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ORIGINAL PAPER

Production of polygalacturonase from *Coriolus versicolor* grown on tomato pomace and its chromatographic behaviour on immobilized metal chelates

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Abstract Tomato pomace and pectin were used as the sole carbon sources for the production of polygalacturonase from a strain of *Coriolus versicolor* in submerged culture. The culture of *C. versicolor* grown on tomato pomace exhibited a peak of polygalacturonase activity (1,427 U/l) on the third day of culture with a specific activity of 14.5 U/mg protein. The production of polygalacturonase by *C. versicolor* grown on pectin as a sole carbon source increased with the time of cultivation, reaching a maximum activity of 3,207 U/l of fermentation broth with a specific activity of 248 U/mg protein. The levels of different isoenzymes of polygalacturonase produced during the culture growth were analysed by native PAGE. Differential chromatographic behaviour of lignocellulosic enzymes produced by *C. versicolor* (i.e. polygalacturonase, xylanase and laccase) was studied on immobilized metal chelates. The effect of ligand concentration, pH, the length of spacer arm and the nature of metal ion were studied for enzyme adsorption on immobilized metal affinity chromatography (IMAC). The adsorption of these lignocellulosic enzymes onto immobilized metal chelates was pH-dependent since an increase in protein adsorption was observed as the pH was increased from 6.0 to 8.0. The adsorption of polygalacturonase as well as other enzymes to immobilized metal chelates was due to coordination of histidine residues which are available at the protein surface since the presence of imidazole in the equilibration buffer abolished the adsorption of the enzyme to immobilized metal chelates. A one-step purification of polygalacturonase from C. *versicolor* was devised by using a column of Sepharose 6B-EPI 30-IDA-Cu(II) and purified enzyme exhibited a specific activity of about 150 U/mg protein, final recovery of enzyme activity of 100% and a purification factor of about 10. The use of short spacer arm and the presence of imidazole in equilibration buffer exhibited a higher selectivity for purification of polygalacturonase on this column with a high purification factor. The purified enzyme preparation was analysed by SDS-PAGE as well as by "in situ" detection of enzyme activity.

Keywords Tomato pomace · Polygalacturonase production · IMAC purification · *Coriolus versicolor* · Lignocellulosic enzymes

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Introduction

A major issue faced by the food processing industries involves the accumulation, handling and disposal of wastes, which gives rise to both economical and environmental problems. Tomato *(Lycopersicon esculentum* Mill.) is a widely cultivated vegetable crop in Mediterranean countries. During tomato processing, a huge amount of a by-product is produced known as tomato pomace, which consists of tomato peel, seeds as well as some pulp. The chemical composition of tomato pomace consists of proteins, lipids, carbohydrates including pectins, amino acids, carotenoids and minerals [1, 13, 20]. Several pectin-rich substrates such as wheat [6], deseeded sunflower head [28], apple pomace [18] and citrus peel [12] have been used for the production of microbial pectinases in both submerged and solid-state fermentation. However, to our knowledge,

tomato pomace has not been used for the production of pectinases. In fact, there are only two reports in the literature on the use of this agricultural waste as a carbon source for production of enzymes (i.e. cutinolytic esterase and xylanase) from bacterial strains in submerged fermentation [15, 29].

Pectins are a family of polysaccharides with a backbone of 1,4-linked a-D-galacturonic acid residues, partially present as their methyl esters. The complete degradation of pectin is due to the synergistic action of methylesterase (EC.3.1.11.1), endo-polygalacturonase (EC.3.2.1.15), exo-polygalacturonase (EC.3.2.1.67), endo-pectate lyase (EC.4.2.2.2), exo-pectate lyase (EC.4.2.2.9) and pectin lyase (4.2.2.10). Pectinases are widely used in biotechnological applications [19], namely, in food industry (i.e. fruit juice extraction, coffee and tea fermentation, oil extraction, improvement of chromaticity and stability of red wines), textile, paper and pulp industries and in waste-water treatment. However, industrial production of pectinases makes use almost exclusively of *Aspergillus niger* strains [31].

Polygalacturonases (EC 3.2.1.15 and EC 3.2.1.67) catalyze the hydrolysis of α-1,4-linkages between α-D-galacturonic acid units in pectin, by endo- and exo-action [19]. There is only one report in the literature on the production of endo- and exo-polygalacturonases from a white rot fungi, *Coriolus trogii* [22]. Polygalacturonases are of industrial interest since they are used in specific applications, such as in baby and functional foods [19, 31].

Since this enzyme is secreted extra celularly by white-rot fungi as well as other lignocellulosic enzymes, it is of great interest to devise a simple and cheap separation method for these enzymes so as to be used in different industrial applications.

