30th International Conference Ecological Truth & Environmental Research 2023

Proceedings

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PROCEEDINGS

30th INTERNATIONAL CONFERENCE

ECOLOGICAL TRUTH AND ENVIRONMENTAL RESEARCH – EcoTER'23

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Publisher: University of Belgrade, Technical Faculty in Bor

For the publisher: Prof. Dr Dejan Tanikić, Dean

Printed: University of Belgrade, Technical Faculty in Bor, 100 copies, electronic edition

Year of publication: 2023

ISBN 978-86-6305-137-9

CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

502/504(082)(0.034.2)
574(082)(0.034.2)

INTERNATIONAL Conference Ecological Truth & Environmental Research (30 ; 2023)
Proceedings [Elektronski izvor] / 30th International Conference Ecological Truth & Environmental Research - EcoTER'23, 20-23 June 2023, Serbia ; organized by University of Belgrade, Technical faculty in Bor (Serbia) ; co-organizers University of Banja Luka, Faculty of Technology – Banja Luka (B&H) ... [et al.] ; [editor Snežana Šerbula]. - Bor : University of Belgrade, Technical faculty, 2023 (Bor : University of Belgrade, Technical faculty). - 1 elektronski optički disk (CD-ROM) ; 12 cm

Sistemski zahtevi: Nisu navedeni. - Nasl. sa naslovne strane dokumenta. - Preface / Snežana Šerbula. - Tiraž 100. - Bibliografija uz svaki rad.

ISBN 978-86-6305-137-9

а) Животна средина -- Зборници б) Екологија – Зборници

COBISS.SR-ID 118723849

30th International Conference
Ecological Truth and Environmental Research – EcoTER'23

is organized by:

UNIVERSITY OF BELGRADE
TECHNICAL FACULTY IN BOR (SERBIA)

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HORSERADISH PEROXIDASE IMMOBILIZATION WITHIN MICRO-BEADS OF OXIDIZED TYRAMINE-ALGINATE FOR PHENOL REMOVAL FROM WASTEWATER

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Abstract

Natural polymers such as alginate, pectin, chitosan etc. were used as carriers for the immobilization of different types of enzymes. Among investigated enzymes, peroxidases hold a special place. Immobilized enzymes are frequently used in phenol removal reactions. In this research horseradish peroxidase was immobilized within alginate micro-beads. This natural polymer was previously oxidized with sodium periodate and modified with tyramine hydrochloride. Percent of oxidation was varied from 2.5 mol% to 10 mol%, and an increase in specific activity was noticed with increasing the oxidation percent. Immobilized peroxidases showed satisfactory stabilities after 10 days of storage. Phenol concentration in a batch reactor decreased during its oxidation with horseradish peroxidase immobilized on tyramine-alginate hydrogels.

Keywords: phenol removal, alginate, horseradish peroxidase, tyramine, immobilization.

INTRODUCTION

Features of natural polymers such as biodegradability, biocompatibility and non-toxicity qualifies them for the immobilization of different enzymes [1]. Among many carriers with natural origin, alginate, chitosan and agarose, can be marked as the most frequently used ones [2], [3]. Each of these polymers can be applied in the immobilization of different enzymes, meanwhile peroxidases are the ones with the widest use. Horseradish peroxidase (HRP) belongs to the group of oxidative enzymes whose role in phenol oxidation is of great importance in various fields. During this process and as a consequence of interactions between enzyme's active site and products of oxidation reaction (phenoxy radicals), inactivation of enzyme might occur [4]. This major issue can be overcome by immobilization or addition of different reagents such as poly(ethylene glycol) (PEG), whose role is in reduction of amount of HRP needed for phenol oxidation reaction [5]. By immobilization, on the other hand, increased activity and operational stability of immobilized enzyme can be achieved [6].

Enzymes immobilized on natural polymers like alginate have wide application in phenol removal. The concentration of phenol and phenol like compounds in wastewater effluents is

increasing daily, making this environmental problem a priority [7]. For these purposes, many methods have been proposed, but among the most effective, those that involve application of immobilized enzymes have been singled out.

In this work alginate was subjected to oxidation with sodium periodate and reductive amination with tyramine hydrochloride in the presence of sodium cyanoborohydride. Micro-beads with immobilized horseradish peroxidase were used for the removal of phenol.

