Popović, N.; Pržulj, D.; Mladenović, M.; Prodanović, O.; Ece, S.; Ilić Đurđić, K.; Ostafe, R.; Fischer, R.; Prodanović, R. Immobilization of Yeast Cell Walls with Surface Displayed Laccase from Streptomyces Cyaneus within Dopamine-Alginate Beads for Dye Decolorization. International Journal of Biological Macromolecules 2021, 181, 1072–1080. https://doi.org/10.1016/j.ijbiomac.2021.04.115.

Research article

# Immobilization of yeast cell walls with surface displayed laccase from *Streptomyces cyaneus* within dopamine-alginate beads for dye decolorization

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## Abstract

High amounts of toxic textile dyes are released into the environment due to coloring and wastewaters treatment processes' inefficiency. To remove dyes from the environment and wastewaters, researchers focused on applying immobilized enzymes due to mild reaction conditions and enzyme nontoxicity. Laccases are oxidases with wide substrate specificity, capable of degradation of many different dye types. Laccase from *Streptomyces cyaneus* was expressed on the surface of *Saccharomyces cerevisiae* EBY100 cells. The specific activity of surface-displayed laccase was increased by toluene-induced lysis to 3.1 U/g of cell walls. For cell wall laccase immobilization within hydrogel beads, alginate was modified by dopamine using periodate oxidation and reductive amination and characterized by UV-Vis, FTIR, and NMR spectroscopy. Cell wall laccase was immobilized within alginate and dopamine-alginate beads additionally cross-linked by oxygen and laccase. The immobilized enzyme's specific activity was two times higher using dopamine-alginate compared to native alginate beads, and immobilization yield increased 16 times. Cell wall laccase immobilized within dopamine-alginate beads decolorized Amido Black 10B, Reactive Black 5, Evans Blue, and Remazol Brilliant Blue with 100% efficiency and after ten rounds of multiple-use retained decolorization efficiency of 90% with Evans Blue and 61% with Amido Black.

**Keywords:** dopamine, laccase, yeast surface display

# 1. Introduction

Laccases (EC 1.10.3.2) are enzymes belonging to the multicopper oxidases family, produced by fungi, higher plants, bacteria, insects [1, 2], lichens and sponges. [3] They use molecular oxygen as the electron acceptor and oxidize a wide range of compounds, such as mono-, di-, poly-, and methoxy-phenols, aromatic and aliphatic amines, hydroxy indoles, benzenethiols, carbohydrates [2], metal ions ($Mn^{2+}$), and organometallics (e.g. $[W(CN)_8]^{4-}$, $[Fe(EDTA)]^{2-}$). [3] Due to their wide substrate specificity, these enzymes have widespread applications in diverse biotechnological and industrial domain, like the forest product industry, the food industry, the pulp and paper industry [3], bioremediation, organic synthesis, pharmaceutical and cosmetic industries [2], bio-bleaching, dye decolorization, juice and wine clarification. [4] The industrial use of laccases is disadvantageous due to sensitivity or loss in enzyme activity in presence of harsh reaction conditions (such as elevated pH, temperature, chelating reagents, organic solvents, inhibitors), instability during storage, etc.. It is also challenging to separate enzymes from reaction system for reuse. [5] One way to improve laccase stability and enable its reusability is enzyme immobilization. Methods for immobilization of an enzyme can be categorized as entrapment, encapsulation, adsorption, covalent binding, and self-immobilization. [6] Various polymeric support materials have been used for immobilization of laccases, such as agar, alginate, gelatin, chitosan, modified cellulose, graphite, etc. [5]

Cell-surface display techniques allow expression of a target protein on the cell's outer surface through linkage with a genetically fused anchor protein. [7] One of the most commonly used host cell systems for enzyme surface display is yeast, Saccharomyces cerevisiae, “generally regarded as safe” (GRAS) by the Food and Drug Administration, making it suitable for industrial food applications. This technique represents a powerful tool for functionalizing yeast to serve as a whole-cell biocatalyst or provide a stable matrix for enzyme immobilization. [8]

For cell entrapment, alginate is the most commonly used polymer because of his nontoxicity, low cost, and mild immobilization procedures. [9] Alginate is natural, unbranched polysaccharide, which is an important component of algae. It consists of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) blocks. [10] The G-blocks of alginate participates in intermolecular cross-linking with divalent cations, such as $Ca^{2+}$, which act as a cross-linker between the functional groups of alginate chains to form a hydrogel. The easy cross-linking gives the alginate possibility to entrap small molecules, enzymes, proteins, and cells. [10] Therefore, alginates have significant potential as a biomaterial for different biomedical science and engineering applications, such as drug delivery, wound healing, tissue engineering, etc. To introduce some new functional groups and create next-generation alginate biomaterials with enhanced or novel properties, chemical modification is the most preferred

method. [11] Till now, the chemical modification of alginate has been achieved using oxidation, sulfation, amidation, esterification, etc. [12]

Much research in recent years has focused on the use of immobilized enzymes for dye decolorization. [13-17] Wastewaters from the textile industry are discharged in the environment in large quantities daily. It has been estimated that approximately 10,000 different types of dyes and about 80,000 tons of dyes are produced worldwide per year, and approximately 5-10% are released into wastewater. [18] Beside the textile industry, synthetic dyes are also used in paper, printing, cosmetics, and pharmaceutical industries. [19] Several physical and chemical methods, including adsorption, coagulation-flocculation, ion exchange, oxidation, and electrochemical methods, have been used for decolorization, but their expensiveness limits their application. [19] An alternative approach for removing organic contaminants from wastewater is enzyme biocatalysis, which has advantages such as high activity under ambient temperature and pressure, low energy requirements, low toxicity, and simple process control. [7] Laccase, an enzyme with high catalytic efficiency and broad substrate specificity, is also tolerant to various physical/chemical parameters [20], making it suitable for decolorization. Decolourization of a number of dyes using laccase has been reported for dyes, such as Remazol Brilliant Blue R (RBBR), Reactive Black 5 (RB5), Bismark Brown R (BBR), Lanaset Grey G (LG) [21], Malachite Green, Methylene Blue, Congo Red [4], Black 10B [22], Methyl Green [19], etc.

The present study aimed to express laccase from *Streptomyces cyaneus* 3335 on the surface of *Saccharomyces cerevisiae* EBY100 cells in an active form and to obtain yeast cell walls with increased laccase activity by cell lysis. Also, dopamine modified alginate prepared by periodate oxidation and reductive amination with ability to form hydrogels using either calcium as a cross-linker or laccase was used for preparation of yeast cell walls with laccase activity immobilized within alginate beads. The immobilized biocatalysts were characterized, compared and the best one was tested for its capability to decolorize different dyes (Amido Black 10B, Methylene Blue, Guinea Green, Reactive Black 5, Methyl green, Evans Blue, Crystal Violet and Remazol Briliant Blue).

# 2. Materials and methods

## 2.1. Chemicals

The *S. cerevisiae* strain EBY100 were kindly provided by Prof. Dane Wittrup and was used in present study as the carrier host cell for laccase surface display. Alginic acid-sodium salt from brown algae and dopamine-hydrochloride were purchased from Sigma Aldrich. (St. Louis, MO, USA). Sodium cyanoborohydride was purchased from Merck (Darmstadt, Germany) and sodium metaperiodate from VWR Chemicals (Leuven, Belgium). Calcium chloride, sodium-chloride and copper (II)-chloride were purchased from Betahem (Belgrade, Serbia), while 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was from AppliChem (Darmstadt, Germany). The synthetic dyes were purchased from Roth (Karlsruhe, Germany) and Sigma (St. Louis, MO, USA). All other chemicals were of analytical reagent grade or higher and supplied by Sigma-Aldrich.

## 2.2. Cloning of laccase from *Streptomyces cyaneus* CECT 3335 into pCTCON2

The laccase coding sequence (GenBank HQ857207), synthesized by Genscript (Piscataway, USA) from pET-22b(+) (Novagen, Darmstadt, Germany) vector was amplified in the PCR reaction (94 °C for 4 min, 1 cycle; 94 °C 1 min/50 °C 1 min/72 °C for 2.45 min, 30 cycles; 72 °C for 10 min, 1 cycle) contained Pfu DNA polymerase (0.04 u/µL), dNTP mix (0.2 mM), forward primer (0.5 µM, reverse primer (0.5 µM), template ScLac-pET22b(+) (30 ng). The purified fragments were restriction digested with NheI and SalI enzyme and ligated in the pCTCON2 (Invitrogen) vector. The ligated gene was used to transform *Echerichia coli* DH5α. Gene length was checked with DNA electrophoresis.