Immobilized metal affinity chromatography (IMAC) has been used for the purification of wild type and recombinant proteins [3, 23, 27]. The interactions between accessible coordination sites of metal ions and electron-donating groups present on the protein surface are responsible for retention of proteins in IMAC. Several factors affect the adsorption process, such as the nature of metal ion involved in coordination, the matrix, the length of spacer arm, the ligand concentration, the pH and buffer used [3]. The purification of microbial pectinases has been recently reviewed [31] and all classical methods of protein purification (i.e. ammonium sulphate and ethanol precipitation, ion-exchange and gel filtration chromatography) have been used to devise isolation schemes for pectic enzymes. Fractionation of pectic enzymes (i.e. pectinlyase, pectinesterase and polygalacturonase) from commercial enzyme preparations has been reported [9–11], by using conventional IMAC as well as IMAC in hollow-fibre membranes. The results published revealed that small fractions of polygalacturonase activity were retained, under certain conditions, in Cu(II)-IDA solid supports [11]. To our knowledge, IMAC has not been reported in the literature for fractionation and purification of polygalacturonase as well as xylanase and laccase from microbial sources.

The present work is concerned with the production of polygalacturonase from *C. versicolor* by using either tomato pomace or pectin as a sole carbon source in culture media. Subsequently, the chromatographic behaviour of this enzyme as well as other lignocellulosic enzymes (i.e. xylanase and laccase) on immobilized metal chelates will be investigated as a function of ligand concentration, pH, length of spacer arm and nature of metal ion. The selective adsorption of polygalacturonase on immobilized metal chelates will be carried out for the purification of this enzyme with high yield and activity.

Materials and methods

Materials

Potato dextrose agar (PDA) was supplied by Biokar Diagnostics. Sephacryl^{TM} S-100 high resolution was obtained from GE Healthcare. Polygalacturonic acid, 1,4-butanediol diglycidyl ether (BDGE) and iminodiacetic acid (IDA) were purchased from Sigma Chemical Company. Epichlorohydrin (EPI) was obtained from Aldrich, and Sepharose 6B from Pharmacia. The gel-support film from Bio-Rad was used to cast ultrathin polyacrylamide gels. The concentration of culture supernatants was performed with a GR 81 PP membrane from Danish Separation Systems (nominal M_r cut-off value of 6,000 Da). Tomato pomace was a kind gift from Sopragol S. A. from Mora, Portugal. All other chemicals used were of analytical grade.

Methods

Growth and maintenance of fungal strain

The strain of *C. versicolor* was isolated from *Quercus suber* stump and it was grown and maintained in solid PDA medium. For enzyme production, the strain was grown in liquid media, containing either 20 g/l of tomato pomace or 10 g/l of pectin as a carbon source, 1 g/l of $(NH_4)_2SO_4$, 0.125 g/l of $CaCl_4$, 1 g/l of $NaH_4PO_4{\cdot}H_4O$ and 0.5 g/l of $MgSO_4{\cdot}7H_4O$. The pH was adjusted to 5.5 prior to autoclaving.

Enzyme assays

Total polygalacturonase activity was assayed by incubating either the culture supernatant or column fractions, for 1 h

at 50°C, with 0.5% (wt/vol) polygalacturonic acid in 50 mM citrate buffer pH 4.8. The enzyme activity was determined from a calibration curve by using galacturonic acid as standard. Reducing sugars in the reaction mixture were determined by the dinitrosalicylic acid method [36]. One enzyme unit is defined as the amount of enzyme required for formation of 1 μmol of reducing sugars per minute at 50°C.

Besides polygalacturonase activity, other lignocellulosic enzymes were also assayed for activity in culture supernatants such as xylanase and laccase. 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 earlier [5]. 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 [4].

Protein assay

Total protein was determined by the Bradford method by using BSA as a protein standard [8].

Fungal growth conditions

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.

Enzyme production

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 3 days. Culture supernatants of fermentation broths were harvested by centrifugation at 10,000×*g* for 5 min. and concentrated by ultrafiltration in an Amicon cell. Concentrated culture supernatants were frozen at −20°C prior to chromatographic analysis.

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 [2]. Under the conditions selected, the agarose matrix contained 30 μmol of epoxide groups/ml of sedimented gel. Subsequently, epoxy-activated agarose was reacted with iminodiacetic acid (IDA) as the chelating agent [2] and the stationary phase thus obtained 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 solution. Chromatographic supports with 10 and 30 μmol of epoxide groups/ml of sedimented gel containing epichlorohydrin (EPI) as spacer arm were also prepared according to the procedure described in the literature [2], and the stationary phases obtained will be referred either as Sepharose 6B-EPI-10-IDA and Sepharose 6B-EPI-30-IDA or as EPI 10 and EPI 30.