MATERIALS AND METHODS

Materials

Alginate from brown algae in the form of sodium salt was purchased from Sigma-Aldrich (St. Louise, Mo, USA), as well as following chemicals: horseradish peroxidase (150−250 U/mg), pyrogallol (98%), tyramine hydrochloride (98%), glucose oxidase-type VII (160 U/mg), Triton X-100 (laboratory grade), Span 80, potassium ferricyanide (≥99%), sodium metaperiodate (≥99.8%) and glycerol (98%). From Fluka (Buchs, Switzerland) were purchased 4-aminoantipyrene (p.a.) and sodium cyanoborohydride (95%). Sodium chloride (p.a.), sodium dihydrogen phosphate (>99%) and phenol (p.a.) were obtained from Centrohem (Stara Pazova, Serbia). Ethanol (96%) and glucose (p.a.) were purchased from Zorka (Šabac, Serbia), while hydrogen peroxide (30% (w/w)) was obtained from AppliChem GmbH (Darmstadt, Germany). Tris buffer and calcium chloride (p.a.) were purchased from Serva (Heidelberg, Germany) and Termohemija (Belgrade, Serbia), respectively.

Periodate oxidation and reductive amination of alginate

Prodanović *et al*. [8] previously described the method for oxidation of alginate with sodium periodate and further modification of this oxidized form with tyramine hydrochloride, which was applied in this study. Briefly, sodium alginate was first dissolved in water to a final concentration of 1% (w/v), and afterwards oxidized with sodium metaperiodate to a concentration of 1.25 mmol/L, 2.5 mmol/L and 5 mmol/L. Hereby it was provided that a molarity ratio of periodate to C6 glycoside units was set to 2.5 mol%, 5 mol% and 10 mol%. The reaction was stopped by adding glycerol to a final concentration of 50 mmol/L. Precipitation of oxidized alginate was performed in ethanol with subsequent addition of sodium chloride at 1% (w/v) concentration. This procedure was repeated twice, after it was dissolved in water. Amination was conducted by adding solid tyramine hydrochloride (final concentration of 1.5% (w/v)) to the solution of oxidized alginate in sodium phosphate buffer pH 6. Sodium cyanoborohydride, as a reducing agent, was added to a final concentration of 0.5%. Modified alginate was precipitated in ethanol with sodium chloride addition (1% (w/v) concentration). This procedure was repeated two times. Tyramine-alginates were stored at −20 °C.

Horseradish peroxidase oxidation onto tyramine-alginates

Alginates oxidized in different degrees with sodium periodate and modified with tyramine were dissolved in Tris buffer pH 7 (final concentration of 15%). Emulsion polymerization reaction was used for horseradish peroxidase immobilization (2.89 µL and 34.9 µL; 0.5 U/mL and 6.98 U/mL) within tyramine-alginate micro-beads. Simultaneously with immobilization, production of hydrogen peroxide from glucose and glucose oxidase, was performed.

Enzyme activity studies

For determination of peroxidase activity pyrogallol and hydrogen peroxide were used as substrates. Bound enzyme activity was determined by using 130 µL of bead suspension and 30 µL of hydrogen peroxide (0.97 mmol/L) were added to the pyrogallol solution (13 mmol/L). Reaction mixture was stirred for 7 min, while every 120 s aliquots were taken from the solution, filtrated and absorbance at 420 nm was measured using UV-VIS spectrophotometer (Shimadzu Corporation, UV-2501PC, Japan). One unit of enzyme activity was defined as the amount of peroxidase that produces 1 mg of purpurogallin in 20 s.

Phenol removal studies

For the removal of phenol from batch reactor, horseradish peroxidase immobilized within alginate-beads was used. Internal generation of hydrogen peroxide was enabled by glucose oxidase catalyzed oxidation reaction of glucose. The best relation of these two chemicals was determined in our previous study [9] and applied in this research. Concentration of phenol was monitored by applying a colorimetric assay with 4-aminoantipyrene (20.8 mmol/L) and potassium ferrocyanide ($K_3Fe(CN)_6$) (83.4 mmol/L). Absorbance at 510 nm was measured using UV-VIS spectrophotometry, and calibration curve of soluble enzyme was used for determination of concentration of removed phenol.

RESULTS AND DISCUSSION

Sodium alginate was oxidized with different amounts of sodium periodate and further subjected to amination with tyramine hydrochloride in the presence of sodium cyanoborohydride as reducing agent. Polymers were used for the immobilization of horseradish peroxidase, by applying emulsion polymerization reaction enzyme was entrapped within crosslinked network of phenolic residues present on the surface of modified alginate. Figure 1 is a schematic representation of described method for enzyme immobilization.

