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ORIGINAL ARTICLE

Covalent Immobilization of *Chondrostereum purpureum* Endopolygalacturonase on Ferromagnetic Nanoparticles: Catalytic Properties and Biotechnological Application

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Abstract

Pectinases are widely used in a variety of industrial processes. However, their application is limited by low catalytic processivity, reduced stability, high cost, and poor re-use compatibility. These drawbacks may be overcome by enzyme immobilization with ferromagnetic nanoparticles, which are easily recovered by a magnetic field. In this work, an endopolygalacturonase from *Chondrostereum purpureum* (EndoPG$_{Cp}$) expressed in *Pichia pastoris* was immobilized on glutaraldehyde-activated chitosan ferromagnetic nanoparticles (EndoPG$_{Cp}$-MNP) and used to supplement a commercial enzyme cocktail. No significant differences in biochemical and kinetic properties were observed between EndoPG$_{Cp}$-MNP and EndoPG$_{Cp}$, although the EndoPG$_{Cp}$-MNP showed slightly increased thermostability. Cocktail supplementation with EndoPG$_{Cp}$-MNP increased reducing sugar release from orange wastes by 1.8-fold and showed a synergistic effect as compared to the free enzyme. Furthermore, EndoPG$_{Cp}$-MNP retained 65% of the initial activity after 7 cycles of re-use. These properties suggest that EndoPG$_{Cp}$-MNP may find applications in the processing of pectin-rich agroindustrial residues.

Keywords Endopolygalacturonase · Enzyme cocktail · *Chondrostereum purpureum* · Citrus pectin · Enzyme synergy · Thermostability

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Introduction

The use of agroindustrial residues for the generation of value-added products is one of the key challenges facing the transition to a sustainable bioeconomy. The fruit juice industry alone generates 5.5 million metric tons (MMT) of underutilized bagasse and pulp every year [1]. These residues are rich in polysaccharides and represent a potentially valuable resource, which could be enzymatically converted into sugars and oligosaccharides to generate a multitude of products within the biorefinery concept. Pectinolytic enzymes, or pectinases, designate a heterogeneous group involved in the degradation of pectin, a complex polysaccharide that is present in the cell wall and middle lamella of all land plants [2, 3]. Pectinases include pectate lyases (PL), polygalacturonases (PG), and pectinesterases (PE), and are used in a variety of applications, such as in the wine, food, and paper industries, and also in animal feed production, extraction of vegetable oil, textile, and for the depolymerization of lignocellulosic biomass for biofuel production [4, 5]. Due to their widespread application, pectinases occupy a leading position among commercially produced industrial enzymes with a 25% share of the global food and beverage enzyme market [4, 5].

Bacteria, fungi, plants, and yeast are the natural sources of pectinases and more than 30 distinct genera have been described as producers of these enzymes [4, 5]. However, the industrial application of pectinases is limited by low productivity, enzyme instability, high cost, and difficulty with enzyme recovery [6, 7]. Many of these limitations can be overcome by the use of immobilized enzymes. Enzyme immobilization is a powerful strategy in biocatalyst design [8] that allows the re-use of a relatively expensive catalyst and improved reaction control since the catalytic process can be interrupted by separating the immobilized enzyme from the reaction medium [9, 10]. Additionally, immobilization can improve the catalytic performance of an enzyme by altering properties such as specific activity, stability, selectivity, and decreased inhibition by reaction products [9]. There are several immobilization techniques, such as simple adsorption on porous substrates, weak chemical interactions (ion exchange and hydrophobic interaction, for example), and immobilization by one-point or multi-point covalent bonding [11]. Recently, the immobilization of pectinolytic enzymes using different methods including adsorption, entrapment, and covalent binding has been reported [6, 7, 12–14]

Covalent immobilization is among the most widely used strategies, with a frequent outcome of reduced enzyme losses and, therefore, long-term process stability. The use of magnetic nanoparticles has attracted attention in recent years due to the ease of recovery of the immobilized enzyme using an externally applied magnetic field and their high surface area that enables a high loading of enzymes per unit mass of support [6, 12, 15, 16]. We have previously demonstrated that glutaraldehyde-activated chitosan ferromagnetic nanoparticles are a promising low-cost alternative for enzyme immobilization. These nanoparticles were coated with chitosan and treated with the cross-linking agent glutaraldehyde that functionalizes the chitosan, stabilizing the nanoparticle coating and providing a chemically reactive surface for enzyme immobilization [17].

In this work, we have immobilized an endopolygalacturonase from *Chondrostereum purpureum* (EndoPG$_{Cp}$) expressed in *Pichia pastoris* [2] on glutaraldehyde-activated chitosan ferromagnetic nanoparticles, and have compared the catalytic properties of the free enzyme with the immobilized enzyme. In addition, we report the capacity of the immobilized EndoPG$_{Cp}$ to generate sugars from agricultural residues derived from the fruit processing industry.