Chromatographic behaviour of polygalacturonase, xylanase and laccase

As described earlier [24], a rapid batch method carried out in ELISA microtiter plates was used to study the adsorption of polygalacturonase, xylanase and laccase on Cu(II), Ni(II), Zn(II) and Co(II) metal chelate supports. Therefore, 20 mM sodium phosphate buffer, containing 1 M NaCl, at the appropriate pH, was used as the equilibration buffer and concentrated enzyme samples were previously diluted twofold with dilution buffer (i.e. 40 mM sodium phosphate buffer, 2 M NaCl at the same pH as the equilibration buffer). The protein desorption from immobilized metal chelates was performed with 20 mM phosphate buffer containing 1 M NaCl and 75 mM imidazole. Three values of pH (pH 6, 7 and 8) were tested 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.

Purification of polygalacturonase from C. versicolor

On the basis of the results of the batch mode on ELISA microtiter plates, a set of experimental conditions were selected to purify polygalacturonase 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 twofold with dilution buffer and applied to the column at a flow rate of 0.5 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 1 ml/min. Column fractions were analysed for protein, polygalacturonase, xylanase and laccase activities.

SDS and native PAGE and "in situ" detection of polygalacturonase activity

SDS-PAGE of enzymes samples was performed in 10% separating gels [21]. Because of enzyme inactivation at high temperature in the presence of SDS, protein samples for "in situ" detection of enzyme activity were applied to gels after a heat treatment for 10 min at 50°C in application buffer (4% SDS, 0.06% bromphenol blue, 10% β-mercaptoethanol, 20% glycerol; 0.1 M Tris hydrochloride pH 6.8). However, the protein samples for silver nitrate staining were denatured at 100°C for 10 min by using the buffer mentioned above. After the electrophoretic run, gels were cut into two halves and one half was stained for protein with silver nitrate [7] and the other half for in situ detection of polygalacturonase activity. To reduce enzyme inactivation due to SDS, gels for zymogram analysis were washed four times with citrate buffer as described earlier [26]. For the detection of polygalacturonase activity, overlay gels with 1% (w/v) agarose containing 0.1% (w/v) polygalacturonic acid were cast by using the gel support film for agarose and the sandwiched gels were incubated for 1 h in a moist chamber at 50°C. Subsequently, the gel was transferred to a 0.05% (w/v) ruthenium red solution for 20 min and the gel was washed with water [30].

Native PAGE of enzyme samples were performed in 7.5% separating gels [17]. For the detection of polygalacturonase activity, overlay gels with 5% polyacrylamide and 0.1% polygalacturonic acid were cast by using the gel support film for polyacrylamide and concentrated culture supernatants from fermentation broths were analysed as described for SDS-PAGE.

Results and discussion

Production of polygalacturonase, reducing sugars and protein content in culture supernatants

The cultivation of *Coriolus versicolor* on tomato pomace and pectin media was followed for 14 days (Fig. 1) and the

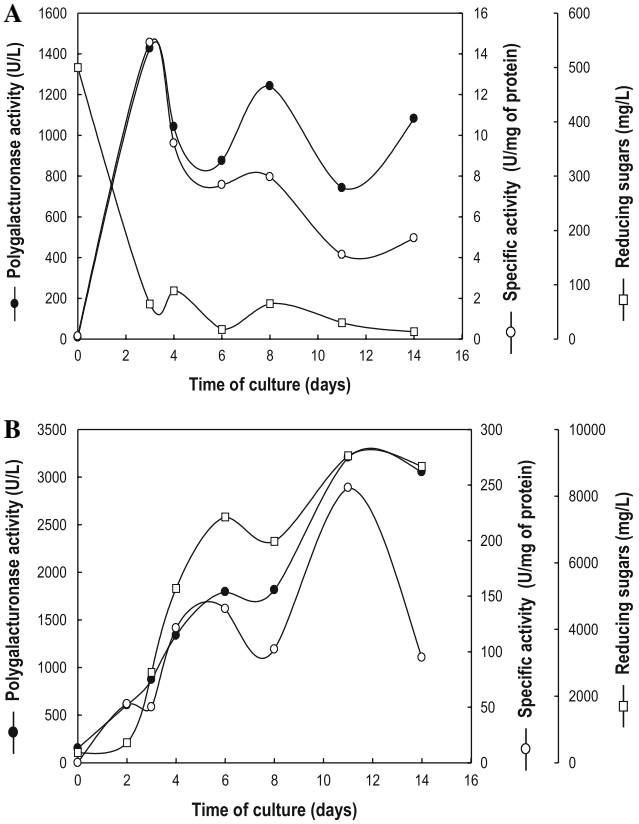

Fig. 1 Total protein and reducing sugars content and polygalacturonase activity assayed in culture supernatant of fermentation broth of *Coriolus versicolor* by using either tomato pomace or pectin as carbon sources. **a** Tomato pomace as a carbon source and **b** Pectin as a carbon source (polygalacturonase activity *filled circle*, specific activity *open circle*) and soluble reducing sugars (*open square*)

