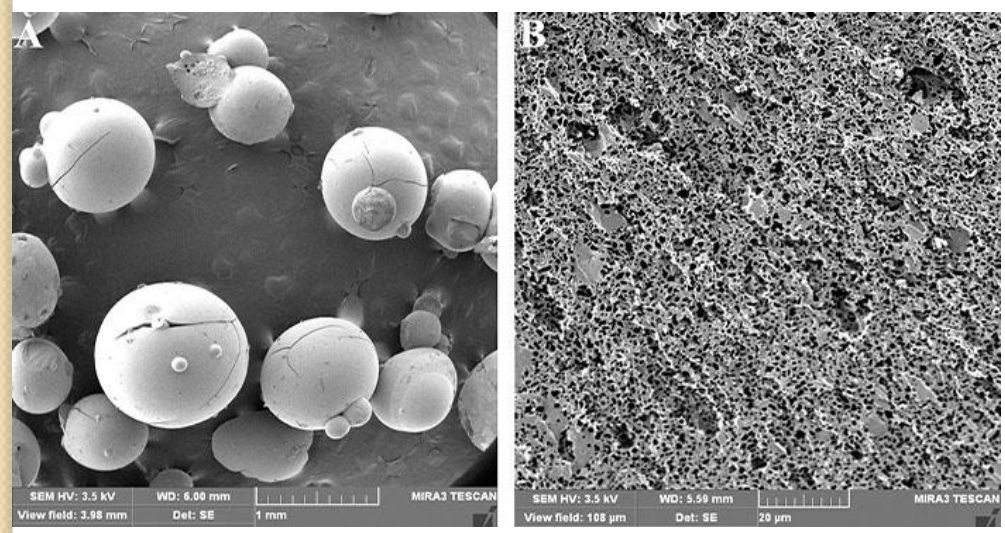
For synthesis of A-LMS_5 and A-LMS_10, the same procedure was applied, the only difference was in the amount of used alginate emulsifier solution:

- A-LMS_5 were obtained by utilization of 5 wt % of emulsifier,
- A-LMS_10 were obtained by utilization of 10 wt % of emulsifier.

A-LMS characterisation

Two types of A-LMS microspheres (A-LMS_5 and A-LMS_10), with different characteristics were selected to be examined in preliminary experiments as supports for two mentioned enzymes.

Sample	Specific surface area SBET, m2/g	Mean diameter, μm	Average pore diameter Dmn, nm	Maximum pore diameter Dmax, nm	Amino group content, mmol/g
A-LMS_5	7.68	800±80	12.36	25-50	7.7
A-LMS_10	2.38	800±80	22.22	20.32-59.77	6.5



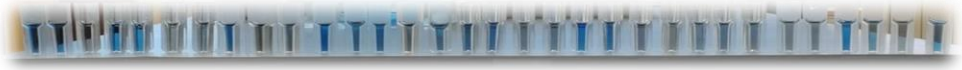
FESEM micrographs of A-LMS_5.

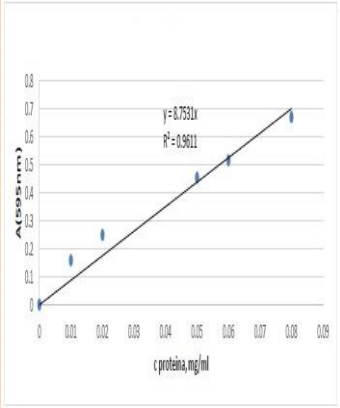
Both types of A-LMS (A-LMS_5 and A-LMS_10), that are used in this study, were kindly provided by our colleagues from Department of Organic Chemistry, Faculty of Technology and metallurgy, University of Belgrade, Serbia.

Experimental

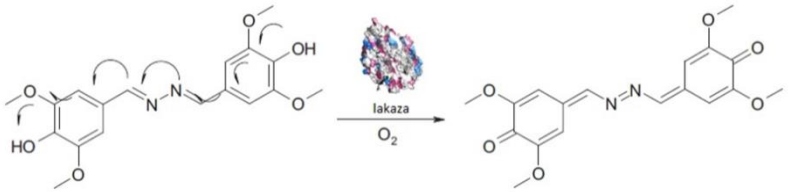
- Methods
- Selection of support
- Immobilization of *β*-galactosidase on A-LMS_5
- Adsorption kinetics and the activity of *β*-galactosidase on A-LMS_5
- GOS synthesis by free and immobilized *β*-galactosidase on A-LMS_5
- Immobilization of laccase onto A-LMS_5
- Adsorption kinetics and the activity of laccase immobilized on A-LMS_5
- Analysis of interactions formed between enzymes and A-LMS_5
- Degradation of lindane by free and laccase immobilized onto A-LMS_5