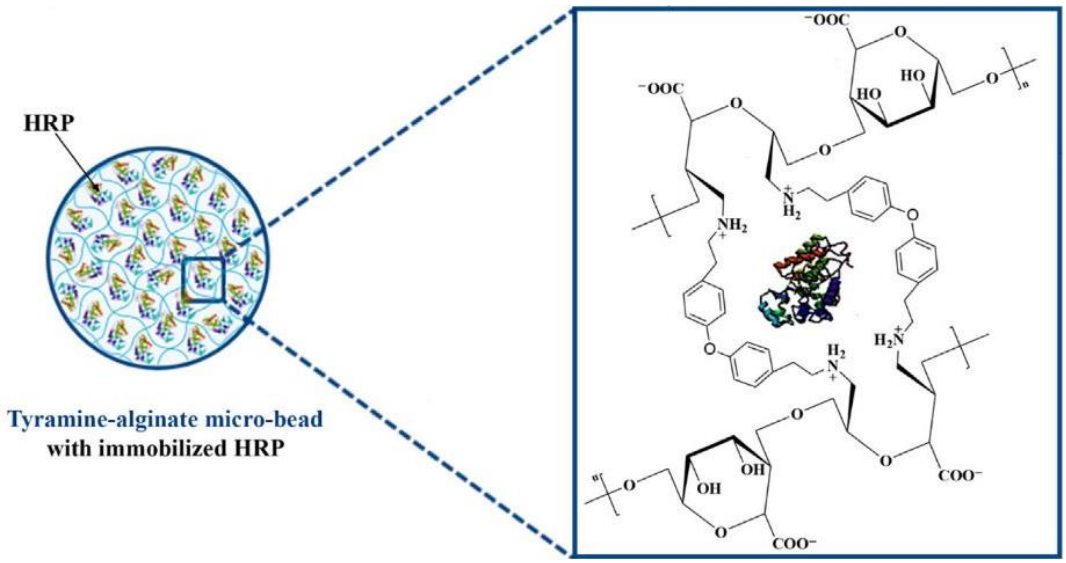

Figure 1 *Entrapment of horseradish peroxidase within tyramine-alginate micro-beads*

Measurements of specific activities of HRP immobilized within tyramine-alginate micro-beads oxidized in different mol% with periodate (2.5, 5 and 10 mol%) showed increasement with the increase of oxidation percent (Figure 2). These findings could be explained with formation of more potential binding sites for enzymes and are corroborated with results previously reported by our group [8].

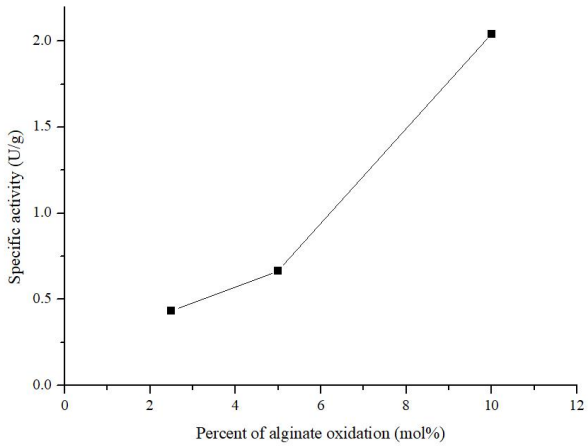

Figure 2 *Specific activity of peroxidase immobilized within tyramine-alginate micro-beads oxidized in different percent with sodium periodate*

Studies of enzyme stabilities in time showed that horseradish peroxidase entrapped within micro-beads of modified alginates oxidized in 2.5 mol% and 5 mol% retains 64.5% and 83% of initial activity, respectively, after 10 days of storage.

HRP immobilized on hydrogel formed with 10 mol% oxidized tyramine-alginate was applied for the removal of phenol in a batch reactor. Results present in Figure 3 show decrease of concentration of remained phenol in a solution with time.

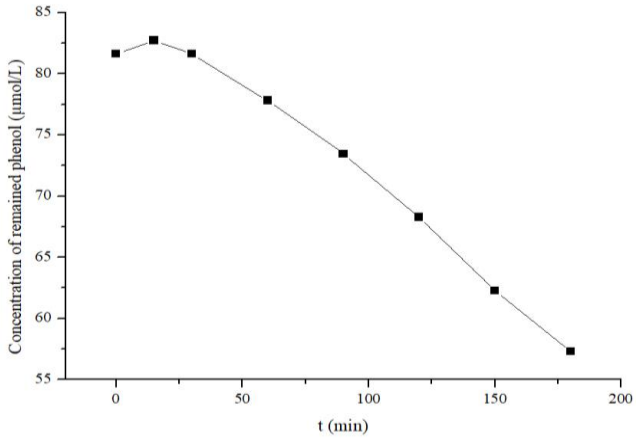

Figure 3 *Dependance of concentration of remained phenol from time*

Within the period of 3 days enzyme was able to remove around 90% of phenol from the reaction mixture.

CONCLUSION

Emulsion polymerization reaction was used for the immobilization of horseradish peroxidase onto micro-beads of modified alginate. Sodium alginate was oxidized with periodate and further subjected to reductive amination with tyramine hydrochloride. Obtained results showed increased specific activity with increasement of oxidation percent. Peroxidase immobilized on 10 mol% oxidized tyramine-alginate was used for the removal of phenol in a batch reactor. Decrease in the concentration of remained phenol was noticed with time.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grant No. 451-03-47/2023-01/200053, University of Belgrade, Institute for Multidisciplinary Research).

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