content of soluble reducing sugars, protein and polygalacturonase activity were measured throughout the cultivation process. Polygalacturonase activity in culture medium containing tomato pomace displayed a "two peaks" time-course curve for this strain of basidiomycete, as shown in Fig. 1a. Such features were observed by other authors, who suggested that they were due to catabolic repression of polygalacturonases in the presence of glucose and other sugars which were released to the medium during fermentation of other polysaccharides [6]. The culture of *C. versicolor* exhibited a peak of polygalacturonase activity (1,427 U/l of fermentation broth) on the third day of culture with a specific activity of 14.5 U/mg protein (Fig. 1a). The data presented in the literature revealed that *C. trogii* secreted polygalacturonase with a lower activity (1,000 U/l of fermentation broth) on the 15th day of culture [22]. On the other hand, several strains of *Aspergillus sp.* have been used for the production of polygalacturonase in submerged culture which exhibited the highest levels in the range of 1,000–6,500 U/l of fermentation broth containing either citrus pectin, dried orange peel or sugar beet as carbon

sources [16]. The highest production of polygalacturonase from *Aspergillus sp.* was observed on either the fifth or sixth day of culture whereas in the present work such levels were obtained on the third day of culture. However, the comparative analysis of these data with the literature is difficult because the specific activity of these enzymes has not been reported and the assay conditions used by these workers were slightly different from the ones used in this work. By contrast, the production of polygalacturonase by *C. versicolor* grown on pectin as a sole carbon source, increased with the time of cultivation, reaching a maximum activity of 3,207 U/l of fermentation broth with a specific activity of 248 U/mg protein (Fig. 1b). Therefore, the highest levels of polygalacturonase activity were obtained by using *C. versicolor* grown in a culture medium containing pectin as a sole carbon source. The levels of polygalacturonase activity obtained in this work are about threefold higher than the levels of this enzyme reported in the literature by using *C. trogii* grown in a culture medium containing pectin [22].

The different levels of polygalacturonase isoenzymes produced during the growth culture of *C. versicolor* strain with tomato pomace was investigated by the detection of enzyme activity on native polyacrylamide gel electrophoresis as a function of time of culture (Fig. 2). According to the data presented in Fig. 2, the specific detection of polygalacturonases from *Corioulus versicolor* revealed a single broad activity band coincident with a protein band with the same M_r which was observed during the entire period of fungal growth. As far as the literature is concerned, three extracellular endopolygalacturonases from extracts of solid-state culture of *Phanerochaete chrysosporium* were isolated and characterized [32].

Chromatographic behaviour of polygalacturonase, xylanase and laccase on immobilized metal chelates

The chromatographic behaviour of these lignocellulosic enzymes is summarized in Table 1, for different stationary phases (Sepharose 6B-BDGE 30-IDA, Sepharose 6B-EPI 30-IDA and Sepharose 6B-EPI 10-IDA) as well as for different immobilized metal ions. The results from adsorption assays did not vary with either different ligand concentration or length of spacer arm, suggesting that protein adsorption takes place through the immobilized metal ion at different pH (Table 1). Xylanase exhibited partial adsorption to immobilized Cu(II)-IDA chelates at pH 7.0 and 8.0 whereas it did not adsorb at pH 6.0. Polygalacturonase and laccase revealed full adsorption to immobilized Cu(II)-IDA chelates at pH 6.0, 7.0 and 8.0 (Table 1). Several X-ray structures of polygalacturonases from *Aspergillus sp.* have been reported in the literature which exhibit common characteristics [34, 35]. Polygalacturonases are monomers with a single polypeptide chain of 362 amino acid residues from *A. niger*. They contain several histidine (His) residues per enzyme molecule (i.e. 4 His for polygalacturonase II from *A. niger* [35]; 2 His for polygalacturonase I from *A. niger* [34]; 7 His for polygalacturonase from *Fusarium moniliforme* [14]). Enzyme adsorption observed in IMAC is essentially governed by the spatial distribution of histidine residues on the protein