Methods

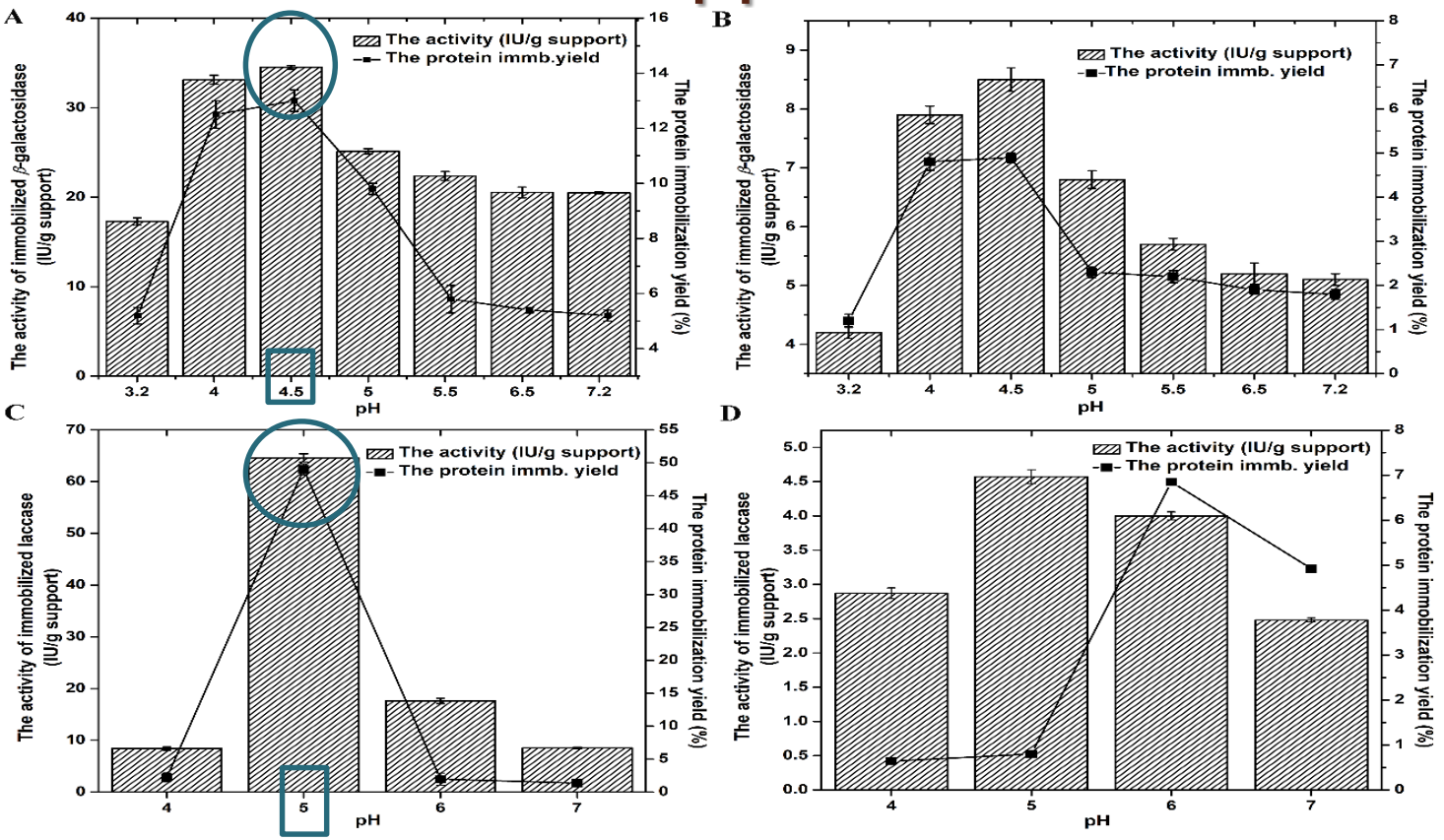




- Enzyme activity was determined spectrophotometrically
- Concentration of proteins in enzyme solutions and supernatants was assayed according to Bradford procedure.
- For the desorption assay, the immobilized preparations were treated separately with 1 % Triton X-100 and 1 M $CaCl_2$.
- The quantitative analysis of GOS was performed by HPLC
- The quantitative analysis of lindane was performed by GC-MS



