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Production of laccase and xylanase from *Coriolus versicolor* grown on tomato pomace and their chromatographic behaviour on immobilized metal chelates

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ABSTRACT

A strain of *Coriolus versicolor* was grown in tomato pomace as sole carbon source for the production of laccase and xylanase. This culture exhibited a peak of laccase (362 U/L of fermentation broth) and xylanase (2565 U/L of fermentation broth) activities on the 3rd and 14th day of cultures with a specific activity of 3.7 and 11.7 U/mg protein, respectively. Differential chromatographic behaviour of xylanase and laccase from *C. versicolor* was investigated on immobilized metal chelates. The effect of pH, length of spacer arm, the presence of imidazole and nature of metal ion was studied for enzyme adsorption on immobilized metal affinity chromatography (IMAC).

PDB survey of solvent accessible histidine residues in laccase and xylanase families was carried out by using bioinformatic tools. A one-step purification for laccase from *C. versicolor* was devised by using Sepharose 6B–EPI 30–IDA–Cu(II) and the purified enzyme was obtained with a specific activity of about 15.0 U/mg protein, a final recovery of enzyme activity of about 60% and a purification factor of about 10. The purified preparation of laccases A exhibited an optimum pH of activity of 5.0 and 3.0 with *o*-dianisidine and ABTS as substrates, respectively. The optimum temperature of activity for this enzyme was found to be 80 °C in acetate buffer at pH 4.5 whereas the half-life ($t_{1/2}$) of 19.4 ± 2.2 h and 0.50 ± 0.012 h was obtained at 45 and 60 °C, respectively. The kinetic parameters (V_{max}, K_m, K_{cat} and K_{cat}/K_m) of the purified enzyme were also obtained with *o*-dianisidine, guaiacol and ABTS as substrates. By using selective experimental conditions in IMAC, it was possible to separate successfully laccase isoenzymes into two groups, one with low and the other with high p*I* values. Kinetic characterization of both groups of isoenzymes was also carried out.

1. Introduction

The food industry produces throughout its chain huge amounts of agro-industrial wastes that cause high commercial losses as well as severe environmental problems.

Tomato processing industries are responsible for annual production of large amounts of a by-product known as tomato pomace which consists of tomato peel, seeds as well as some pulp [1,2]. The chemical composition of tomato pomace includes proteins, lipids, carbohydrates, aminoacids, carotenoids and minerals [3]. Therefore, this agricultural waste could be used as fermentation media for production of fine chemicals such as enzymes and vitamins [4,5]. Recently, tomato pomace has been used for production of enzymes from white-rot fungi by submerged fermentation [6].

Laccases (benzenodiol:oxygen oxidoreductase, EC 1.10.3.2) occur in fungi, plants and some bacteria and are multicopper enzymes which catalyze the oxidation of polyphenols with oxygen as final electron acceptor. It is an industrial enzyme with high economical interest since it plays important roles in several industrial applications, namely in the pulp and paper industry, food industry, textile industry and in the bioremediation of soils [7]. However, some novel applications of this enzyme in medicinal and nanobiotechnology fields require highly purified enzyme preparations. Therefore, there is an increasing interest to devise simple and specific isolation schemes for this enzyme in order to obtain purified preparations with high purity and recovery of enzyme activity [7,8]. To our knowledge, there are no reports in the

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literature about the use of either pseudo-affinity or affinity chromatographic techniques for specific purification of laccase either from fungal or bacterial strains. In fact, laccases [9] have been purified through classical methods of protein purification, namely a purification scheme for a laccase from *Trametes* sp. that makes use of ion-exchange chromatography followed by two steps of gel filtration chromatography [10]. With this purification scheme, laccase was purified 33.8-fold with a final recovery of activity of 21.2% [10].

Endoxylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) are a component of the xylanolytic system that accounts for the degradation of xylan and other hemicelluloses by catalyzing the cleavage of glycosidic bonds in the xylan backbone [11]. In recent years, important applications of xylanases in different industrial processes have been found. One major area of application involves their use as bleaching agent in the pulp and paper and textile industries. Other applications of xylanases include the saccharification of lignocellulosic materials, clarification of juices, improvement in the digestibility of animal feed and manufacture of bread, food and drinks [8,12]. However, some applications, namely in biobleaching, require that xylanases should be free of cellulolytic enzymes [11]. To our knowledge, there are no reports in the literature about the differential chromatographic behaviour of xylanases on immobilized metal chelates.