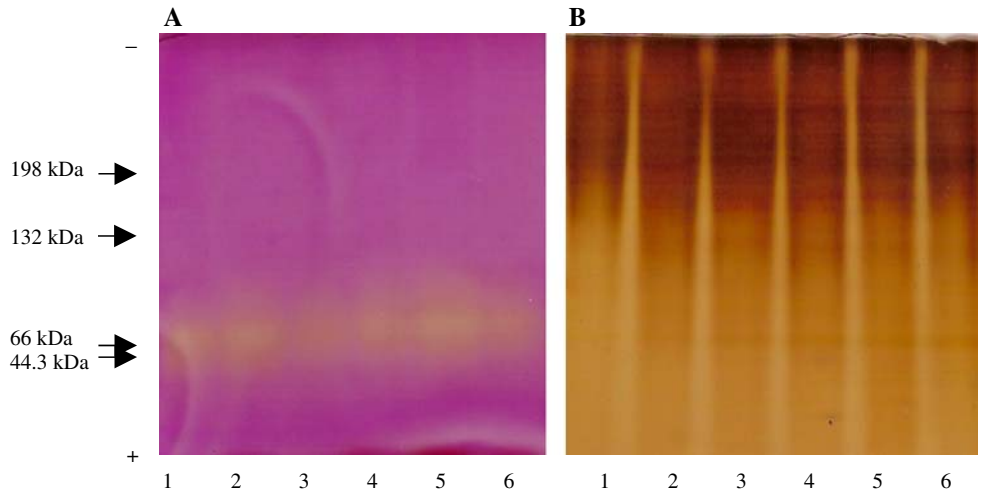

Fig. 2 Detection of polygalacturonase activity by native PAGE (7.5% separating gel). Samples from *Coriolus versicolor* with different times of culture by using tomato pomace as a carbon source were used for in situ detection of polygalacturonase activity (**a**). *Lane 1* Third day of growth (2.25 μg protein); *lane 2* fifth day of growth: (10.65 μg); *lane 3* seventh day of growth (9.45 μg); *lane 4* ninth day of growth (10.5 μg); *lane 5* 11th day of growth (12.15 μg); *lane 6* 14th day of growth (13.5 μg). On the left margin are indicated protein markers: BSA (monomer, dimer and trimer) and ovalbumin. **b** Corresponding protein stain of samples with silver nitrate

Table 1 Differential chromatographic behaviour of lignocellulosic enzymes from *Coriolus versicolor* on immobilized metal chelates

	pH	Stationary phase	Cu(II)	Ni(II)	Zn(II)	Co(II)
Xylanase	6	BDGE-30	–	–	–	–
		EPI-30				
		EPI-10				
	7	BDGE-30	±	–	–	–
		EPI-30				
		EPI-10				
	8	BDGE-30	±	–	–	–
		EPI-30				
		EPI-10				
Polygalacturonase	6	BDGE-30	+	–	–	–
		EPI-30				
		EPI-10				
	7	BDGE-30	+	–	–	–
		EPI-30				
		EPI-10				
	8	BDGE-30	+	–	–	–
		EPI-30				
		EPI-10				
Laccase	6	BDGE-30	+	–	–	–
		EPI-30				
		EPI-10				
	7	BDGE-30	+	–	–	–
		EPI-30				
		EPI-10				
	8	BDGE-30	+	–	–	–
		EPI-30				
		EPI-10				

All chromatographic runs were carried out in triplicates as described in Materials and methods section

– denotes that enzyme activity did not bind to the chromatographic support since total enzyme activity was recovered at the washing step in five column volumes; ± denotes partial binding since enzyme activity was detected both at the washing and elution steps; + denotes that enzyme activity bound to the chromatographic support

surface. Therefore, the differential chromatographic behaviour of polygalacturonase, xylanase and laccase from *C. versicolor* is due to the presence of available histidine residues on their surfaces.

On the basis of the data obtained from the quantitative adsorption tests, several chromatographic runs were carried out on columns packed with 10 ml of Sepharose 6B-BDGE 30-IDA which was charged with Cu(II) ions at different pH values. The concentration of imidazole required for protein desorption from the column was found to increase as a function of the pH of the mobile phase as predicted by the IMAC theory. For instance, polygalacturonase from *C. versicolor* was desorbed from the column packed with

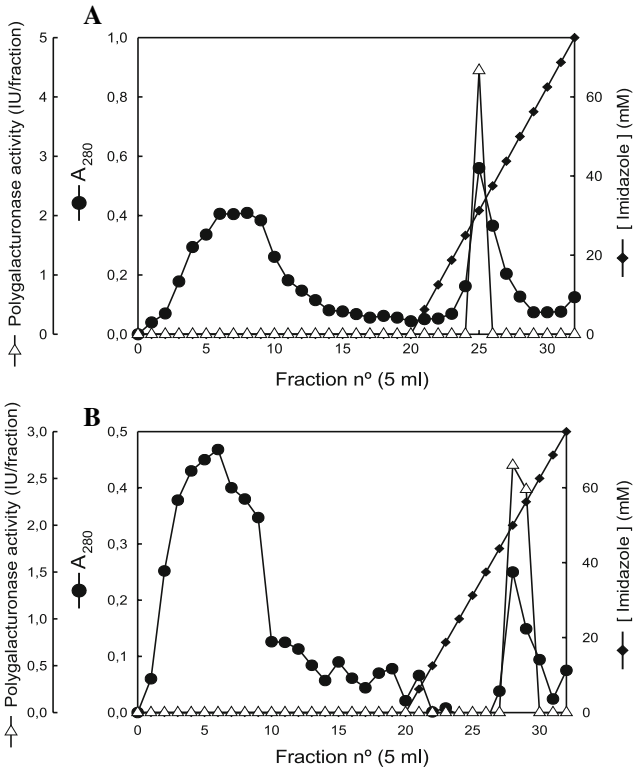

Fig. 3 Chromatographic behaviour of polygalacturonase from *Coriolus versicolor* on Sepharose 6B-BDGE 30-IDA column, with different immobilized metal ions and pH values of the mobile phase. Elution was carried out with a linear gradient of imidazole (increments of 6.25 mM imidazole per fraction), as described in Materials and methods section. **a** Cu(II) pH 6.0 and **b** Cu(II) pH 8.0