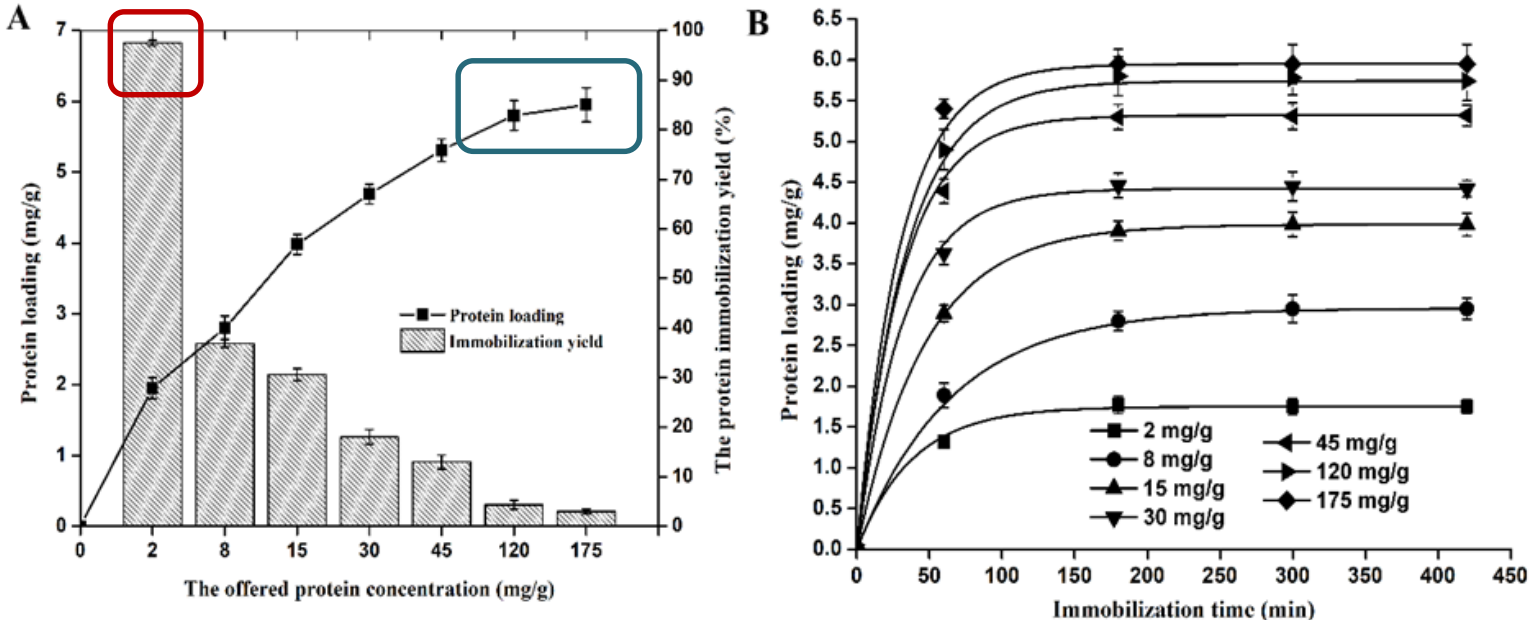
Selection of support



Preliminary screening of the most efficient immobilization carrier (A-LMS_5 (A, C) and A-LMS_10 (B, D)) for immobilization of β-galactosidase and laccase. Examined parameters were: immobilization buffer (pH 3.2-7.2), the activity of immobilized preparation (bars) and the protein immobilization yield (■)

For both enzymes, the highest yields of immobilized proteins per mass of microspheres and activities of immobilized preparations were achieved when immobilization was performed on the A-LMS_5 microspheres, that have 3 times higher specific surface area, and higher concentration of amino groups on the surface.