Material and Methods

EndoPG$_{Cp}$ Production and Purification

The recombinant pectinase from *C. purpureum* was overexpressed in *Pichia pastoris* GS115 as previously described [2]. Briefly, a single colony of *P. pastoris* GS115 transformed with the pPIC9Kf1CT_*endopg* plasmid was inoculated into 100 mL of MG medium (3.4 g/L yeast nitrogen base without amino acids and ammonium sulfate, 10 g/L ammonium sulfate, 0.0004 g/L biotin, 1% (v/v) glycerol, 100 mmol/L potassium phosphate buffer, pH 6.0). The culture was incubated at 30 °C for 16 h in an orbital shaker at 250 rpm. Cells were harvested by centrifugation at 5000 g for 5 min, and the cell pellet was resuspended to an $OD_{600\ nm}$ of 1 in 100 mL MM medium (MG medium in which glycerol was substituted with 1% (v/v) methanol) and the culture was grown for 3 days at 30 °C. Every 24 h, methanol was added to a final concentration of 1% and the pH was corrected by the addition of 1 mL of 1 mol/L potassium phosphate buffer, pH 8.0. After 3 days, the cells were centrifuged at 5000 g for 20 min and the recombinant EndoPG$_{Cp}$ was purified from the culture supernatant by nickel affinity chromatography.

Estimation of Protein Concentration

The protein concentrations were determined according to Read and Northcote [18] using bovine serum albumin as standard.

Preparation of Ferromagnetic Nanoparticles (MNP)

The glutaraldehyde-treated magnetic nanoparticles were prepared using a protocol adapted from that previously described by Carneiro and Ward [17]. Briefly, the Fe_3O_4 ferromagnetic nanoparticles were prepared after controlled co-precipitation of Fe^{2+} and Fe^{3+} under alkaline conditions in the presence of Pluronic F127, a non-ionic surfactant that prevents spontaneous aggregation of the newly formed particles. The particles were then coated with chitosan 0.1% dissolved in acetic acid and the mixture was stirred for 30 min at room temperature. The MNPs coated with chitosan were treated with the cross-linking agent glutaraldehyde. The initial reaction was performed for 1 h at room temperature and the particle suspension was subsequently incubated at 4 °C for 16 h. The nanoparticles were separated with a neodymium magnet and repeatedly washed with water to remove excess reagent to remove excess glutaraldehyde. The glutaraldehyde-activated chitosan MNPs were stored in water at 4 °C until further use.

Enzyme Immobilization

EndoPG$_{Cp}$ was immobilized on MNP by covalent bonding to produce EndoPG$_{Cp}$-MNP. Concentrations (C_i) of 50, 100, and 200 µg of pure EndoPG$_{Cp}$ were prepared in 100 mmol/L sodium phosphate buffer; pH 8.0 in a final volume of 100 µL was incubated with 100 µL of a MNP suspension (10% w/v) at 4 °C for 16 h. The MNP were washed 4 times with 1.0 mL of wash buffer (50 mmol/L sodium phosphate, pH 8.0) to remove non-adsorbed proteins using a neodymium magnet (11-mm diameter, 3-mm thickness). The

supernatants from the initial immobilization (S) and the subsequent washes (W_1, W_2, W_3, and W_4) were collected for protein quantifications. The amount of protein retained on the support was estimated using Eq. (1):

$$Immobilizedproteincontent(\mu g) = C_i - (S + W_1 + W_2 + W_3 + W_4) \quad (1)$$

where C_i, S, and $W_{1\text{-}4}$ were the amount of protein measured in the initial solution and in the supernatants from the immobilization and washing steps, respectively.

The resultant EndoPG$_{Cp}$-MNP were resuspended in 200 µL of the wash buffer and stored at 10 °C. The immobilization yields and efficiencies of the immobilized enzymes were calculated according to Eqs. (2) and (3), respectively [15]. All immobilization tests were performed in triplicate.

$$\frac{Amountofproteinretainedonthesupport(mgprotein)}{\text{Amount of offered protein to immobilization (mg protein)}} \times 100 \quad (2)$$

$$\frac{Specificofimmobilizedenzyme(U/mg)}{\text{Specific activity of free enzyme (U/mg)}} \times 100 \quad (2)$$

Scanning Electron Microscopy (SEM)

Analysis of the surface topology characteristics of the magnetic nanoparticles both in the absence and presence of immobilized EndoPG$_{Cp}$ was performed by scanning electron microscopy (SEM). A 5-µL drop of the particle suspension at 0.5% (w/v) was applied to a glass microscope slide and dried under vacuum prior analysis, and images were obtained using a JEOL model JSM 6610LV instrument (JEOL, Akishima, Tokyo, Japan) operated at 30 kV and at 1×10^4 and 2×10^4 magnification.

Determination of the Free and Immobilized Endopolygalacturonase Activity

Unless otherwise stated, the activities of the free and immobilized EndoPG$_{Cp}$ were determined at 60 °C in McIlvaine buffer [19], pH 4.5, containing 0.5% (w/v) citrus pectin (Sigma-Aldrich Chem. Co.) as substrate in a final volume of 60 µL, as previously described [2]. The total released reducing sugars were quantified using the dinitrosalicylic acid (DNS) method [20]. The experimental conditions (reaction times, enzymatic units) of all enzymatic assays were adjusted to guarantee the reliable estimation of initial velocities. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the formation of 1 µmol of galacturonic acid per min under optimal conditions and converted to a specific activity (U/mg protein). Controls with heat-inactivated enzyme were included in all enzymatic assays, and all the experiments were performed in triplicate.