## 2.3. Transformation of *S. cerevisiae* EBY100 cells and expression of laccase on cell surface

The coding sequence of laccase from Streptomyces cyaneus CECT 3335 (ScLac; UniProt F6L7B5) was obtained from Genscript (Piscataway, USA) as a codon-optimized synthetic gene. ScLac sequence was amplified from the synthetic gene using Sc1 and 2 primers containing NdeI-XhoI sites for ligation into the pET-22b (+) vector (Novagen, Darmstadt, Germany). The ligation product was transformed into electro-competent *E. coli* DH5α cells and colonies were selected on LB plates supplemented with 100 µg/ml ampicillin for the residual vectors overnight at 37°C. Colony-PCR was carried out to test recombinant colonies

after transformation of ligation products into the electro-competent cells. Backbone-specific primers T7 pro and T7 ter were used for colony-PCRs and the protocol was applied as follows: The colonies were picked with a sterile toothpick, streaked on an LB agar plate (master plate) containing appropriate antibiotic and subsequently swirled into the PCR mix. The PCR products were resolved by 1.2 % (w/v) agarose gel electrophoresis in 1 x TAE buffer. The corresponding ScLac_pET22b(+) construct was used as template for the preparation of pCTcon2 construct. The primers pCT7-8 were used in the PCR reaction and the insert was ligated into the pCTcon2 vector via NheI-SalI sites. The preparation of the competent EBY100 cells and transformation with pCTcon2 plasmid was carried according to the protocol described by (Gietz & Schiestl, 2007) via heat shock at 42°C. After transformation, the cells were grown directly in YNB- CAA glucose medium for 48 h at 30°C, 160 rpm. For short term storage cells were kept on YNB-CAA agar plates containing glucose and for long term storage, cell stocks were prepared with 20% (v/v) glycerol and stored at -80°C. The construct was checked by applying colony PCR following ligation reaction using backbone-specific pCT fw and pCT rev primers for initial confirmation of the colonies with correct insert. Sequences of the primers were given in Supplementary.

Plasmid pCTcon-lac was introduced into *S. cerevisiae* EBY 100 cells (provided by Dane Wittrup, Massachusetts Institute of Technology) as described previously in LiAc/PEG method [23], using a 42°C heat-shock step for 1h. *S. cerevisiae* EBY 100 cells were transformed with the control plasmid pCTCON2 (empty plasmid, without laccase gene*)* too, to obtain the control strain without laccase surface display. Transformed *S. cerevisiae* EBY 100 cells were grown in YNB-CAA (yeast nitrogen base-casamino acid) glucose medium (2% (w/v) glucose) at 30°C, 160 rpm (final $OD_{600}$~5 in glucose medium). Laccase expression was then induced by transferring to YNB-CAA galactose (2% (w/v) galactose) medium (final $OD_{600}$~0.5 in galactose medium) under the same conditions for 24h. The optimal time of fermentation is determined after monitoring the enzyme activity during 24 hours. The cells were harvested by centrifugation (3000 g, 15 min, 25°C), washed three times with Na-acetate (NaAc) buffer (pH 4.5,100mM) and resuspended in solution for cell lysis (3% toluene in water, v/v).

## 2.4. Laccase enzyme activity assays

The enzyme activity of cells with expressed laccase was determined by measuring the oxidation rate of 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS, a model substrate for laccase, $\varepsilon_{420}$=36 000 $M^{-1}$ $cm^{-1}$) using a spectrophotometric method. The reaction was set up by mixing

13 µl of the cell suspension (cells in NaAc buffer pH 4.5, concentration of cells is 250mg/ml) with 200 µl of 20 mM $CuCl_2$, 1087 µl of NaAc buffer pH 4.5 and 200 µl of 20 mM ABTS (final concentration was 2.7 mM). 200 µl of obtained mixture was transferred to microtiter plate well and the absorbance was measured every 2 min for 15 min at 420 nm, at Elisa Microplate Reader, at room temperature. The oxidation rate of ABTS by laccase could be calculated by determining the ABTS absorbance change over time. One unit (U) of laccase activity is defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute. [24] All measurements were conducted in triplicate.

## 2.5. Cell wall laccase preparation (lysis of *S. cerevisiae* cells)

Lysis of *S. cerevisiae* cells was performed at room temperature, at 170 rpm, using 3% toluene (v/v). The cell lysis was monitored for 48 h, and every couple of hours were taken sample of 2 ml for further analyze. The samples were centrifuged 2 minutes on 13000 rpm and in obtained supernatant (lysate) were determined concentration of proteins (measured spectrophotometrically on 280 nm) and enzyme activity. Enzyme activity was measured spectrophotometrically, absorbance was followed on 405 nm, every 15 min, for 2 hours. Assay mixture was made by mixing 825 µl of supernatant, 150 µl of 20 mM $CuCl_2$ and 525 µl of 20 mM ABTS.

Harvested yeast cell walls were diluted with water, to remove toluene. To measure activity of obtained yeast cell walls, 13 µl of the yeast cell walls in NaAc buffer pH 4.5 (concentration of cell walls solution is 150 mg/ml) are mixed with 200 µl of 20 mM $CuCl_2$ solution, 1087 µl of NaAc buffer pH 4.5 and 200 µl of 20mM ABTS (final concentration of ABTS was 2.7 mM). 200 µl of obtained mixture was transferred to microtiter plate well. Change of absorbance was monitored spectrophotometrically on 405 nm, every 2 min for 15 min.

## 2.6. Modification of alginate with dopamine

Alginate was dissolved in distilled water at final concentration of 1.12% (w/v). In alginate solution was added sodium metaperiodate at final concentration of 1.25, 2.5 mM, 5.0 mM and 10 mM so that molarity ratio of periodate to alginate glycoside units in alginate was set to 2.5 mol%, 5 mol%, 10 mol% and 20 mol%. The reaction of periodate oxidation was carried out in the dark for 24 h at +4 °C and was stopped by adding glycerol at final concentration of 500mM and stirring for 30 minutes. The oxidized alginate was precipitated from reaction mixture with

1% sodium chloride (w/v) and two volumes of 96% (v/v) ethanol. Afterward, alginate was dissolved in water (1%, w/v) and precipitation procedure was repeater two more times using same procedure. Finally obtained precipitate was separated, dried on air and dissolved in 0.1M Na-acetate buffer pH 4.5, at final concentration of 1% (w/v). Solid dopamine-hydrochloride was added to obtained solution at 1.5% (w/v) final concentration (79 mM). The reaction mixture was stirred for half an hour and after that was added solid sodium cyanoborohydride at 0.5% (w/v) final concentration. Reaction mixture was left in the dark with stirring for 24 h at room temperature. At the end of the reaction, modified alginate was precipitated by adding sodium chloride to 1 M final concentration and two volumes of 96% (v/v) ethanol. Afterward, precipitate was dissolved in water (1%, w/v) and precipitated two more times using the same procedure. Modified dopamine-alginate was lyophilized and stored at −20°C.

## 2.7. Spectral characterization of modified alginate

UV-Vis, NMR and FTIR spectra were recorded for modified alginates. For UV-Vis spectra modified and unmodified alginates were prepared by dissolving in distilled water at final concentration of 0.1% (w/v). UV-Vis spectra were recorded using a LLG-uniSPEC2 Spectrophotometer in a range of 200 to 330nm. For NMR spectra, deuterium oxide was used as a solvent. The NMR spectra of alginate were acquired on a Bruker Avance III 500 MHz instrument. (Chemical shifts are given in parts per million (δ) downfield from tetramethyl silane as the internal standard). IR spectra of alginates were recorded on a Thermo Scientific Nicolet 6700 FT-IR spectrometer, using the attenuated total reflectance (ATR) technique. Spectral data were collected in the mid-IR range (1800–600 $cm^{-1}$)

## 2.8. Immobilization of cell wall laccase in alginate and dopamine-alginate beads

Beads with native and dopamine-alginate were prepared by dropping a mixture of 2.7% alginate (w/v) and various amounts of cell walls in final concentration of 20, 40, 60, 100, 120, 150, 200 and 250 mg/ml of cell walls in 0.1 M NaAc buffer with 2 mM $CuSO_4$ in 300 ml of 2% $CaCl_2$ (w/v) solution with continuous stirring. The beads were stirred for 2h and washed with 50ml of 2% $CaCl_2$ solution (w/v). Obtained beads were kept in 50 mM NaAc buffer (pH 4.5) containing 5 mM $CaCl_2$ (w/v), at 4°C.

## 2.9. Characterization of the cell wall laccase and immobilized cell wall laccase

### 2.9.1. Immobilized enzyme activity assay

Spectrophotometric assay for measuring the enzyme activity of alginate beads was done using alginate beads (two beads per reaction) with 550 µl of 0.1M NaAc buffer pH 4.5, 100 µl of 20 mM $CuCl_2$ and 350 µl of 20 mM ABTS (final concentration 7 mM). ABTS concentration was increased due to diffusion limitations. [25] The absorbance was measured every 5 min at 420 nm in a Elisa Microplate. The immobilization yield of cell wall laccase in alginate/dopamine-alginate beads was calculated as a ratio of the activity of immobilized laccase to the activity of the free laccase.

### 2.9.2. pH optimum

The effect of pH on cell wall laccase and immobilized cell wall laccase in Ca-dopamine-alginate beads was compared and studied. The pH optimum was determined by incubating the samples in 100 mM buffers, ranging from pH 2.0 to 7.5, and measuring the activity of cell walls laccase and immobilized cell walls laccase against ABTS as described above. The buffers used were: citrate buffer, pH 2.0-3.5; acetate buffer pH 4.0-5.5, PIPES buffer pH 6.0-7.5. The relative activity at various pH was measured and normalized by the maximal activity (at optimal pH). Relative activity is presented in percentage.

### 2.9.3. pH stability

The enzyme stability in optimal buffer system for cell walls laccase and immobilized cell walls laccase in Ca-dopamine-alginate beads was determined by incubating 2 mg of cell walls/~20 mg of alginate beads (two beads) in 0.1 mM NaAc buffer pH 5 (optimal buffer) for 24h and 48h at room temperature. The residual activity was estimated after incubating period.