immobilized Cu(II) chelate, with imidazole concentrations of 35 and 50 mM at pH 6.0 and 8.0, respectively (Fig. 3a, b). According to previous reports that have observed the same effect in retention of peptides and proteins lacking histidyl groups on immobilized metal chelates, this result may be due presumably to the binding of unprotonated α-amino group to metal chelates at higher pH [33, 37]. As the pH of the mobile phase was decreased to 6.0, selective interaction with available histidine residues on protein surface apparently takes place, and thus protein desorption was achieved with a lower concentration of imidazole in the mobile phase. The pH of the mobile phase was found to affect the degree of purification of polygalacturonase since purification factors of 5.3 and 3.4 were obtained at pH 6.0 and 8.0 by using Sepharose 6B-BDGE 30-IDA-Cu(II) columns (data not shown). In order to confirm that the interaction of polygalacturonase with immobilized Cu(II) chelates was due to the coordination between available histidine residues on enzyme molecules and chelated transition metal ions, chromatographic runs were carried out with imidazole in the equilibration buffer. The results revealed that polygalacturonase did not adsorb to immobilized Cu(II) chelates in the presence of 4 mM imidazole

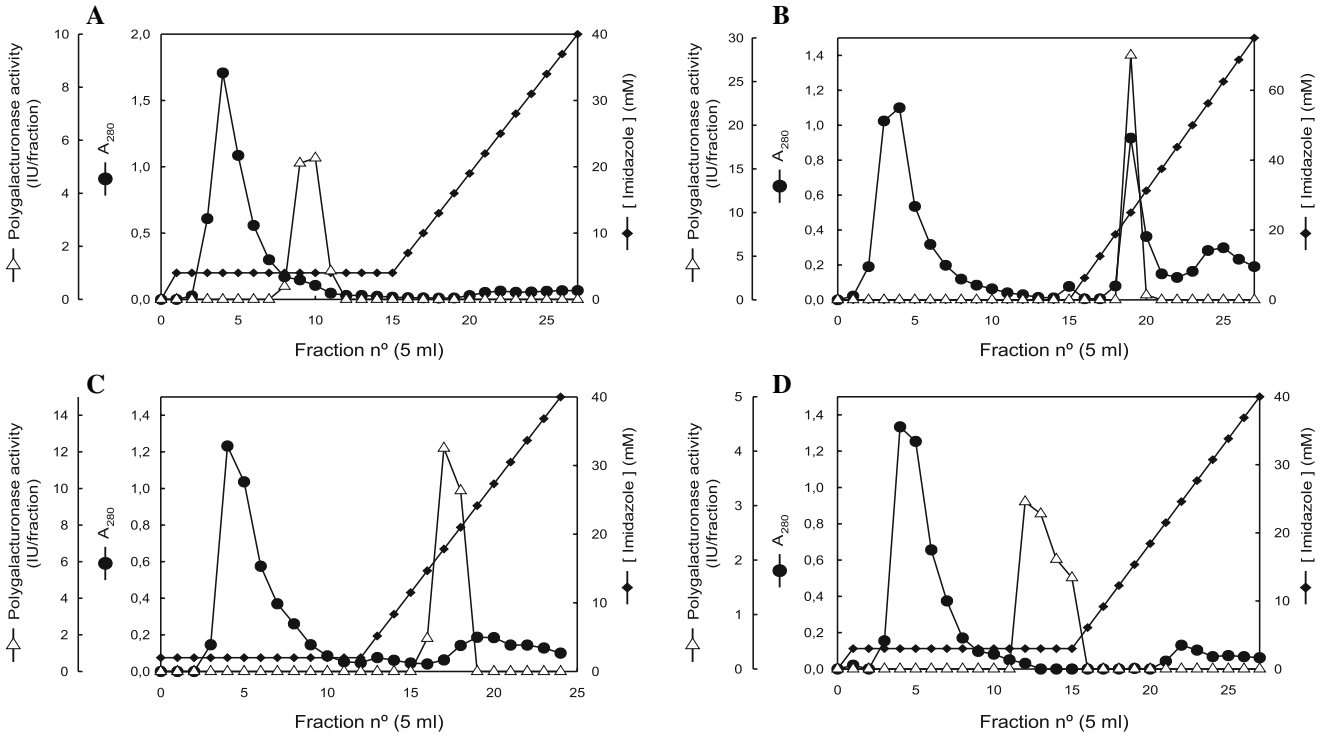

Fig. 4 Chromatographic behaviour of polygalacturonase from *Coriolus versicolor* on a Sepharose 6B-EPI-30-IDA-Cu(II) column at pH 6.0. Elution was carried out with a linear gradient of imidazole, as described in Materials and methods section. **a** 4 mM imidazole in the equilibration buffer (increments of 3 mM imidazole per fraction); **b** No imidazole in the equilibration buffer (increments of 3.33 mM imidazole per fraction); **c** 2 mM imidazole in the equilibration buffer (increments of 3.17 mM imidazole per fraction); **d** 3 mM imidazole in the equilibration buffer (increments of 3.08 mM imidazole per fraction)