Effect of temperature and pH on immobilized EndoPG$_{Cp}$

The effect of temperature on the activity of the free and immobilized EndoPG$_{Cp}$ was determined in the range from 45 to 85 °C in McIlvaine buffer [19], pH 4.5. The activation energy (Ea) for the hydrolysis of pectin by free and immobilized EndoPG$_{Cp}$ was determined using the Arrhenius equation following Segel [21]. The thermal stability of the enzymes was

evaluated by pre-incubating the free and immobilized EndoPG$_{Cp}$ diluted in Millipore MilliQ ultrapure water for 30 min at different temperatures (30–80 °C). After cooling in an ice bath for 1 min, the residual activities were estimated at 60 °C as described in the previous section. The effects of pH on the activity of EndoPG$_{Cp}$ and EndoPG$_{Cp}$-MNP were determined at 60 °C in McIlvaine buffer [19] over the pH range 3.0–8.0.

Estimation of Kinetic Parameters and Data Fitting

Maximum velocity (V_{max}) and Michaelis–Menten constant (K_M) for the hydrolysis of citrus pectin (concentrations ranging from 0.1 to 10 mg/mL) by EndoPG$_{Cp}$-MNP were estimated by non-linear regression using the SigrafW software [22]. The protocols of expression, purification, and immobilization of EndoPG$_{Cp}$ were repeated three times, resulting in three independent preparations of EndoPG$_{Cp}$-MNPs. The experimental kinetic curves were repeated three times using independent enzyme preparations, with duplicate enzyme assays for each preparation. The kinetic parameters are presented as the mean ± SD for the three different enzyme preparations ($n = 3$).

Evaluation of Reusability of the EndoPG$_{Cp}$-MNps

The reusability of the immobilized EndoPG$_{Cp}$ was determined over 10 reaction/washing cycles using the activity assay as described in the section describing the enzyme activity assay. At the end of each cycle, the immobilized enzyme was magnetically separated from the reaction media, washed with 1.0 mL of wash buffer, and then added to a fresh reaction mixture to start a new reaction cycle. The reducing sugar concentration in the supernatant was assayed using the DNS method [20].

Supplementation of a Commercial Enzyme Cocktail by Free and Immobilized EndoPG$_{Cp}$ for Hydrolysis of Processing Fruits

The passion fruit and orange residues were prepared from the fruit peels after the removal of the pulps. The peels were washed with distilled water and dried for 72 h at 50 °C and subsequently milled in a Wiley-type mill (SL 31 – SOLAB, Piracicaba – BRA) using a 30-mesh sieve. The powders were washed 2 times with ethanol 100% to remove soluble sugars, dried to remove the ethanol, and used in the hydrolysis experiments.

The hydrolysis of lignocellulosic residues was performed at 45 °C and 200 rpm in hermetically sealed conical plastic tubes containing 50 mmol/L sodium acetate buffer, pH 5.5, 10 mmol/L sodium azide, and 10 mg of the passion fruit or orange residue in a final volume of 1 mL. The reaction was initiated by the addition of an aliquot of either free or immobilized EndoPG$_{Cp}$, or a cellulase-rich commercial enzyme preparation (Celluclast 1.5 L), or of a mixture of both. At defined time intervals, aliquots of the reaction medium were centrifuged at 10,000 *g* for 5 min, and total reducing sugars were determined by the DNS method [20]. In all experiments, the enzyme loading was constant at 10 FPU/g substrates with respect to Celluclast 1.5 L and 50 U/g substrates with respect to EndoPG$_{Cp}$ (free or immobilized). Each hydrolysis experiment was repeated three times using three independent EndoPG$_{Cp}$ preparations.

Statistical Analysis of the Enzyme Activity Data

All data were expressed as the mean and standard deviation of at least three independent experiments.

Results and Discussion

MNP Synthesis and Enzyme Immobilization Efficiency and Yields

Optimizing the initial EndoPG$_{Cp}$ concentration for immobilization suggested that the ideal enzyme loading was 100 µg of protein for 100 µL of a MNP suspension (10% w/v) (data not shown). The immobilization yield of the EndoPG$_{Cp}$ on the glutaraldehyde-treated magnetic nanoparticle support was approximately 20% and the specific activity of the immobilized enzyme was similar to that observed for a free enzyme, giving an efficiency yield of approximately 100%. The yield of covalent immobilization using glutaraldehyde as a spacer arm for most pectinases is in the range of 65–98% [13, 23–25]. However, similar to that observed for EndoPG$_{Cp}$, the polygalacturonase from *Aspergillus niger* [26] and *Aspergillus ustus* [27] also showed a low immobilization yield, of 37 and 20%, respectively. Low immobilization yields can be associated with different factors such as initial protein concentration, type of support, and the glutaraldehyde concentration used for enzyme cross-linking to the support [28–30].