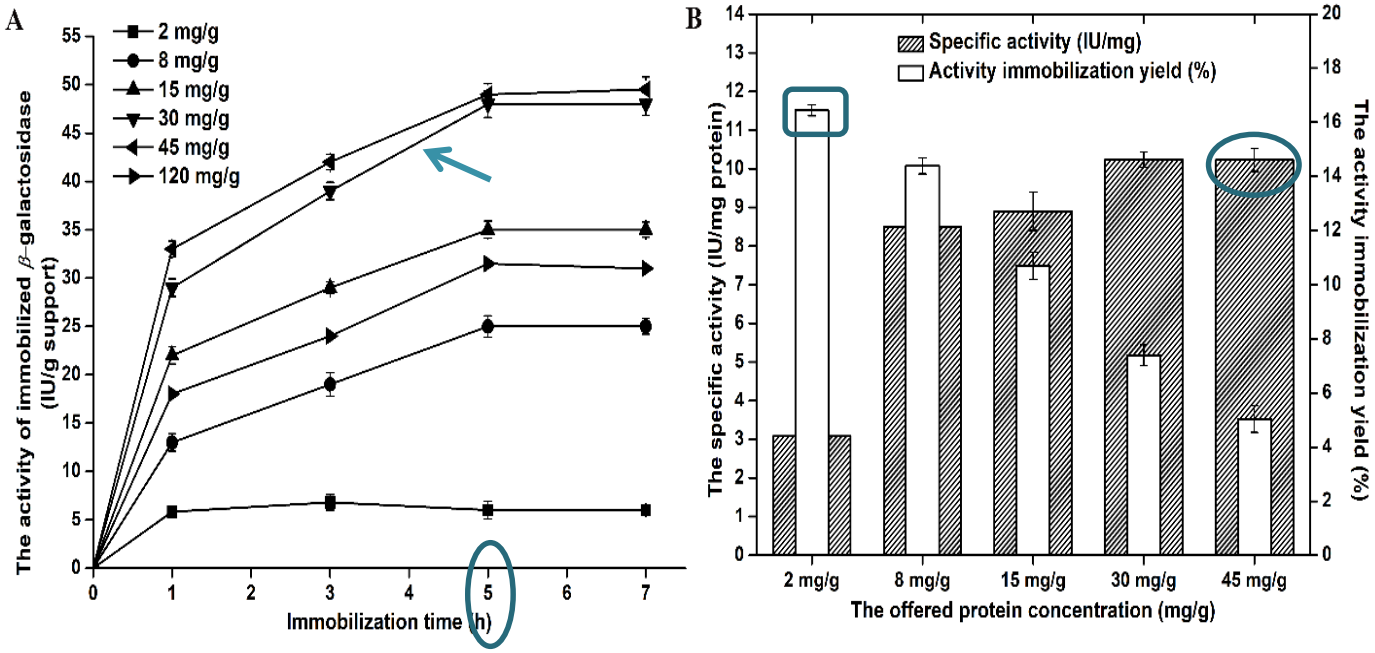
Immobilization of β-galactosidase on A-LMS_5 - Adsorption kinetics



The effect of the offered protein concentration on protein loading and the protein immobilization yield (A). Adsorption kinetics of β-galactosidase at different protein/support ratios (B). The obtained experimental results for the adsorption kinetics of β-galactosidase on the A-LMS_5 were fitted by pseudo-first-order kinetic model (plotted curves).

Maximum binding capacity for was 5.5 mg proteins per g of support, at the offered protein concentration of 2 mg/g support, A-LMS_5 bound almost all offered proteins (98 %) on its surface. Langmuir adsorption model of unrestricted monolayer enzyme adsorption was proven, obtained high values of regression coefficient R^2 (0.999-1) of pseudo-first-order kinetic model indicate that the applied model successfully describes the rate of β-galactosidase diffusion from solution onto A-LMS_5, with two steps, first being bulk and film diffusion, second step, much slower, was pore diffusion.