### 2.9.4. Influence of temperature on laccase enzymatic activity

The temperature stability was determined by incubating the cell wall laccase and immobilized cell wall laccase simultaneously at 25, 30, 60 and 90°C for 1h in optimal buffer, then chilling

on ice for 5min and measuring enzyme activity. Residual enzyme activity was measured using ABTS as described above for cell walls and beads. Relative activities were calculated by assigning the highest value of activity in the dataset representing each enzyme as 100%.

#### 2.9.5. Determination of $K_m$ and $V_{max}$

The kinetics parameters $K_m$ and $V_{max}$ were determined for free and immobilized cell wall laccase. This test was carried out at 25°C in optimal buffer using ABTS as the substrate. Used substrate concentration was in range 0.0007-0.2 mM for free cell walls and 1.5-12 mM for immobilized cell walls. These parameters were calculated using GraphPad Prism v6 software.

## 2.10. Dye decolorization

Eight different dyes were selected to study the decolorization ability of the yeast cell wall laccase immobilized into Ca-dopamine-alginate beads. Selected dyes are Amido Black 10B, Methylene Blue, Guinea Green, Reactive Black 5, Methyl green, Evans Blue, Crystal Violet and Remazol Brilliant Blue. Stock solutions of the dyes in 0.1 M NaAc buffer pH 5 were stored in the dark at room temperature. For decolorization were used ~20mg of dopamine-alginate beads with immobilized cell wall laccase. As a control reaction of decolorization test was used Ca-dopamine-alginate beads without cell wall laccase, which was prepared under the same conditions to detect possible removal of dye, due to dye adsorption onto the alginate beads. Additional control has done with dyes in mentioned buffer, without beads. All the experiments were performed in triplicate. Concentrations for every dye were prepared in order to obtain around 0.8 absorbance units at the maximum wavelength of each dye (maximum wavelength for all dyes are 620 nm) and the final concentrations for Amido Black 10B, Methylene Blue, Guinea Green, Reactive Black 5, Methyl green, Evans Blue, Crystal Violet and Remazol Brilliant Blue were 0.027, 0.020, 0.102, 0.129, 2.895, 0.008, 0.009 and 0.038 mM, respectively. The reaction mixture, containing ~20 mg of beads (two beads) with cell wall laccase and 1ml of every selected dye with 2 mM $CuCl_2$ and 5 mM $CaCl_2$, was incubated for 48 h at room temperature, without shaking. Reaction of decolorization was performed in a 2 ml Eppendorf tubes. The initial and final dyes absorbance was spectrophotometrically measured at 620nm for every dye.

Relative decolorization (%) was calculated by the following equation:

$$Relative\ decolorization\ (\%) = \frac{Ainitial - Afinal}{Ainitial} \times 100$$

Relative decolorization of selected dyes was calculated with control beads too. Calculated value for control beads were subtracted from decolorization value for beads with cell wall laccase.

### 2.11. Reusability

For testing reusability of cell wall laccase entrapped in beads, Ca-dopamine-alginate beads with immobilized cell wall laccase was used ten times for dye decolorization. Reusability was investigated in 100 mM NaAc buffer solution of different dyes (pH 5, with 5 mM $CaCl_2$ and 2 mM $CuCl_2$) at room temperature. Beads were incubated in dyes solution for 48 h and at the end of each cycle the beads were filtered and washed three times with NaAc buffer with 5mM $CaCl_2$ (100 mM, pH 5). The procedure was repeated with fresh solution of dyes. The initial and final dye absorbance were spectrophotometrically measured at 620 nm for every cycle. The activity of first, freshly prepared beads in the first run was defined as 100%. Ten decolorization cycles of 48 h were done for each of the different dyes. This study has done in triplicate too.

## 3. Results and discussion

### 3.1. Yeast surface display of laccase

In this study, functional expression and surface display of laccase from *Streptomyces cyaneus* CECT 3335 in a strain of *S. cerevisiae* EBY 100 was done successfully for the first time. The recombinant laccase was functionally expressed on the yeast cell surface, and its activity was detected with ABTS as a substrate. Maximal laccase activity was obtained after 16 hours of fermentation (Fig S1). The activity was detected only on yeast cell transformed with pCTCON2-lac construct and no background activity was detected in the control cells transformed with empty pctCON2 plasmid. Since copper ions are present in laccase active center, addition of copper in the reaction mixture was necessary for activation of the enzyme and activity assay. When no copper was added to reaction mixture laccase activity could not be detected, as was shown in previous report on expression of the same enzyme in *E.coli*. [26] The surface displayed laccase showed enzyme activity of 0.01±0.001 IU/g wet cell biomass.

In literature, only a few studies reported the production of laccase in *S. cerevisiae* cells. [8, 27] Yingying et al. showed that surface display of laccase significantly improved the stability of this enzyme. [8] The activity of the surface display laccase that was 0.1 U/g dry cell weight at the beginning, after 25 days was retained more than 90%, in contrast to the activity of free laccase, which declined to 60% of its initial activity after the same period. The advantages of surface-displayed laccase are high longevity and stability with the possibility of reuse in multiple treatment processes and simple production procedures by cell cultivation. [8] Laccase from *Streptomyces cyaneus* has not been previously expressed on the yeast cell surface, but was expressed in *E. coli*. [26]

## 3.2. Cell wall laccase preparation

To further increase activity of surface displayed laccase yeast cells were removed from ballast proteins by lysing with 3% toluene (v/v) at room temperature. The goal was to disrupt yeast cells and release their cellular content, but not to remove laccase from the cell wall. Therefore, lysis was monitored for 48h to determine the optimal time of lysis. Laccase activity within yeast cell walls and in the lysate was measured, while protein concentration was measured only in the lysate. Laccase activity within cell walls increased during lysing and reached maximum after around 20-30h, while lysate protein concentration reached a plateau after 30h of lysis, Figure 1.

Laccase activity in lysate could not be detected during 48 h, probably because laccase remained attached to the cell wall. The optimal time for cell lysis was 24 h when we obtained cell walls with the highest specific activity of laccase.

After lysing, the specific activity of cell wall laccase increased significantly, from 0.010±0.001 IU/g to 3.1± 0.2 U/g (310 times). This could result from a decreased mass of cell walls compared to the yeast cells and the release of non-secreted enzyme from the cell wall's internal cell compartments after toluene lysis.

## 3.3. Modification of alginate with dopamine and its characterization

To introduce new functional groups (bi-phenol groups) and make alginate more suitable for biocatalysts immobilization and hydrogel formation, alginate was for the first time modified with dopamine using periodate oxidation with various molar ratios of sodium periodate to repetitive glycoside: 2.5, 5.0, 10.0, and 20.0 mol% and the reaction of reductive amination with

sodium cyanoborohydride in the presence of dopamine, thus having the final product with stable secondary amino groups, Figure 2.

Dopamine modified alginate was characterized using UV-Vis spectroscopy, FTIR, and $^{1}H$ NMR spectra to confirm modification and determine the degree of modification. UV-Vis spectra show the presence of phenol groups in modified alginate, at a wavelength maximum of 280 nm that originates from dopamine. In contrast, native alginate doesn't have that wavelength maximum in lack of chromophore that can absorb it. Also, it could be seen from UV-Vis spectra that oxidation degree increases with higher molar ratios of oxidation reagent, which leads to more introduced dopamine groups and higher absorption at 280 nm (Figure 3).

FTIR spectra of native and dopamine modified alginate showed dopamine aromatic structures within modified alginate by the appearance of a peak at 1532 $cm^{-1}$ from C-C in-ring aromatic stretching vibrations (Figure S2). [28] Successful modification of alginate with phenol compound was also confirmed by $^{1}H$ NMR spectra, with distinct peaks from three aromatic H atoms at 6.65, 6.75. and 6.85 ppm present in dopamine and not present in native alginate (Figure S3).

## 3.4. Immobilization of cell walls

For use in batch and bed packed reactor, laccase immobilization was done by alginate bead immobilization using entrapment, with calcium ions as cross-linking agent. With this method, spherical regular-shaped alginate beads were obtained. Concentrations of alginate and cell walls were optimized for the highest laccase activity. Beads were prepared using a mixture of 2.7% native alginate (w/v) and 150 mg/ml cell walls (CW) and dropped in 2% $CaCl_2$ (w/v), exhibited mechanical stability and optimal laccase activity, in comparison to lower alginate concentrations.

Hydrogel encapsulation of cell wall laccase has also been done using dopamine-alginate in the same concentration as native alginate. Encapsulation was done firstly by ionic cross-linking with calcium ions and then additionally enzymatically by cross-linking of dopamine using oxygen and laccase attached on the immobilized cell walls. For immobilization, dopamine-alginate oxidized with various molar ratios of periodate (2.5-20 mol%) was tested, and the most mechanically stable beads were obtained using five mol% dopamine-alginate at 2.7% (w/v) concentration, 150 mg/ml cell walls (CW), dropped in 2% $CaCl_2$ (w/v). During additional

cross-linking with laccase, dopamine-alginate beads changed the whitish color to grey-brown color due to oxidized and cross-linked dopamine molecules.