The immobilization efficiency is a measure of the change in specific activity of the enzyme after immobilization, and few pectinases showed efficiency above 80% [13, 27, 31]. Alagöz and collaborators [25] observed a 90% reduction in the specific activity of an *Aspergillus aculeatus* pectinase after covalent immobilization on glutaraldehyde-activated magnesium silicate. However, the same enzyme presented ~ 75% of the specific activity as compared to the free enzyme after covalent immobilization on nanosilica activated with glutaraldehyde, demonstrating the influence of the support on the enzymatic activity after immobilization. The maintenance of the catalytic activity and the stability of the enzyme after the immobilization process are crucial for the viability of the operation. In general, this is governed by the orientation of the enzyme on the support, which largely depends on the distribution of the amino acid residues that participate in the covalent bond between enzyme and support. Techniques using glutaraldehyde as a coupling agent are probably the most used to enzyme immobilization. They can react mainly with the protein primary amino groups and eventually thiols, phenols, and imidazoles, and this could allow the EndoPG$_{Cp}$-MNP to be fully functional after the process [32–34]. Mohamad et al. [35] suggested that the enzyme activity is preserved when residues at the active site are not involved in binding to the support, and this effect may explain the high efficiency of immobilization in the present study.

Morphological Characterization of EndoPG$_{Cp}$-MNP by SEM

Scanning electron microscopy showed the formation of aggregates in the MNPs both in the presence and absence of the immobilized enzyme (Supplementary Information (SI)). These results could be related to the tendency of the ferromagnetic nanoparticles to agglomerate spontaneously [17, 36]. The nanoparticle aggregation effect was also observed in SEM by

Carneiro and Ward [17]. The resulting particle sizes are in the range of 2–10 μM and present a highly irregular and porous surface. Immobilization of the EndoPG$_{Cp}$ has no discernable effect on the morphology of the aggregates. Andrade et al. [37] observed the formation of aggregated structures in magnetic nanoparticles and immobilized β-galactosidase, relating this with magnetism. Podrepšek et al. [38] reported an increase in particle size caused by chitosan coating compared to the bare nanoparticle (22.7 nm). They found coated particles with an average size of 68.5 μm using a microemulsion process, 44.2 μm in the cross-linking process, and 58.8 nm by the covalent binding method. Gennari et al. [39] showed that magnetic nanoparticles coated with nanocellulose presented an average diameter of 74 nm that is about 2 × larger than the non-coated nanoparticles.

Influence of pH and Temperature on the Activity and Stability of Free and Immobilized EndoPG

The effect of temperature on the free and immobilized EndoPG$_{Cp}$ is presented in Fig. 1a. The EndoPG$_{Cp}$-MNP presented a similar profile to that previously described for free EndoPG$_{Cp}$ [1], and in both cases, the catalytic activity gradually increased from 45 to

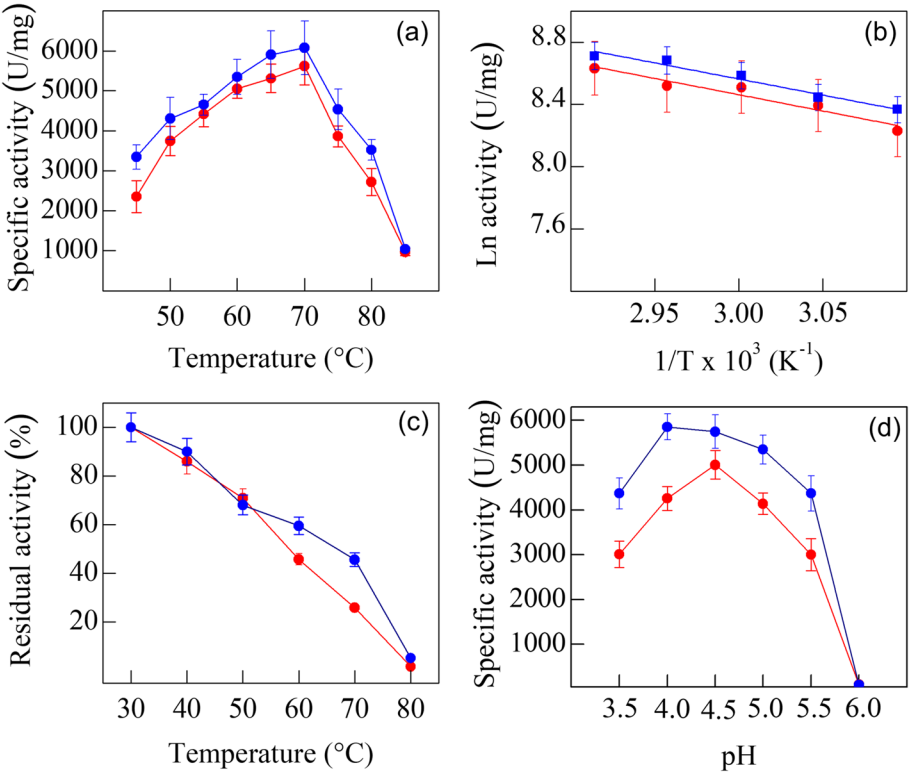

Fig. 1 Catalytic properties of immobilized (blue) and free (red) EndoPG$_{Cp}$. **a** Influence of temperature on the enzymatic activity. **b** Arrhenius plots for the effect of temperature on the enzymatic activity. **c** Influence of temperature on the stability of the enzymes. **d** Influence of pH on the enzymatic activity. One hundred percent specific activity corresponded to 4890.4 ± 368.3 U/mg for free EndoPG$_{Cp}$ and 5085.9 ± 217.1 U/mg for EndoPG$_{Cp}$-MNPs