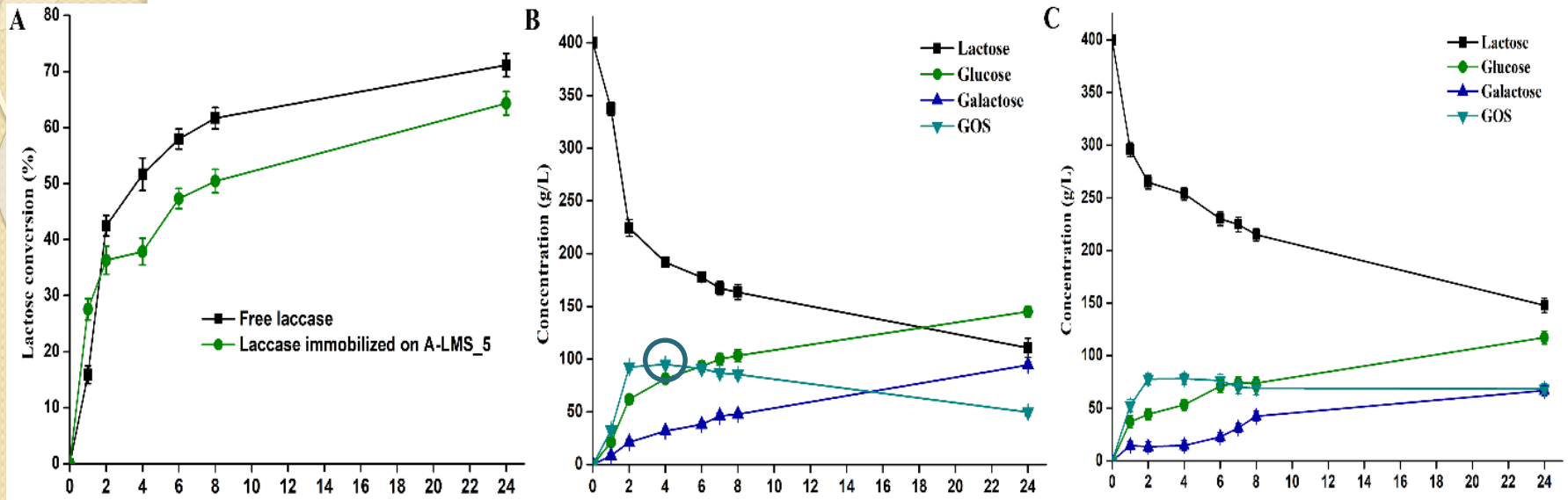
The activity of β-galactosidase immobilized on A-LMS_5



The effect of immobilization time on the activity of β-galactosidase immobilized on A-LMS_5 at different offered protein concentrations (A). The effect of offered protein concentration on specific activity (IU/mg protein) and activity immobilization yield (%) of immobilized β-galactosidase on A-LMS_5 (B).

The best results when the immobilization was carried with the offered protein concentration of 30 mg/g support for 5 h. The highest activity immobilization yield (16 %) was obtained at lowest offered protein concentration (2 mg/g), and specific activity of 10.22 IU per mg of attached proteins was obtained when immobilization is performed at offered protein concentrations 30 mg/g and 45 mg/g, indicating that almost all attached enzyme molecules on A-LMS_5 occupy active conformation.

GOS synthesis by free and immobilized β-galactosidase



Time course of lactose conversion using β-galactosidase immobilized on A-LMS_5 and free β-galactosidase (A). Reaction mixture changes in the product stream at different reaction time for free β-galactosidase (B) and immobilized β-galactosidase on A-LMS_5 (C).

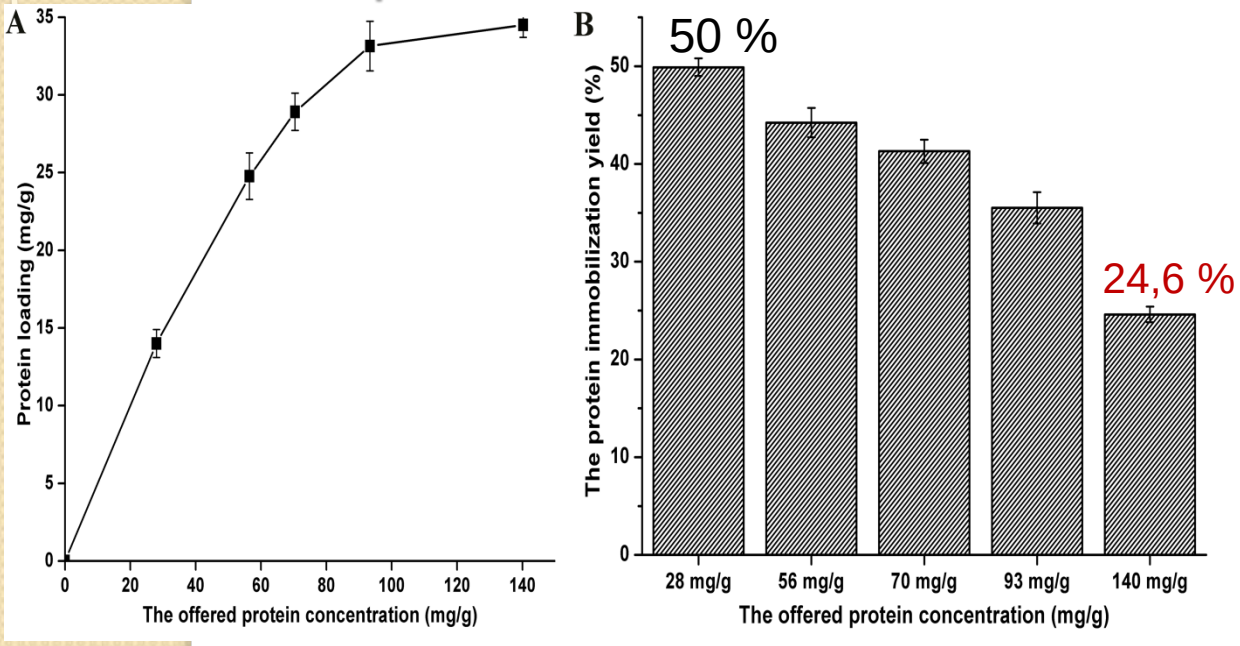
The developed biocatalyst was used in the synthesis of galacto-oligosaccharides (GOS) from lactose and compared with the soluble enzyme. The maximum amount of total GOS of 78.2 g/L for the immobilized enzyme was achieved at lactose conversion of 36.5 % after 4 h, hydrolysis and transgalactosylation occur simultaneously during first two hours, transgalactosylation being dominat compared to glucose production.