The optimal amount of added cell walls for immobilization was determined by testing different amounts of cell walls for specific activity of the immobilized biocatalyst. The highest specific activity of immobilized biocatalysts was obtained at 150 mg/ml of added cell walls and did not increased further by adding higher amounts of cell walls (Figure 4).

It could also be seen that the enzyme activity of immobilized laccase cell walls within dopamine-alginate was two times higher than when using native alginate. This fact suggests that additional cross-linking of alginate chains by oxidizing dopamine retained better laccase molecules or that the porosity of dopamine-alginate was better than the native one.

It has been reported that optimal conditions for the crude enzyme's alginate encapsulation were 2% (w/v) alginate and 0.05 M $CuSO_4$ as a hardening agent. [29] In another study, it was observed that optimal conditions were 2% (w/v) sodium alginate, 2% (w/v) $CaCl_2$, and 1:4 Enzyme/Alginate ratio, with the loading efficiency 88.1±2.4% for proteins and 93.3±1.1% for the enzyme. [21] In the present study, immobilization of the cell walls with laccase was done, and therefore loading efficiency was 100% since leakage of the enzyme within filtrate from the beads was not detected, probably because enzyme was already covalently bound to the cell walls.

## 3.5. Immobilization yield (%)

The immobilization of cell wall laccase was analyzed in native and modified alginate with respect to immobilization yield that has been defined as the ratio of specific activity of the immobilized enzyme and total amount of encapsulated enzyme. Therefore, it shows the efficiency of the use of immobilized enzymes and loss of activity due to diffusional limitations or enzyme inactivation. It could be seen that by increasing amount of added laccase cell walls immobilization yield was decreased due to increased diffusional limitations within hydrogel beads, (Figure 5).

Results also showed that the immobilization of cell wall laccase within dopamine-alginate gave a 2.5 to 16-fold increase in yield compared to native alginate, depending on the concentration of added cell walls. (Figure 5)

Reasons for such higher immobilization yield could be much better porosity of cross-linked dopamine alginate resulting from a decrease in the molecular weight of periodate oxidized alginate and better swelling properties caused by introduced positively charged amino groups

within alginate molecules. Also, the presence of novel charged groups and aromatic rings compared to native alginate could lead to better retainment of immobilized enzyme or increased local concentrations of substrates due to interactions with dopamine within alginate. All of these factors could lead to higher enzyme and substrate concentrations within hydrogel beads and decreased diffusional limitation that often occurs with alginate immobilized enzymes.

## 3.6. Characterization of free and immobilized cell wall laccase

Since cell wall laccase immobilized within dopamine-alginate beads had significantly higher activity compared to the same biocatalysts immobilized within native alginate for further studies we used only dopamine-alginate.

### 3.6.1. Influence of pH on laccase enzymatic activity

As mentioned previously, the activity of free and immobilized cell wall laccase in dopamine-alginate beads was determined in a pH range from 2.0 to 7.5 in different buffers. Results showed that the optimal pH of immobilized cell wall laccase and free cell wall laccase is at pH 5 (Figure 6).

The optimal pH for immobilized enzymes is usually slightly shifted towards more acidic regions than free enzymes presumably due to the negative charge present in the alginate. [30] Immobilization of cell wall laccase within dopamine-alginate beads did change significantly, probably because of the presence of both negative and positive charge.

Enzyme stability at pH 5 in 0.1 M Na-acetate buffer was determined by preincubating the free and immobilized cell wall laccase for 24 h and 48 h at room temperature. As shown in Figure 7, dopamine-alginate immobilized cell wall laccase displayed higher pH-stability than its free counterpart.

Greater pH stability of immobilized enzyme was explained by binding enzyme on support, which gives a lower probability to pH change induce conformational change of enzyme. [4]

### 3.6.2. Influence of temperature on laccase enzymatic activity

The thermostability of free and immobilized cell wall laccase in dopamine-alginate beads was determined by incubating cell wall laccase beads for 1 h at 25, 30, 60 and 90°C, and at pH 5.

Residual enzyme activity was measured using ABTS as described above. Free and immobilized cell wall laccase kept maximal enzyme activity till 30°C (Figure 8).

At 60°C free cell wall, laccase retained 69% of enzyme activity, while its immobilized counterpart retained 93%. Immobilization of cell wall laccase had increased temperature stability, like in the case of free laccase in other research results. [4, 21, 31]

### 3.6.3. Effect of substrate concentration: Determination of Km and Vmax

The enzyme kinetic parameters were determined for immobilized and free cell wall laccase using ABTS as a substrate. The Michaelis Menten kinetics constant ($K_m$) increased from 0.0125 mM for the free cell wall laccase to 8.36 mM for the immobilized cell wall laccase, as expected. Maximal velocity ($V_{max}$) decreased from 0.661 µmol/min for the free cell wall laccase to 0.0340 µmol/min for the immobilized cell wall laccase. The higher $K_m$ value of immobilized enzyme is caused by diffusion limitations and this data are in agreement with other reports of decreased affinity of Ca-alginate immobilized laccase for substrate. [31, 32]

## 3.7. Decolorization of dyes

Several dyes were examined as a model substrate to assess the potential of the cell wall laccase for dye degradation. Dyes were selected from different groups of textile dyes (Amido Black 10B, Methylene Blue, Guinea Green, Reactive Black 5, Methyl green, Evans Blue, Crystal Violet, and Remazol Brilliant Blue) to evaluate the dye-decolorizing potential of immobilized cell wall laccase within alginate beads. The efficiency of the laccase enzyme for removal and degradation of dyes has been previously reported and discussed. [4, 19, 21, 22, 33-35] In our study, dye decolorization was done without the use of a redox mediator and was monitored for 48h and time course of decolorization reaction is presented on Figure 9.

We used relative decolorization value that was calculated by subtracting the value of the control beads without laccase from the value of the beads with cell wall laccase. The dye removal by beads with immobilized enzymes could result not only of enzymatic reaction but also of alginate adsorption. Because of that we used alginate beads with cell walls without laccase, as a control. Another control was done without beads, only dyes in buffer. We determined that an acidic pH of buffer has not effect on dye decolorization. (after 48 h no absorbance change has been noticed)

The results showed that Amido Black 10B and Evans Blue exhibited maximum (60%) relative decolorization, while Methyl Green exhibited minimum (0%) relative decolorization (the decolorization ability for Methyl Green has not been demonstrated). The immobilized cell wall laccase was also highly effective against Reactive Black 5 and Remazol Brilliant Blue, removing 58% and 61% of dyes after 48 h, respectively. The RB5 and RBBR were also tested for decolorization by laccase from *M. thermophila*, immobilized on Sepabeads EC-EP3. There was no decolorization with immobilized enzyme, without the addition of redox mediator when decolorization reached 60%. [19] They revealed that redox mediator is essential for decolorization of RB5. The probable reason is RB5 has steric hindrance that could reduce the accessibility of $–NH_2$ and –OH groups, which are the most vulnerable by laccase attack. In present study, decolorization of Evans Blue dye had shown as the most effective. This dye had already been used for this purpose in some studies. [36-38] The free *Trameter villosa* laccase decolorized 49% of EB for 3h, and Pardo et al. determined that the presence of two hydroxyl groups favor rapid oxidation of this dye. [10] All of this shows that our dopamine-alginate immobilized cell wall laccase from *Streptomyces cyaneus* CECT 3335 shows good catalytic properties for dye decolorization.

## 3.8. Reusability

In the end, we investigated whether the immobilized cell wall laccase in dopamine-alginate beads could be successfully recycled for multiple treatment processes of dye decolorization. Dyes that have been degraded in higher percent in the previous experiment were selected for multiple decolorization processes. The selected dyes were Amido Black 10B, Evans Blue, Remazol Brilliant Blue and Reactive Black 5. Reusability was investigated in ten cycles of 48 h duration per cycle. The relative decolorization rates are shown in Figure 10.

After being used ten times, laccase immobilized in dopamine-alginate beads retained 90% of its activity with Evans Blue dye, 61% with Amido Black 10B and 33% with Reactive Black 5. Immobilized laccase lost all the activity after $5^{th}$ cycle of decolorization of Remazol Briliant blue dye. The gradual decrease of decolorization rate from $1^{st}$ to $10^{th}$ cycle may be related with enzyme inactivation, or with difficulties with the diffusion of substrate and product, caused by decrease in pore sizes of support matrix. [22]

Reusability of immobilized enzymes is the most relevant aspect for industrial use, due to their continuous reuses, which decrease the cost of process. In the literature, laccase from various sources had been immobilized in different support materials in order to be reused in process of

decolorization of dyes. [19-22, 29, 33, 34, 39, 40] Thus, a laccase from *C. gallica* immobilized on Ca-alginate beads was tested in ten cycles of decolorization Remazol Brilliant Blue R (RBBR), Reactive Black 5 (RB5), Bismark Brown R (BBR) and Lanaset Grey G (LG) dye. After 7 cycle of 24 h, RB5 and RBBR were decolorized about 50% and 85%, respectively. [21]

# 4. Conclusions

In this article we report development of biocatalysts immobilized within dopamine-alginate beads that is suitable for multiple cycles of dye decolorization like Evans Blue dye, Amido Black 10B and Reactive Black 5. For this purpose, for the first time, laccase from *Streptomyces cyaneus* CECT 3335 was displayed on the surface of *Saccharomyces cerevisiae* EBY 100 cell walls and periodate oxidized alginate was modified using reductive amination reaction in the presence of dopamine. By lysing yeast cells with toluene and removing ballast proteins within cells, and increasing permeability of membranes and cell walls, the specific activity of laccase within cell walls was increased 300 times. Modification of alginate with dopamine was confirmed by UV-VIS, FTIR, and H1 NMR spectroscopy. Obtained dopamine-alginate was able to form hydrogels in the presence of calcium or by cross-linking with laccase. Cell walls with laccase were immobilized within dopamine-alginate beads by cross-linking of dopamine-alginate with calcium ions and laccase.