65 °C, reaching the maximum at 70 °C and decreasing abruptly at higher temperatures. Previous reports demonstrate that the enzyme immobilization process may have positive [7] or negative [12] effects on the optimal reaction temperature. However, maintained optimal reaction temperature is a common feature for pectinases that are covalently immobilized with glutaraldehyde [13, 23–25]. Indeed, a commercial pectinase was immobilized on magnetic nanoparticles coated with silica and functionalized with glutaraldehyde [40] also showed an optimum temperature identical to the free enzyme. However, for this same enzyme, the immobilization process favored activity at higher temperatures and the enzyme showed a 30% increase in activity at temperatures above the optimum temperature for the free enzyme [40]. These effects are influenced by the immobilization method, the nature of the enzyme, and the support and cross-linker used for immobilization [9, 28, 30, 41]. Analysis of the Arrhenius plots (Fig. 1b) of the hydrolysis of pectin by both free and immobilized EndoPG$_{Cp}$ yield values for Ea of 17.3 ± 1.3 kJ/mol (free EndoPG$_{Cp}$) and 17.1 ± 1.1 kJ/mol (EndoPG$_{Cp}$-MNPs), demonstrating that the activation energy (Ea) was unchanged, confirming that the immobilization process had no effect on the temperature dependence of the catalytic reaction rate.

The EndoPG$_{Cp}$-MNP retained about 50% of its initial activity after incubation at 70 °C for 30 min and is more stable than the free enzyme, which presented about 25% of the initial activity under the same conditions (Fig. 1c). The covalent bonds between EndoPG$_{Cp}$ and the MNP are likely to decrease the thermal motions of the polypeptide chain leaving the enzyme less susceptible to thermal denaturation. This effect is widely observed in enzymes that are covalently immobilized to the support [6, 13, 14, 42].

The activity of the free and immobilized pectinase in various pH at a constant temperature (60 °C) is presented in Fig. 1d. The maximum activity of the free pectinase was obtained at a pH of 4.5 [2], and after immobilization, the optimum pH was shifted to 4.0. Furthermore, at pH 5.5, approximately 75% of the maximum activity is maintained for the immobilized enzyme, whereas the free enzyme maintains only 50% activity. Equally subtle effects of immobilization on the optimal pH of catalysis have been observed with other pectinases [31, 43–45] and may be associated with changes in enzyme conformation or pH effects on the concentration of substrate, product, charged species, hydrogen, and hydroxyl ions both in the immediate microenvironment of the immobilized enzyme [9, 28, 30, 41].

Kinetic Parameters

The EndoPG$_{Cp}$-MNP hydrolyzed citrus pectin with a V_{max} = 5250.60 ± 315.59 U/mg and K_M = 2.93 ± 0.30 mg/mL, giving in a catalytic efficiency (k_{cat}/K_M) of 2178.84 ± 105.20 mL/mg s (Table 1). These values are similar to those previously observed for the free EndoPG$_{Cp}$ [2], indicating that the conformational flexibility of pectinase was maintained

Table 1 Kinetic parameters for pectin hydrolysis by free and immobilized EndoPG

	K_M (mg/mL)	V_{max} (U/mg)	k_{cat}/K_M (mL/mg s)	References
EndoPG$_{Cp}$	2.45 ± 0.23	4947.10 ± 393.63	2052.90 ± 193.54	[1]
EndoPG$_{Cp}$-MNPs	2.93 ± 0.30	5250.60 ± 315.59	2178.84 ± 105.20	This work

The kinetic parameters are presented as the mean ± SD of the values calculated from data from three separate experiments (n = 3)

after immobilization. Although immobilization may limit the diffusion of the substrate to the active site of the enzyme or alter the dynamics of the enzyme molecule resulting in a compromised catalytic cycle, such effects would alter the kinetic parameters, as has been observed in the increased K_M values and decreased in V_{max} of immobilized enzymes previously reported for other pectinases immobilized by different methods [6, 16, 46, 47].

Application of EndoPG$_{Cp}$-MNP as Supplementation of a Commercial Enzyme Cocktail for Hydrolysis of Citrus Fruit Residues

Brazil is the largest producer of sweet orange and passion fruit in the world and is responsible for over three-quarters of global orange juice exports [48, 49]. Processing these fruits generates peel, seed, and bagasse by-products, and the re-use of these residues is a challenge for the food industry. These by-products are lignocellulosic materials rich in polysaccharides, including pectin, which could potentially also be utilized for the production of value-added bioproducts [50]. The application of immobilized EndoPG$_{Cp}$-MNP as a supplement to commercial enzymatic cocktails for the hydrolysis of by-products of the fruit processing industry was evaluated and compared with the results obtained using the free enzyme (Fig. 2). The total reducing sugar released by a commercial enzyme cocktail supplemented with EndoPG$_{Cp}$-MNP using orange residues (Fig. 2b) gradually increased over time and reached about 1.8-fold higher as compared to the hydrolysis by the unsupplemented cocktail. The EndoPG$_{Cp}$ alone (Fig. 2b) released approximately 45% of the total