The present work is concerned with the production of xylanase and laccase from culture supernatant of *Coriolus versicolor* which was grown in tomato pomace as sole carbon source. Subsequently, differential chromatographic behaviour of these enzymes will be investigated on immobilized metal chelates and a one-step purification of laccase will be carried out by immobilized metal affinity chromatography (IMAC) as well as partial characterization of purified laccase isoenzymes will be presented.

2. Materials and methods

2.1. Materials

Potato Dextrose Agar (PDA) was supplied by Biokar Diagnostics. 1,4-butanediol diglycidyl ether (BDGE), *o*-dianisidine, guaiacol, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and iminodiacetic acid (IDA) were purchased from Sigma Chemical Company. Epichlorohydrin (EPI) was obtained from Aldrich whereas Sepharose 6B and Sephacryl HR-100 were purchased from GE Health Care. Concentration of culture supernatants was performed with a GR 81 PP membrane from Danish Separation Systems (nominal M_r cut-off value of 6000 Da). Tomato pomace was a kind gift from Sopragol S. A. from Mora, Portugal. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Enzyme assays

Laccase (EC 1.10.3.2) was assayed by using *o*-dianisidine as a substrate. Assays were performed in 0.2 M sodium acetate buffer at pH 4.5 containing 17 mM *o*-dianisidine and the oxidized product was read at 450 nm as described previously [13]. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmole substrate per min. Total xylanase (EC 3.2.1.8) activity was assayed by incubating the extract, for 1 h at 50 °C, with 0.5% (w/v) Birchwood xylan in 0.05 M citrate buffer pH 4.8. The increase in reducing sugars in the reaction mixture was determined by the dinitrosalicylic acid method by using xylose as standard [14].

2.2.2. Protein assay

Total protein was determined by Bradford method by using BSA as a protein standard [15].

2.2.3. Fungal growth conditions and enzyme production

The strain of *C. versicolor* was grown and maintained in solid potato dextrose agar (PDA) medium. For enzyme production, the strain was grown in liquid medium containing 20 g/L of tomato pomace, 1 g/L of $(NH_4)_2SO_4$, 0.125 g/L of $CaCl_2$, 1 g/L of $NaH_2PO_4{\cdot}H_2O$ and 0.5 g/L of $MgSO_4{\cdot}7H_2O$. The pH was adjusted to 5.5 prior to autoclaving. As far as the chemical composition of tomato pomace is concerned [1], the following main components are present: protein (17.66%), lipids (9.66%), total carbohydrates (59.07%) and several micronutrients [1].

For submerged fermentation, *C. versicolor* was first grown in 250 mL Erlenmeyer flasks containing 110 mL of culture media for 5 days. These cultures were then used as inocula to prepare several 60 mL flasks containing 11 mL of culture media. Incubations were performed in an orbital shaker at 25 °C and 150 rpm and samples were collected at regular intervals for 14 days. Two culture samples were withdrawn at each interval and assayed for enzyme production, reducing sugars and protein content. All cultures were grown in triplicates. For enzyme production and purification, *C. versicolor* was first grown in 250 mL Erlenmeyer flasks containing 110 mL of culture media for 5 days. These cultures were then used as inocula to prepare several 1 L flasks containing 200 mL of culture media which were incubated in an orbital shaker at 25 °C and 150 rpm for 7 days.

2.2.4. Preparation of chromatographic matrices

Epoxy-activated agarose gel containing 1,4-butanediol diglycidyl ether (BDGE) as spacer arm was prepared as described in the literature [16]. Under the conditions selected, the agarose matrix contained 30 μmole of epoxy groups/mL of sedimented gel. Subsequently, epoxy-activated agarose was reacted with iminodiacetic acid (IDA) as the chelating agent [16] and these stationary phases will be referred as Sepharose 6B–BDGE 30–IDA, or simply as BDGE-30. The chromatographic matrix was thoroughly washed with water and kept at 4 °C in 0.01% (w/v) sodium azide. Chromatographic support with 30 μmole of epoxy groups/mL of sedimented gel containing epichlorohydrin (EPI) as spacer arm was also prepared according to the procedure described in the literature [16], and this stationary phase will be referred as Sepharose 6B–EPI 30–IDA or simply as EPI-30.