Obtained immobilized biocatalysts had two times higher activity and 16 times higher immobilization yield than the same one immobilized within native alginate using only calcium as a cross-linker. Compared to cell wall, laccase pH optimum did not change significantly, while temperature stability slightly increased. The immobilized biocatalyst showed 100% percent decolourization efficiency of Evans Blue dye, Amido Black 10B, and Reactive Black 5 dye. After 10 cycles of repeated use decolourization efficiency decreased only to 90% for Evans Blue dye.

All of these results indicate that the obtained cell surface displayed laccase biocatalysts could be used for textile dye decolourization from waste waters and that developed dopamine-alginate hydrogels could be very useful for biocatalysts immobilization due to the superb properties compared to native alginate.

In summary, we report the first application of laccase-coated yeast cell walls with 300-fold increased activity per unit of mass compared with previously used whole cells. We also report the application of novel dopamine-modified alginate polymer with additional *in situ* cross-

linking catalysed by immobilized enzyme. Application of new polymer significantly improved both immobilization yield (16-fold) and enzyme activity (2-fold) when compared with application of native polymer. In addition to that, laccase-coated yeast cell walls immobilized within dopamine-alginate beads may be applied for multiple cycles of dyes decolourization with only slight loss of activity after 10 consecutive 48-h degradation cycles.

**Conflict of interest:** The authors declare that they have no competing interests.

**Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

**Funding:** This study was funded by project No. ON173017 and project No. ON172049 granted from the Ministry of Education and Science, Republic of Serbia.

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## Figure captions

**Figure 1** A) Specific activity of laccase within cell walls and B) protein concentration in cell lysate, during the 48 hours of lysis.

**Figure 2** Synthesis of dopamine-alginate in reaction of periodate oxidation and reductive amination.

**Figure 3** UV-Vis spectra of 0.1% (w/v) native alginate and dopamine-alginate oxidized with various molar ratio of periodate (2.5-20 mol%)

**Figure 4** Specific activity dependence on the amount of added cell walls in immobilization mixture for beads with native and dopamine-alginate. Concentration of alginate was 2.7% (w/v) and 2% (w/v) calcium chloride was used.

**Figure 5** Immobilization yield dependence on the concentration of added cell wall laccase. Immobilization in native alginate and dopamine-alginate.

**Figure 6** Effect of pH on the activity of free and immobilized cell wall laccase in Ca-dopamine-alginate beads

**Figure 7** The pH stability of free and immobilized cell wall laccase in Ca-dopamine-alginate beads, after 24 and 48 h of incubating in optimal buffer

**Figure 8** The temperature stability of free and immobilized cell wall laccase after incubating for 1 h at 25, 30, 60 and 90°C

**Figure 9** Time course of decolorization reaction (during 48h) of several dyes: Amido Black 10B, Methylene Blue, Guinea Green, Reactive Black 5, Methyl green, Evans Blue, Crystal Violet, Remazol Brilliant Blue.

**Figure 10** Reusability of cell walls laccase immobilized in Ca-dopamine-alginate beads. Beads were used for ten decolorization cycles of 48 hours. After each cycle, the beads were filtrated and washed three times with 100 mM NaAc buffer pH 4.5 and replaced with fresh solution of dyes.

Figure 1



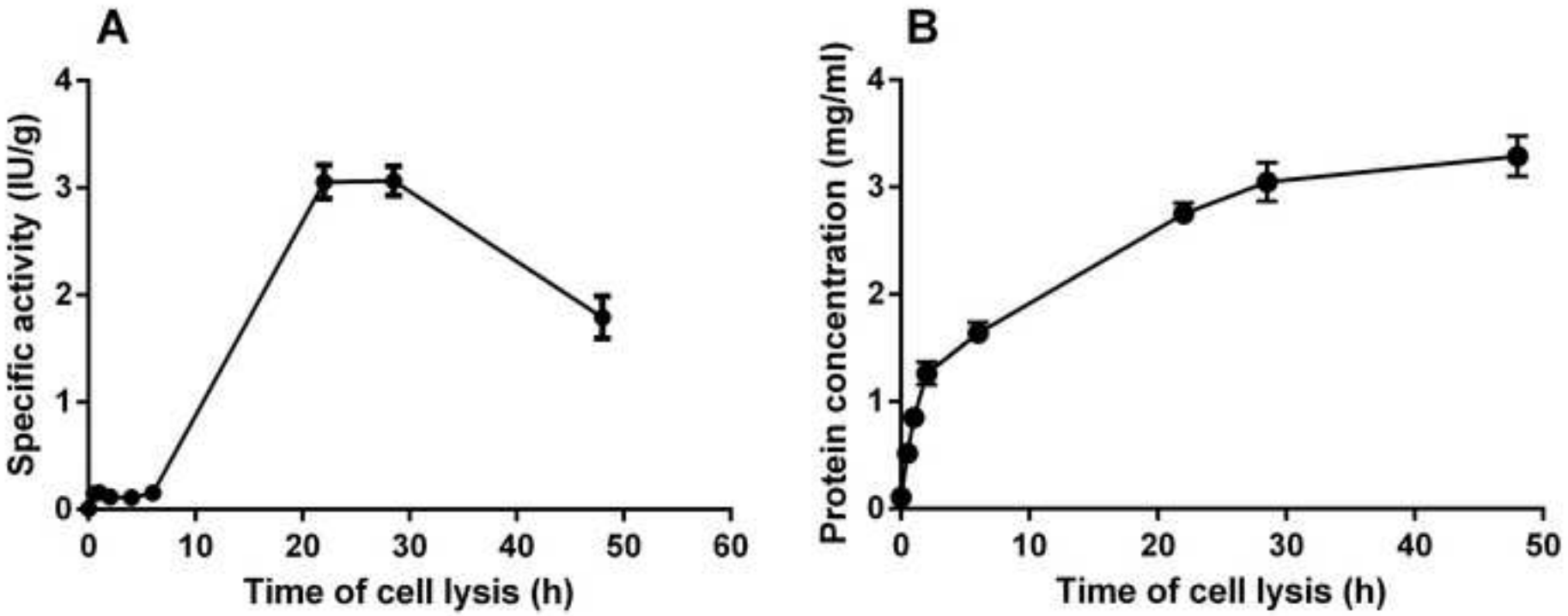

Figure 2

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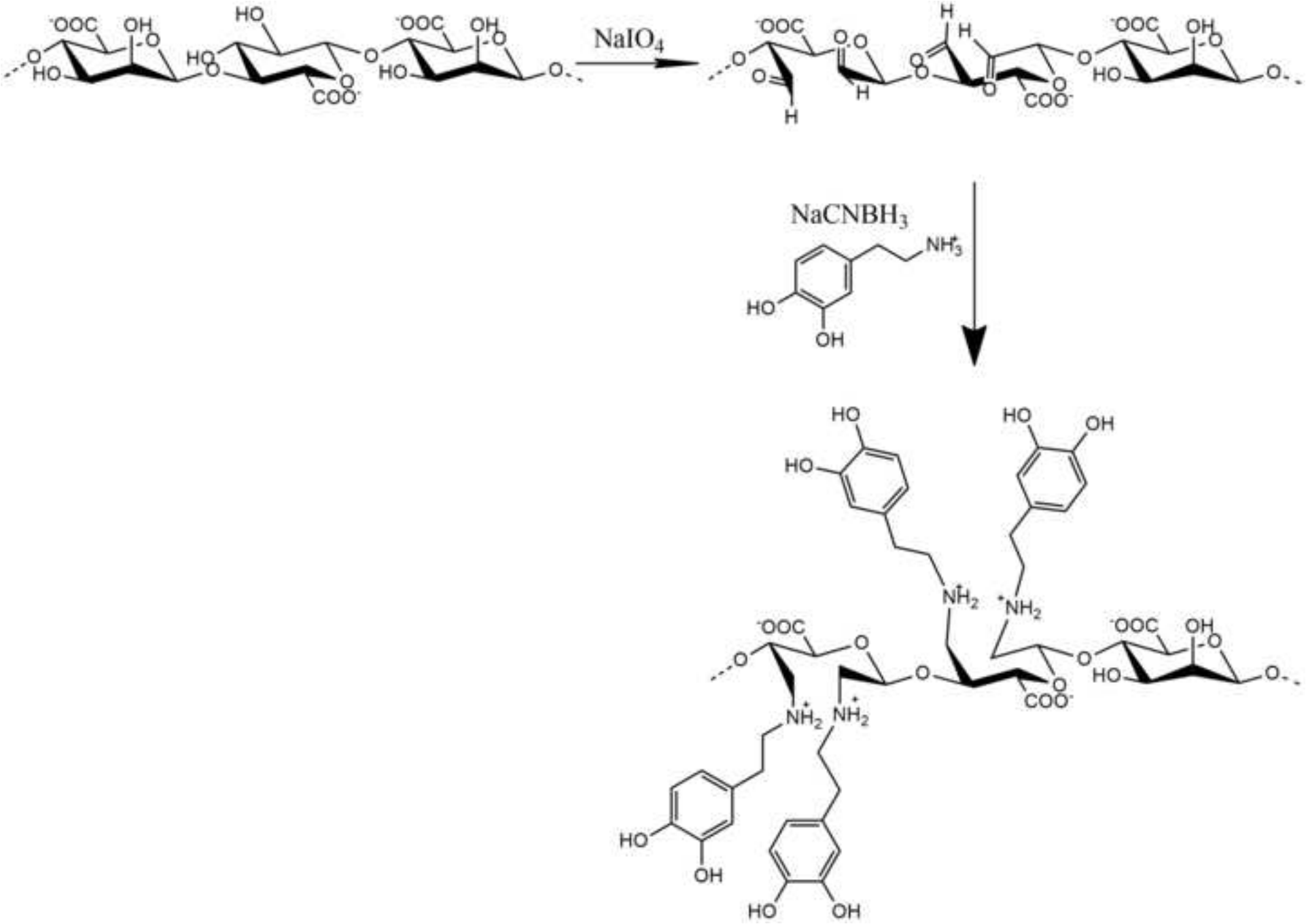