in the equilibration buffer (Fig. 4a). In fact, the enzymes revealed different affinities for immobilized metal chelates (as explained in Fig. 4) which obeys the following order: polygalacturonase > laccase > xylanase, since the presence of 2 mM imidazole in the equilibration buffer abolished the adsorption of laccase (data not shown) to Cu(II)-IDA chelate at pH 6.0 (Fig. 4c).

Purification of polygalacturonase by using immobilized metal chelates containing different lengths of spacer arms

Chromatographic runs were carried out with two different spacer arms (EPI and BDGE with 3 and 10 carbon atoms, respectively) which contained a ligand concentration of 30 μmol of immobilized Cu(II)/ml of sedimented stationary phase. The concentration of imidazole required for protein desorption from the column was found to increase as a function of the length of spacer arm. For instance, polygalacturonase from *C. versicolor* was desorbed from the column packed with immobilized Cu(II) chelate containing EPI and BDGE spacer arms, with imidazole concentrations of 25 and 35 mM at pH 6.0, respectively (Figs. 3a, 4b). These data suggest apparently that protein–metal chelate interactions on longer spacer arm are stronger and some non-specific hydrophobic interactions may also occur [33]. Moreover, polygalacturonase exhibited purification factors of 7.2 and 5.3 with short (i.e. EPI) and long (i.e. BDGE) spacer arms, respectively (data not shown). Longer spacer arms seem to interact apparently with slightly hidden histidine residues in protein molecules. Furthermore, these spacer arms may be also responsible for some undesirable non-specific hydrophobic interactions with protein molecules [33]. The use of shorter spacer arms such as EPI appears to be advantageous since only histidine residues exposed on protein surface will coordinate to immobilized metal chelates.

Purification of polygalacturonase by using imidazole in equilibration buffer

The combination of tailor-made stationary phases for IMAC and a correct choice of the adsorption conditions permitted to design a one-step purification procedure for polygalacturonase from *C. versicolor*. The chromatographic behaviour of polygalacturonase on Sepharose 6B-EPI 30-IDA-Cu(II) column at pH 6.0 (Fig. 4b) resulted in partially purified enzyme according to the electrophoretic

analysis (data not shown). However, the presence of 2 mM imidazole in the equilibration buffer abolished the adsorption of several protein contaminants to Sepharose 6B-EPI 30-IDA-Cu(II) column including laccase and xylanase (data not shown). Hence, polygalacturonase was desorbed from the column with a linear gradient of imidazole (Fig. 4c) with a specific activity of about 150 U/mg protein, a final recovery of enzyme activity of about 100% and a purification factor of about 10 (Table 2). An extracellular polygalacturonase from *Trichoderma harzianum* has been reported in the literature [25] which was purified in a two-step procedure with a specific activity of 276 U/mg protein, a purification factor of 13 and a final recovery of activity of about 55%. Interestingly, in the present work, the peak of polygalacturonase activity was eluted with 3 and 4 mM imidazole in the equilibration buffer after the broad peak due to protein impurities (Fig. 4d, a, respectively). The degree of purification of this polygalacturonase activity peak was high and exhibited no contamination (data not shown) with either laccase or xylanase activities (Table 3). However, the final recovery of enzyme activity was only about 50% with a specific activity of about 180 U/mg protein in the presence of 4 mM imidazole in the equilibration buffer (Table 3).

The purity of chromatographic fractions from Sepharose 6B-EPI 30-IDA-Cu(II) was analysed by SDS-PAGE (Fig. 5) which revealed that the purified fractions exhibited three protein bands and three activity bands of polygalacturonase (Fig. 5) with M_r of 63.3, 43 and 30 kDa. The presence of two additional activity bands of polygalacturonase may be due to other forms of this enzyme in white-rot fungi. However, the M_r value of 41.7 kDa was also reported in the literature for purified preparations of endopolygalacturonases from *P. chrysosporium* [32]. On the other hand, an extracellular polygalacturonase from *T. harzianum* exhibited an M_r of 31 kDa on SDS-PAGE [25].