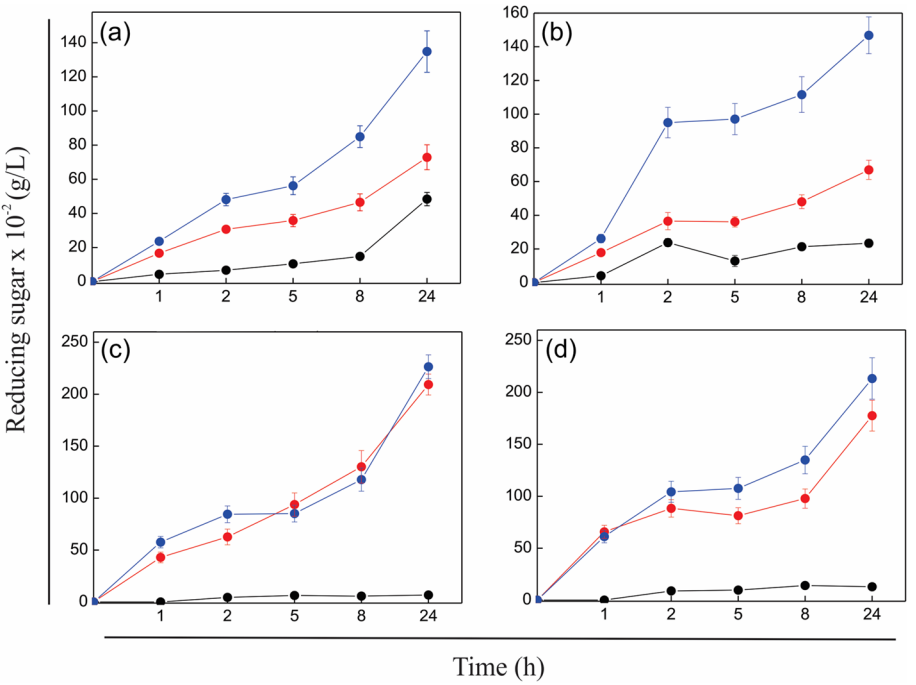

Fig. 2 Time course analysis of the hydrolysis of orange (**a** and **b**) and passion fruit (**c** and **d**) residues by EndoPG$_{Cp}$-MNPs (**a** and **c**) or free EndoPG$_{Cp}$ (**b** and **d**). Conditions: Celluclast + EndoPG$_{Cp}$ (blue), Celluclast only (red), EndoPG$_{Cp}$-MNPs, or free EndoPG$_{Cp}$ only (black)

when the enzyme was added to the commercial cocktail, and the cocktail alone released 15% of the total. The sum of the separate activities of the EndoPG$_{Cp}$ and the commercial cocktail was therefore only 60% of the total activity of the mixture, indicating a synergistic effect of the combined enzymes.

In experiments using passion fruit waste, no reducing sugar release was detected on treatment with either the immobilized (Fig. 2c) or free pectinase (Fig. 2d). Although the addition of the commercial cocktail resulted in a gradual increase in reducing sugar release over time to reach final levels that were 2 times higher than with the orange residues, the addition of the pectinase did not significantly alter the total reducing sugar release as compared to commercial enzyme cocktail. The increased reducing sugar release from passion fruit residues may reflect the higher pectin content in passion fruit residues than in orange residues [51]. Furthermore, the degree of esterification is higher in the pectin from passion fruit [51], and the differences in reducing sugar release by the EndoPG$_{Cp}$ may indicate that the enzyme preferentially hydrolyses non-esterified substrates.

The synergistic effect observed between EndoPG$_{Cp}$ and the enzymes that make up the commercial cocktail may be related to the structure of the orange plant cell wall, which presents the type I plant cell wall architecture [52]. In this type of cell wall, the content of the three main polysaccharides is 15–30% cellulose, 20–25% hemicelluloses, and 20–35% pectin. In addition, although the lignin content is extremely low, the recalcitrance of the material is related to the high concentration of pectin [52, 53]. The results obtained in this work for the orange pomace hydrolysis indicate that the EndoPG$_{Cp}$ improves the saccharification efficiency by reduction of the recalcitrance of the cell wall, due to the hydrolysis of the pectin and increasing the access of other hydrolytic enzymes.

Reusability of EndoPG$_{Cp}$-MNP

The possibility of reusing immobilized enzymes is one of the main advantages that directly affects the cost reduction associated with their industrial application [6, 11]. The reusability of the immobilized pectinase is presented in Fig. 3. EndoPG$_{Cp}$-MNP retained more than 65% of the initial activity even after seven re-use cycles, and after