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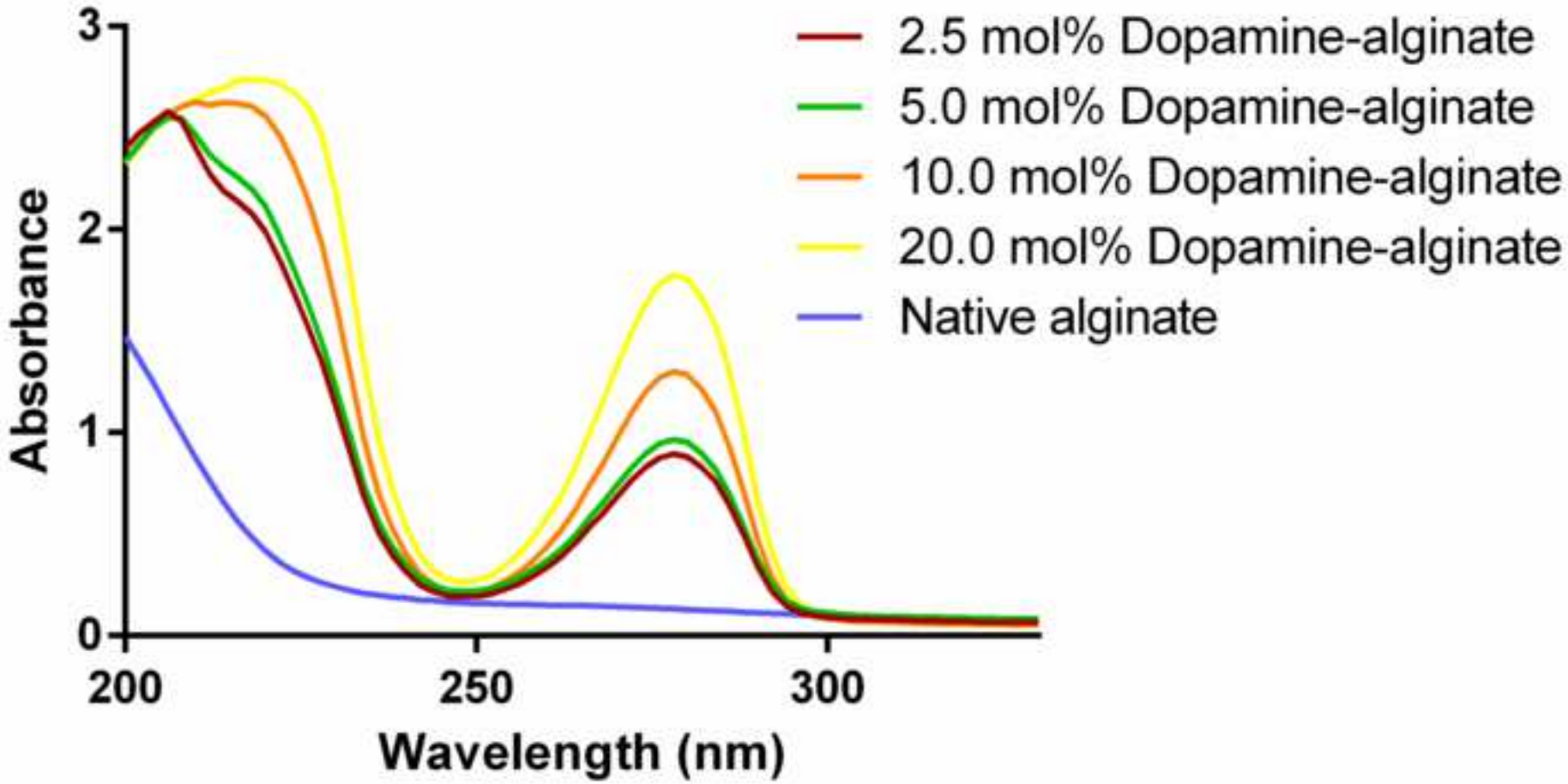

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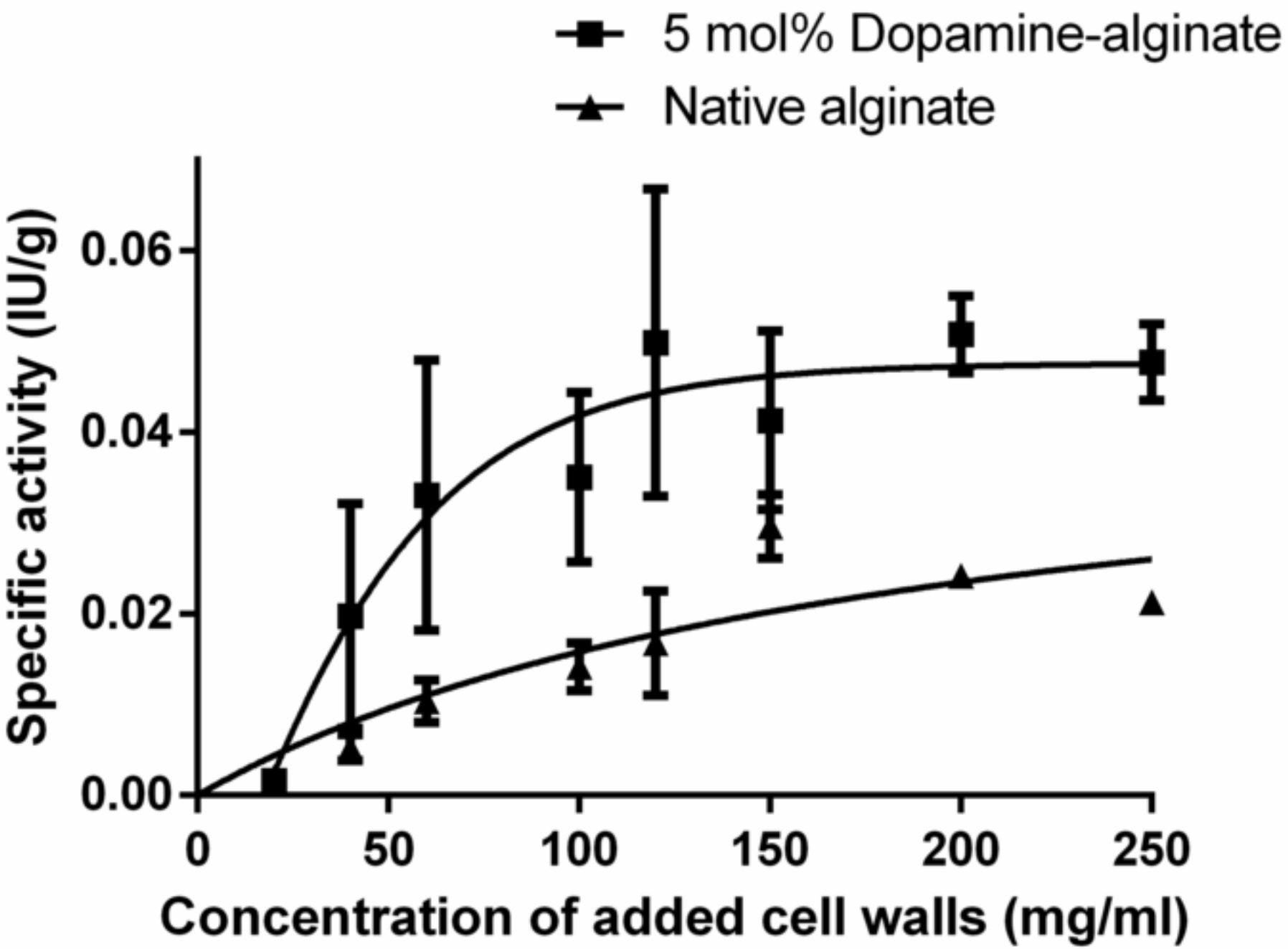