2.2.5. Chromatographic behaviour of xylanase and laccase

As previously described [17], a rapid batch method carried out in ELISA microtiter plates was used to study the adsorption of xylanase and laccase on Cu(II), Ni(II), Zn(II) and Co(II) metal chelate supports. The batch method was carried out as follows: sedimented metal chelate supports (100 μL) were transferred to ELISA microtiter plates which were loaded with 4 volumes of either 50 mM $CuSO_4$, $NiCl_2$, $ZnCl_2$ or $CoCl_2$.

Subsequently, the agarose gel was washed with distilled water and equilibrated with 20 mM sodium phosphate buffer, containing 1 M NaCl, at the appropriate pH. The concentrated culture supernatant containing laccase and xylanase activities, diluted 2-fold with the dilution buffer (i.e., 40 mM sodium phosphate buffer, 2 M NaCl at the same pH as the equilibration buffer), was then applied at room temperature to metal chelate supports. After 10 min of incubation, the first fraction was collected after resin sedimentation, to another ELISA microtiter plate. The agarose gel was washed with the same buffer system and four washing fractions were collected and the adsorbed enzymes were then eluted from the metal chelate supports by using 20 mM phosphate buffer containing 1 M NaCl and 75 mM imidazole. A total of three elution fractions were obtained and all fractions were then assayed for all enzyme activities. Three values of pH (6, 7 and 8) were tested in order to identify the best conditions to promote the adsorption of enzymes to the immobilized metal chelates. All experiments were carried out at room temperature in triplicates.

2.2.6. Purification of laccase from C. versicolor by IMAC

Based on the results of the batch mode on ELISA microtiter plates, a set of experimental conditions were selected to purify laccases from this fungal strain. Chromatographic columns containing 10 mL of either sedimented Sepharose 6B–BDGE-30–IDA–M(II) or Sepharose 6B–EPI-30–IDA–M(II) were washed with four volumes of equilibration buffer. The enzyme samples were diluted 2-fold with dilution buffer and applied to the column at a flow rate of 0.6 mL/min. The columns were then washed with equilibration buffer until A_{280} was less than 0.05 and enzyme desorption was carried out with a linear gradient of imidazole (either 0–40 mM or 0–75 mM), by using the same buffer system at a flow rate of 2 mL/min. Column fractions were analyzed for protein content as well as for xylanase and laccase activities.

2.2.7. SDS and Native PAGE and in situ detection of xylanase and laccase activity

Native PAGE of enzymes samples was performed in 7.5 and 10% separating gels [18]. Gels were stained for laccase and xylanase activities. *In situ* visualization of laccase activity was performed with 17 mM *o*-dianisidine in 0.2 M sodium acetate buffer at pH 4.5. Xylanase zymogram gel was prepared by incorporating 0.1% (w/v) Birchwood xylan to the separating gel and concentrated culture supernatants were analyzed. After electrophoresis, the gel was washed with 50 mM citrate buffer pH 4.8, and incubated in this buffer for 30 min at 50 °C. Subsequently, the gel was transferred to a solution of 0.1% (w/v) Congo Red and washed with 1 M NaCl. Zones of clearance were visualized with 5% (v/v) of acetic acid as described previously [19]. Purity of chromatographic fractions from IMAC column was also checked by native PAGE as well as by SDS-PAGE. After the electrophoretic run, gels were cut into two halves and one half was stained for protein with silver nitrate [20] and the other half for *in situ* detection of laccase activity.

2.2.8. Isoelectric focusing

Analytical isoelectric focusing (IEF) in the pH range 3–10 was performed with a Model 111 Mini IEF cell (Bio Rad), according to the manufacturer's instructions. For