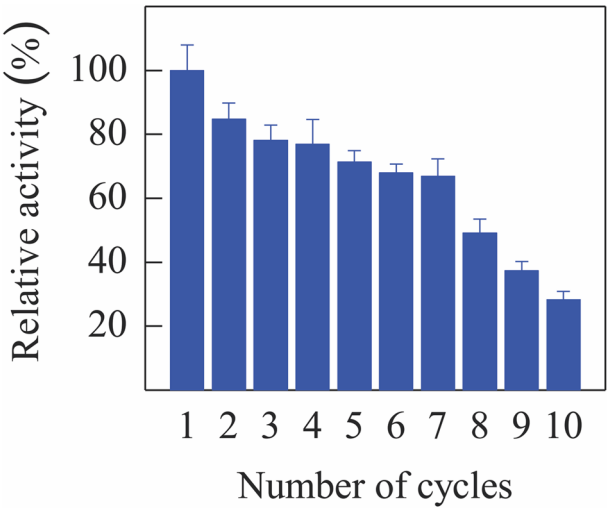

Fig. 3 Re-use of EndoPG-MNP determined by repeated use-wash cycles of the hydrolysis assay

10 cycles, the enzyme still retained about 30% of its initial activity. Similar results have been reported for commercial pectinase immobilized onto magnetic nanoparticles via glutaraldehyde activation [40, 54]. The possibility of easy separation of the enzyme from the reaction medium using a magnetic field is an added advantage compared to enzymes immobilized on other types of non-magnetic supports [17] where the biocatalyst is normally separated by filtration or centrifugation [29, 30, 55]. However, in order to recover the immobilized enzyme by filtration, larger particles are required which tends to result in substantial loss of enzyme activity due to the increased diffusion limitation through larger particles [29, 30, 55].

Conclusions

This study has demonstrated that the use of immobilized pectinase on chitosan-coated ferromagnetic particles (EndoPG$_{Cp}$-MNPs) can be advantageous for the hydrolysis of pectinolytic-rich residues as compared to the free enzyme. The immobilization process does not alter enzyme activity since the EndoPG$_{Cp}$-MNP retained essentially the same biochemical properties of the free enzyme such as optima temperature, activation energy, and kinetic parameters, and even demonstrated a slight increase in the thermostability. The use of the pectinase preparations to supplement the commercial cocktail Celluclast for the hydrolysis of orange residues increased the release of reducing sugars by about 1.8-fold and in the case of the EndoPG$_{Cp}$ showed a synergistic effect compared with controls using Celluclast and pectinase alone. The immobilization allowed repeated use of the EndoPG$_{Cp}$-MNPs, which retained about 30% of the initial activity after 10 re-use cycles. The use of ferromagnetic particles allowed the easy recovery of the EndoPG$_{Cp}$-MNP from reaction media using a magnetic field. Taken together, our results showed that the pectinase immobilized on ferromagnetic particles has advantages that are compatible with industrial applications.

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Author Contributions Sibeli Carli: data curation, formal analysis, investigation, methodology, figure preparation, visualization, writing—review & editing; Jose Carlos Santos Salgado: data curation, formal analysis, investigation, methodology, figure preparation, visualization, writing—review & editing; Luana Parras Meleiro: data curation, formal analysis, investigation, methodology, figure preparation, visualization, writing—original draft; Richard John Ward: conceptualization, funding acquisition, project administration, resource management, team supervision, writing—review & editing.

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Declarations

Competing Interests The authors declare no competing interests.