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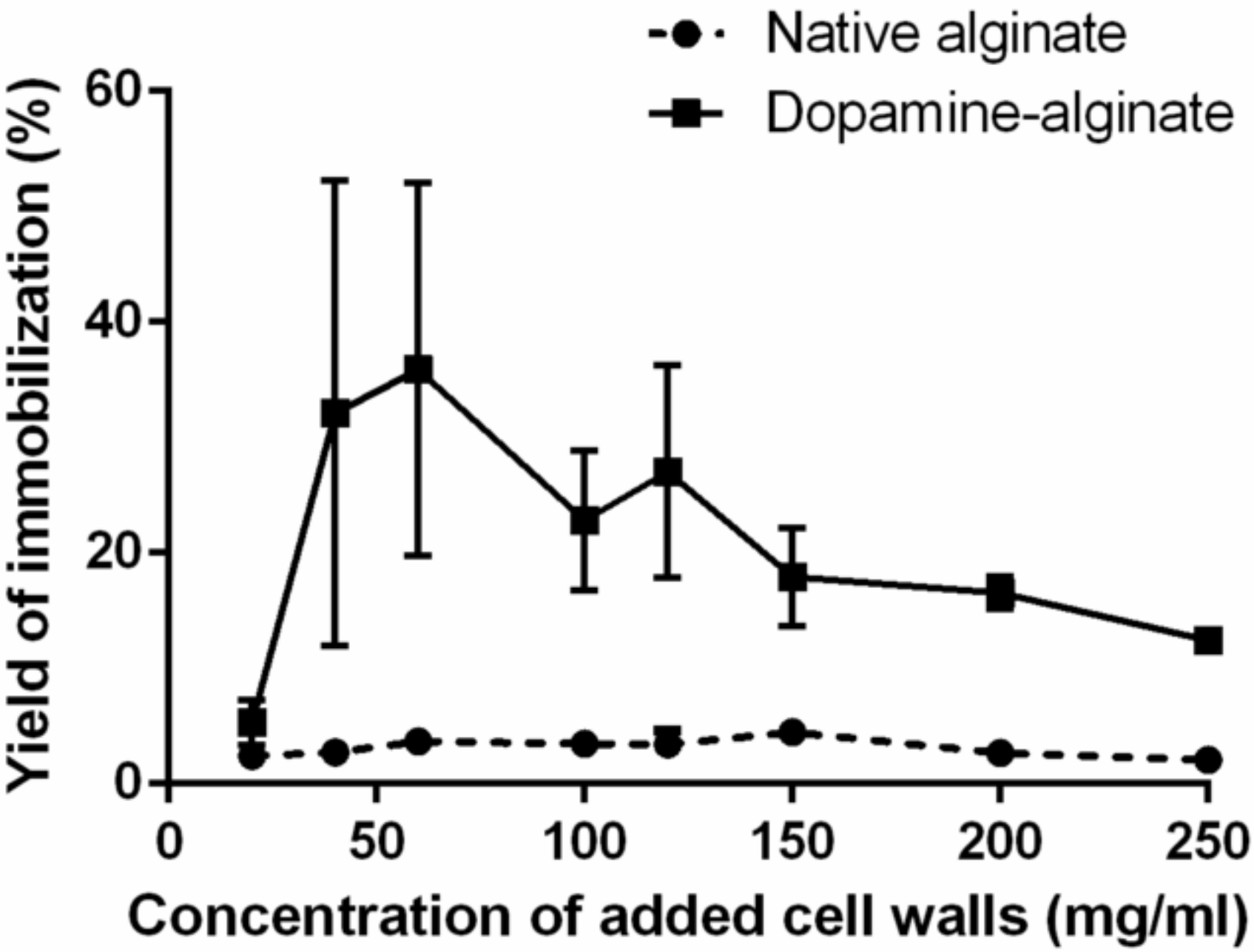

Figure 6

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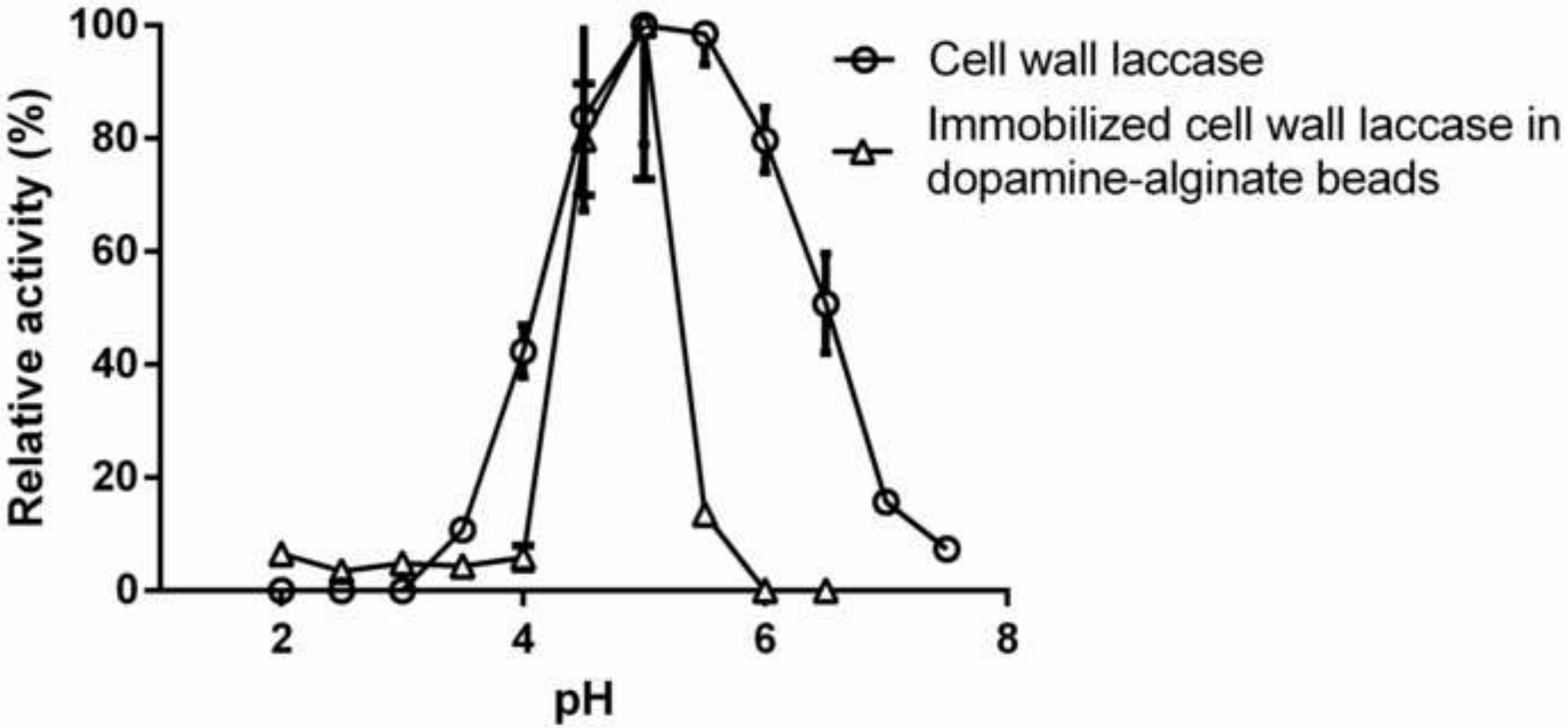