The perspective for utilization of β-galactosidase immobilized on A-LMS_5 in GOS synthesis lies in the fact that the high GOS productivity of 19.5 g/L/h was achieved.

Developed immobilized preparation provides the means for facilitated GOS downstream processing and multiple reuse of the same batch of the biocatalyst, including the application in different bioreactor configurations.

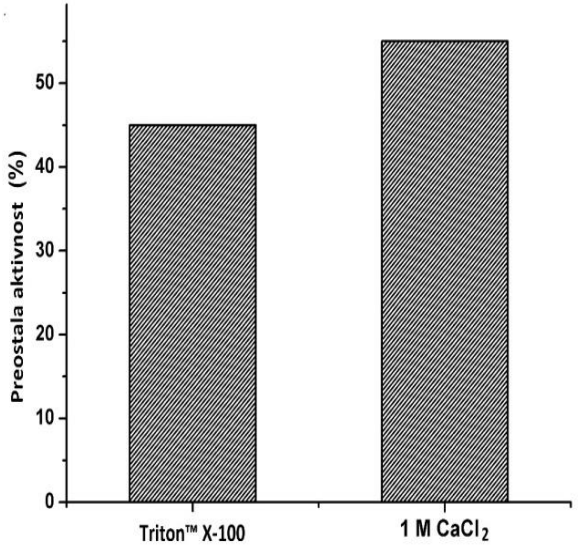
Immobilization of laccase onto A-LMS_5

Adsorption kinetics



- Maximum binding capacity of A-LMS_5 is arround 35 mg/g support, for Novozym® 51003 laccase, which is approximately 6 times higher than in case of *β*-galactosidase.
- The highest protein immobilization yield was 50 % for the offered protein concentration of 28 mg/g