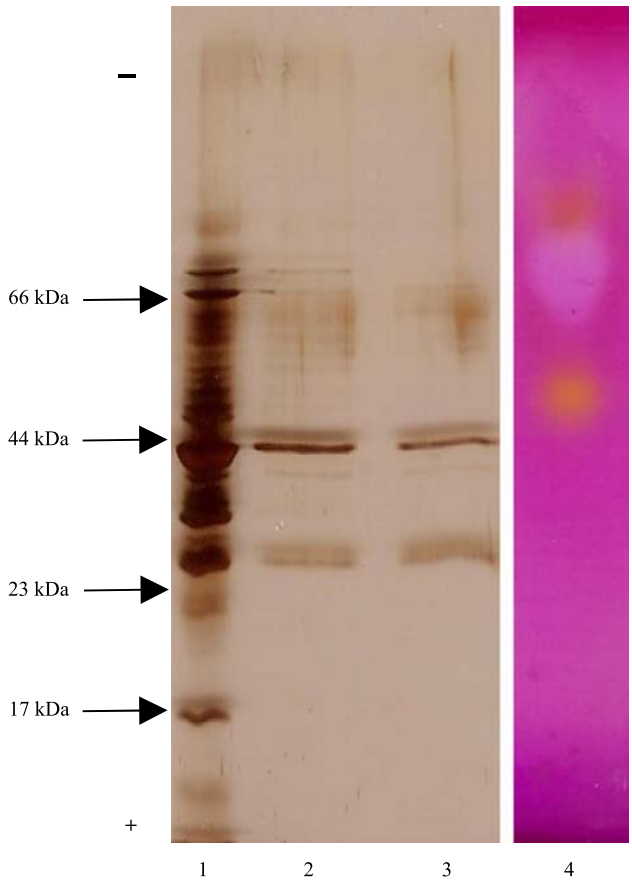

Fig. 5 SDS-PAGE (10% separating gel) of concentrated culture supernatants from *Coriolus versicolor* as well as purified fractions from Sepharose 6B-EPI-30-IDA-Cu(II) column in the presence of 2 mM imidazole at pH 6.0. *Lanes*: *1* concentrated culture supernatant from *Coriolus versicolor* (1.8 μg); *2* purified fraction from Sepharose 6B-EPI 30-IDA-Cu(II) column (0.3 μg); *3* purified fraction from Sepharose 6B-EPI 30-IDA-Cu(II) column (0.2 μg); *4* In situ detection of polygalacturonase activity of concentrated culture supernatant from *Coriolus versicolor* (1.0 μg). On the left margin are indicated protein markers: bovine serum albumin (66,500), ovalbumin (44,000), chymotrypsin (23,000) and myoglobin (17,000)

Table 2 Purification of polygalacturonase from *Coriolus versicolor* by immobilized metal affinity chromatography on Sepharose 6B-EPI 30-IDA-Cu(II) at pH 6.0 in the mobile phase containing 2 mM imidazole

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification factor
1. Culture supernatant	1.4	20.62	14.73	100	1
2. Cu(II) column eluate	0.140	20.58	147.0	99.8	9.97

Table 3 Purification of polygalacturonase from *Coriolus versicolor* (CV) by immobilized metal affinity chromatography on sepharose 6B-EPI 30-IDA-Cu(II) at pH 6.0 in the mobile phase containing 4 mM imidazole

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification factor
1. Culture supernatant	1.4	20.22	14.44	100	1
2. Cu (II) column eluate	0.0587	10.46	178.19	51.7	12.34

Conclusions

To our knowledge, there are only two reports in the literature on the production of enzymes from bacterial strains by using tomato pomace as a sole carbon source. On the other hand, there are also no reports in the literature on the production of polygalacturonases from *C. versicolor*.

The present work reports the production of polygalacturonases from *C. versicolor* by using either tomato pomace or pectin as sole source of carbon. The results presented strongly suggest that the amount of pectin in tomato pomace is high enough to obtain high levels of polygalacturonase from *C. versicolor* when cultured on this substrate.

Zymogram analysis of polygalacturonase activity revealed that the culture of *C. versicolor* exhibited a single broad activity band coincident with a protein band, which was observed during the entire period of fungal growth. To our knowledge, this is the first report describing the chromatographic behaviour of polygalacturonase, xylanase and laccase from *C. versicolor* on immobilized metal chelates. Therefore, the combination of selective stationary phases for IMAC and a correct choice of adsorption variables allowed fractionation of xylanase, laccase and polygalacturonase from culture supernatants of *C. versicolor*. A suitable one-step purification scheme for polygalacturonase from *C. versicolor* was devised by IMAC on Sepharose 6B-EPI 30-IDA-Cu(II) column at pH 6.0 and in the presence of 2 mM imidazole in the equilibration buffer. Therefore, IMAC can be considered as a cost-effective and process-compatible alternative to other type of the chromatographic technique for fractionation and isolation of non-denaturing and biologically active lignocellulosic enzymes.

Although several polygalacturonases from *Aspergillus sp.* have been described in the literature, this enzyme from white- rot fungi may become an attractive alternative at industrial levels. In fact, basidiomycete strains can be grown in several agricultural wastes with a high enzyme productivity and specific activity which could be purified in a one-step procedure by IMAC with high enzyme recovery and purity.

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