Figure 7



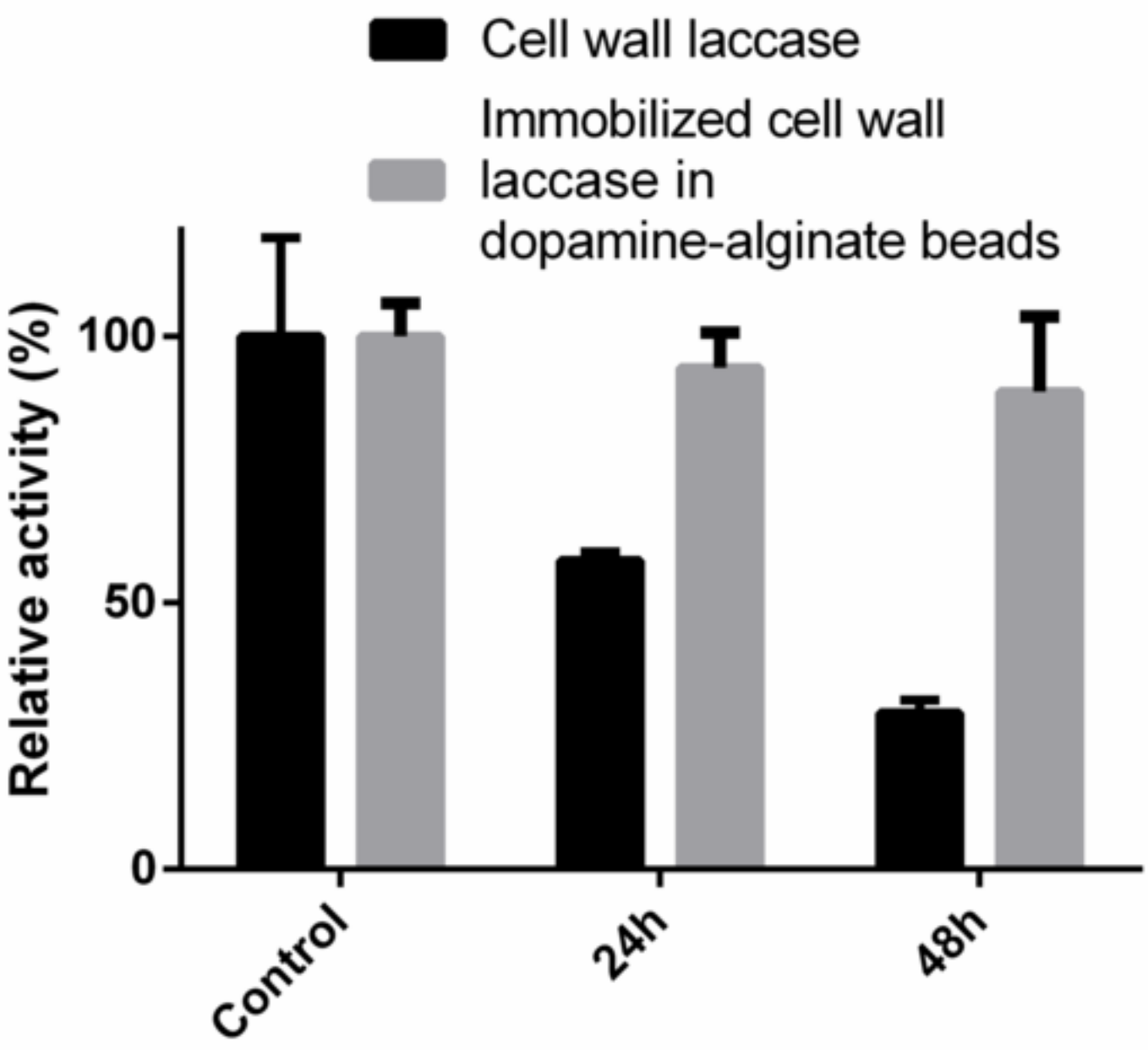

Figure 8



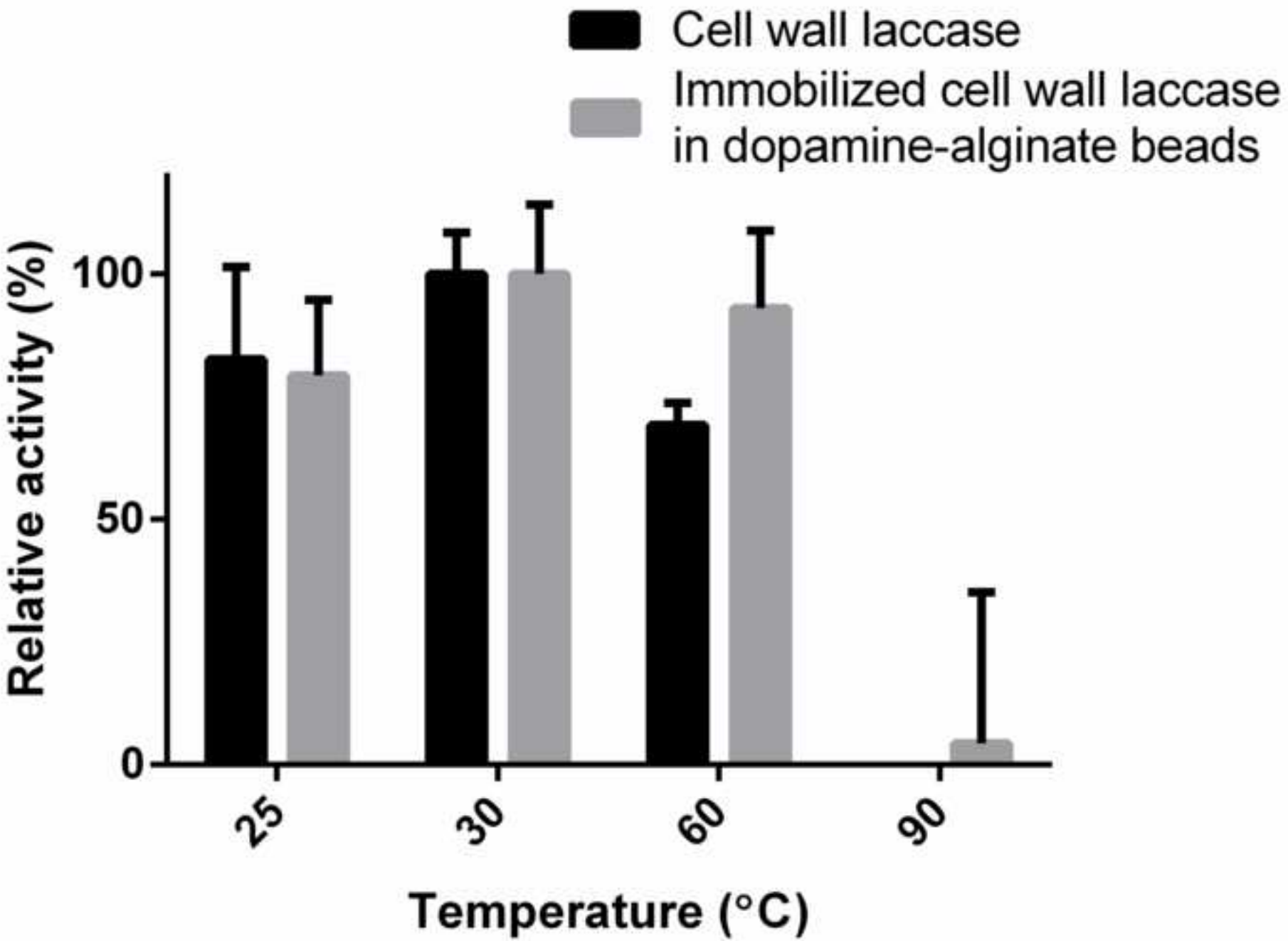

Figure 9

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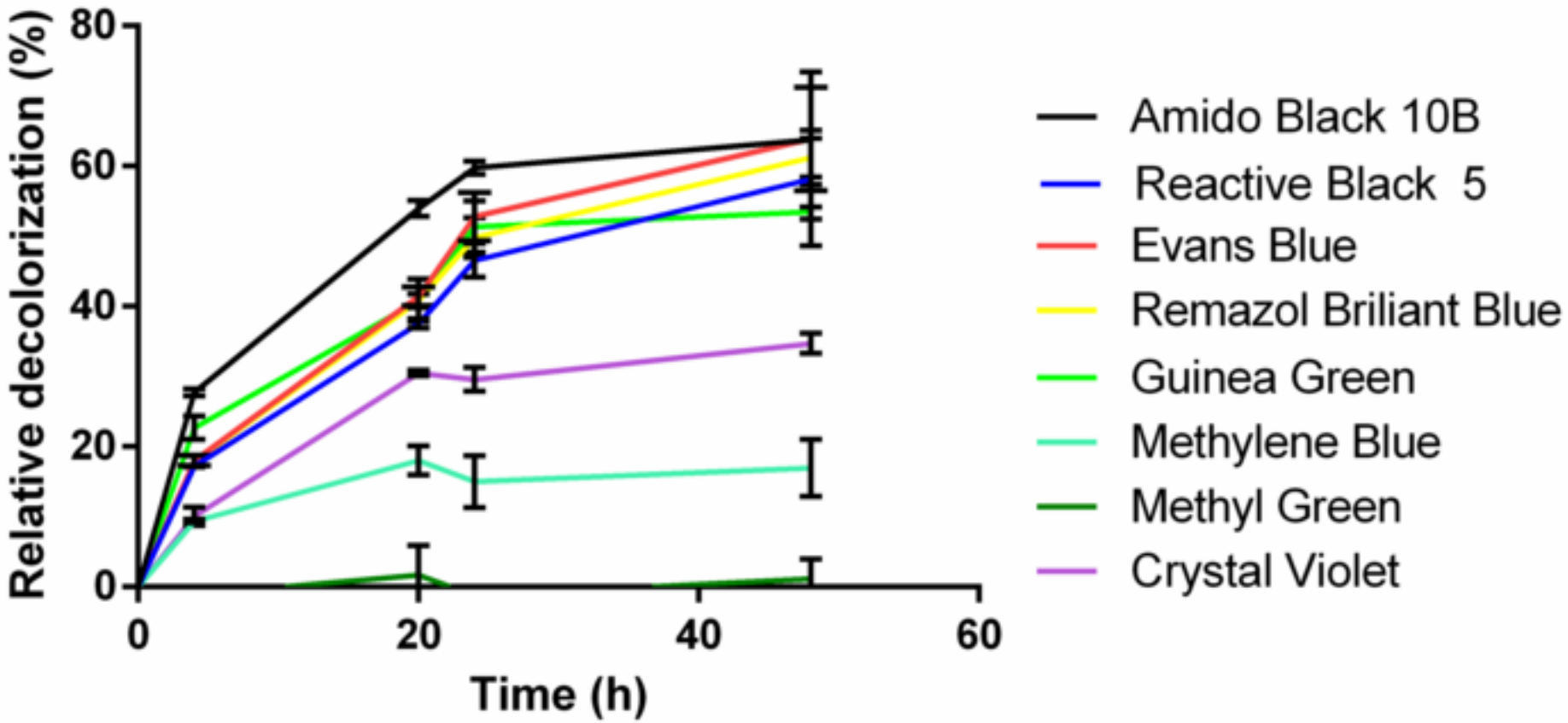

Figure 10