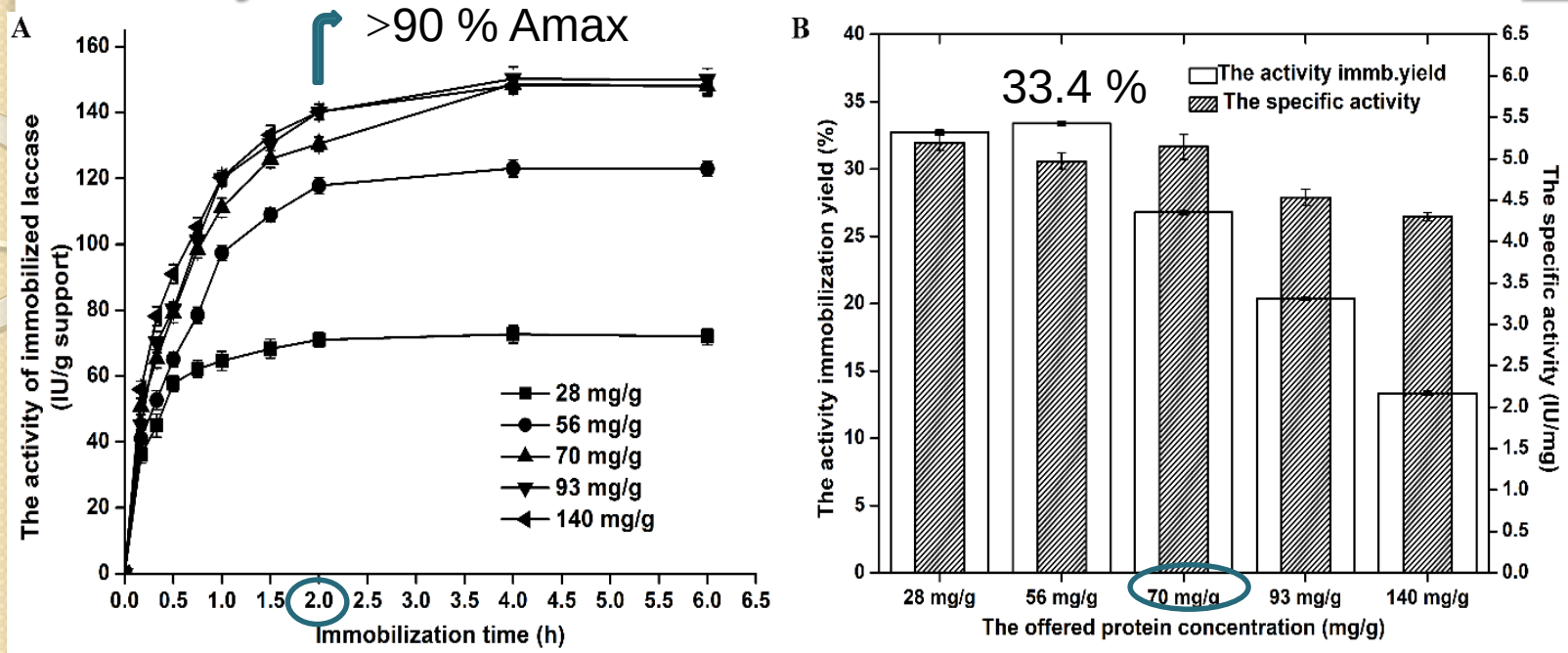
Analysis of interactions formed between enzymes and A-LMS_5



Desorption assay→ **laccase** binding to A-LMS_5 is governed almost equally with both hydrophobic and electrostatic interactions.

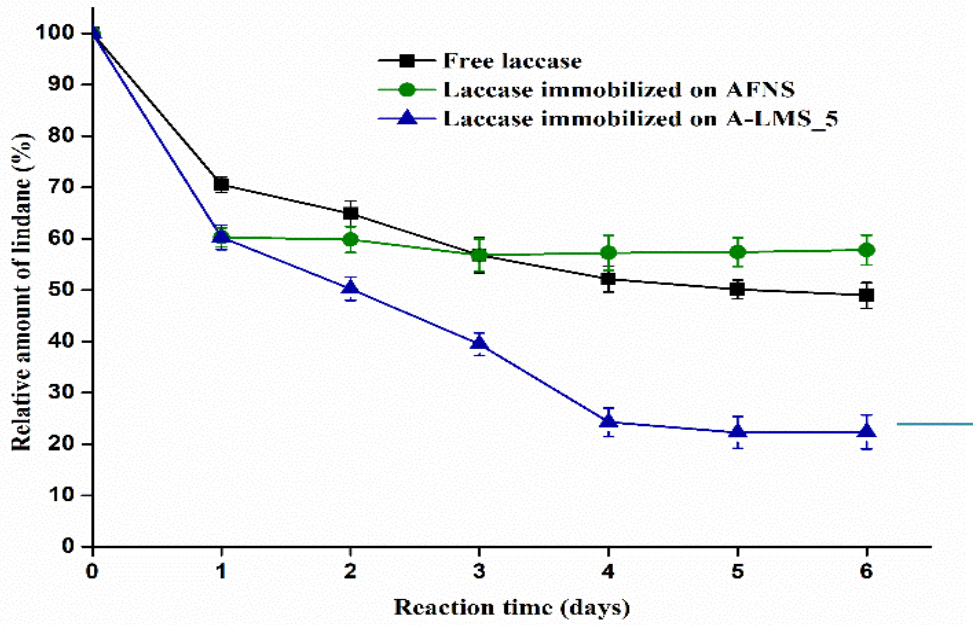
In case of ***β*-galactosidase** the result of treatment with Triton-X100 and 1 M $CaCl_2$ showed that almost all enzyme molecules (90 %) are predominantly attached on A-LMS_5 surface *via* electrostatic interactions

The activity of laccase immobilized on A-LMS_5



- At the offered protein concentration of 70 mg/g, the highest activity of 149 IU / g support after 4 hours, was achieved.
- More than 90 % of maximum activity obtained after first 2 hours of the experiment
- Specific activity obtained was 5 IU per mg of attached proteins at 70 mg/g of offered proteins concentration, and the highest activity yield (33.4 %) was obtained at offered protein concentration of 56 mg/g.
- Optimization of laccase immobilization onto A-LMS_5 at pH 5,0, with 70 mg/g offered protein concentration, during the period of 4 h.

Degradation of lindane by free and immobilized laccase onto A-LMS_5



22,4 %

The relative amount of lindane according to the initial amount (controls) after treatment with free laccase, laccase immobilized on AFNS and laccase immobilized on A-LMS_5.

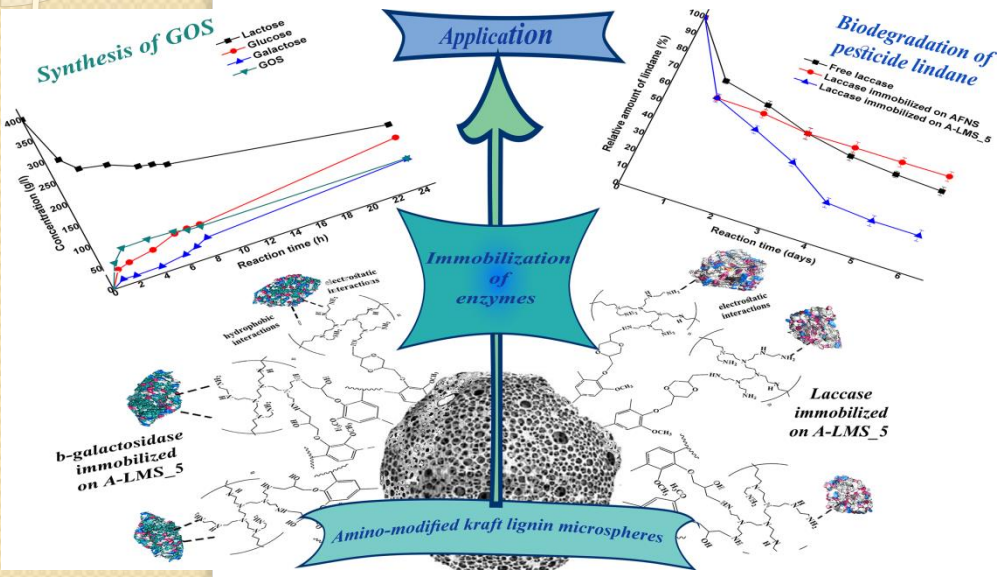
Pesticide lindane (γ-HCH) is a member of organochlorine (OC) pesticide class, and it has been used mainly in plant protection and pest control. It belongs to a group of chemicals known as POPs, due to their durability in the environment, toxicity and tendency to bioaccumulation in the soil and water.

- Adsrption of lindane on A-LMS_5 was also detected, 66 % relative
- Laccase immobilized onto A-LMS_5 exhibited prolonged activity, while lindane degradation was more efficient than in the case of free laccase or laccase immobilized onto amino-fumed nanosilica (AFNS).

Conclusions

- The effect of emulsifier concentration on morphological and physical characteristics of A-LMS was considered regarding the efficiency of immobilization process, and it has been shown that microspheres produced using 5 wt % of emulsifier (A-LMS_5) have pore shape, size and distribution for enzyme attachment by far better than microspheres produced using 10 wt % of emulsifier (A-LMS_10).
- *β*-galactosidase immobilized on A-LMS_5 demonstrated good specificity towards GOS synthesis from lactose, while immobilized laccase preparation exhibited good activity in reaction of pesticide lindane degradation.
- From all the results presented, it can be concluded that A-LMS, has good perspective in various large-scale industrial applications.

Acknowledgements



Jelena Bebić, Katarina Banjanac, Jelena Rusmirović, Marija Ćorović, Ana Milivojević, Milica Simović, Aleksandar Marinković and Dejan Bezbradica, *Amino-modified kraft lignin microspheres as a support for enzyme immobilization,* June 2020, RSC Advances 10(36):21495-21508

DOI: 10.1039/D0RA03439H

- This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Contract No. 451-03-68/2020-14/200135 and Contact No. 451-03-68/2020-14/200287).
- The authors are also grateful to Directorate of Measures and Precious Metals for the technical support.
- The contribution of COST Action LignoCOST (CA17128), supported by COST (European Cooperation in Science and Technology), in promoting interaction, exchange of knowledge and collaborations in the field of lignin valorisation is gratefully acknowledged.

THANK YOU FOR YOUR ATTENTION !

QUESTIONS?