References

1. Sagar, N. A., Pareek, S., Sharma, S., Yahia, E. M., & Lobo, M. G. (2018). Fruit and vegetable waste: Bioactive compounds, their extraction, and possible utilization. *Comprehensive Reviews in Food Science and Food Safety, 17*(3), 512–531. https://doi.org/10.1111/1541-4337.12330
2. Carli, S., Meleiro, L. P., & Ward, R. J. (2019). Biochemical and kinetic characterization of the recombinant GH28 *Stereumpurpureum*endopolygalacturonase and its biotechnological application. *International Journal of Biological Macromolecules, 137*, 469–474. https://doi.org/10.1016/J.IJBIOMAC.2019.06.165
3. Samanta, S. (2019). Microbial pectinases: A review on molecular and biotechnological perspectives. *Journal of Microbiology, Biotechnology and Food Sciences, 9*(2), 248–266. https://doi.org/10.15414/JMBFS.2019.9.2.248-266
4. Amin, F., Bhatti, H. N., & Bilal, M. (2019). Recent advances in the production strategies of microbial pectinases—A review. *International Journal of Biological Macromolecules, 122*, 1017–1026. https://doi.org/10.1016/J.IJBIOMAC.2018.09.048
5. Garg, G., Singh, A., Kaur, A., Singh, R., Kaur, J., & Mahajan, R. (2016). Microbial pectinases: An ecofriendly tool of nature for industries. *Biotech, 6*(1), 1–13. https://doi.org/10.1007/S13205-016-0371-4
6. Hosseini, S. S., Khodaiyan, F., Seyed, S. M., Azimi, S. Z., & Gharaghani, M. (2020). Immobilization of pectinase on the glass bead using polyaldehyde kefiran as a new safe cross-linker and its effect on the activity and kinetic parameters. *Food Chemistry, 309*, 125777. https://doi.org/10.1016/J.FOODCHEM.2019.125777
7. Wu, R., He, B. H., Zhao, G. L., Qian, L. Y., & Li, X. F. (2013). Immobilization of pectinase on oxidized pulp fiber and its application in whitewater treatment. *Carbohydrate Polymers, 97*(2), 523–529. https://doi.org/10.1016/J.CARBPOL.2013.05.019
8. Boudrant, J., Woodley, J. M., & Fernandez-Lafuente, R. (2020). Parameters necessary to define an immobilized enzyme preparation. *Process Biochemistry, 90*, 66–80. https://doi.org/10.1016/J.PROCBIO.2019.11.026
9. Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology*. Elsevier. https://doi.org/10.1016/j.enzmictec.2007.01.018
10. Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., & Fernández-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews, 42*(15), 6290–6307. https://doi.org/10.1039/C2CS35231A
11. Guisan, J. M. (2013). *Immobilization of Enzymes and Cells* (1st ed.). HUMANA.
12. de Oliveira, R. L., Dias, J. L., da Silva, O. S., & Porto, T. S. (2018). Immobilization of pectinase from *Aspergillusaculeatus* in alginate beads and clarification of apple and umbu juices in a packed bed reactor. *Food and Bioproducts Processing, 109*, 9–18. https://doi.org/10.1016/J.FBP.2018.02.005
13. Seenuvasan, M., Malar, C. G., Preethi, S., Balaji, N., Iyyappan, J., Kumar, M. A., & Kumar, K. S. (2013). Immobilization of pectinase on co-precipitated magnetic nanoparticles for enhanced stability and activity. *Research Journal of Biotechnology, 8*(5), 24–30.
14. Ramírez Tapias, Y. A., Rivero, C. W., Gallego, F. L., Guisán, J. M., & Trelles, J. A. (2016). Stabilization by multipoint covalent attachment of a biocatalyst with polygalacturonase activity used for juice clarification. *Food Chemistry, 208*, 252–257. https://doi.org/10.1016/J.FOODCHEM.2016.03.086
15. Carli, S., de Carneiro, L. A. B., & C., Ward, R. J., & Meleiro, L. P. . (2019). Immobilization of a β-glucosidase and an endoglucanase in ferromagnetic nanoparticles: A study of synergistic effects. *Protein Expression and Purification, 160*, 28–35. https://doi.org/10.1016/J.PEP.2019.03.016
16. Lei, Z., Bi, S., Hu, B., & Yang, H. (2007). Combined magnetic and chemical covalent immobilization of pectinase on composites membranes improves stability and activity. *Food Chemistry, 105*(3), 889–896. https://doi.org/10.1016/J.FOODCHEM.2007.04.045
17. Carneiro, L. A. B. C., & Ward, R. J. (2018). Functionalization of paramagnetic nanoparticles for protein immobilization and purification. *Analytical Biochemistry, 540–541*, 45–51. https://doi.org/10.1016/J.AB.2017.11.005
18. Read, S. M., & Northcote, D. H. (1981). Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Analytical Biochemistry, 116*(1), 53–64. https://doi.org/10.1016/0003-2697(81)90321-3
19. McIlvaine, T. C. (1921). A buffer solution for colorimetric comparison. *Journal of Biological Chemistry, 49*(1), 183–186. https://doi.org/10.1016/S0021-9258(18)86000-8
20. Miller, G. L. (2002). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426–428. https://doi.org/10.1021/AC60147A030

21. Segel, I. H. (1975). *Enzyme kinetics behavior and analysis of rapid equilibrium and steady state enzyme systems* (2nd ed.). Wiley-Blackwell.
22. Leone, F. A., Baranauskas, J. A., Furriel, R. P. M., & Borin, I. A. (2005). SigrafW: An easy-to-use program for fitting enzyme kinetic data. *Biochemistry and Molecular Biology Education, 33*(6), 399–403. https://doi.org/10.1002/BMB.2005.49403306399
23. Dal Magro, L., Kornecki, J. F., Klein, M. P., Rodrigues, R. C., & Fernandez-Lafuente, R. (2019). Optimized immobilization of polygalacturonase from *Aspergillusniger* following different protocols: Improved stability and activity under drastic conditions. *International Journal of Biological Macromolecules, 138*, 234–243. https://doi.org/10.1016/j.ijbiomac.2019.07.092
24. Mohammadi, M., KhakbazHeshmati, M., Sarabandi, K., Fathi, M., Lim, L. T., & Hamishehkar, H. (2019). Activated alginate-montmorillonite beads as an efficient carrier for pectinase immobilization. *International Journal of Biological Macromolecules, 137*, 253–260. https://doi.org/10.1016/j.ijbiomac.2019.06.236
25. Alagöz, D., Tükel, S. S., & Yildirim, D. (2016). Immobilization of pectinase on silica-based supports: Impacts of particle size and spacer arm on the activity. *International Journal of Biological Macromolecules, 87*, 426–432. https://doi.org/10.1016/j.ijbiomac.2016.03.007
26. Saxena, S., Shukla, S., Thakur, A., & Gupta, R. (2008). Immobilization of polygalacturonase from *Aspergillusniger* onto activated polyethylene and its application in apple juice clarification. *Acta Microbiologica et Immunologica Hungarica, 55*(1), 33–51. https://doi.org/10.1556/AMicr.55.2008.1.3
27. Rao, M. N., Kembhavi, A. A., & Pant, A. (2000). Immobilization of endo-polygalacturonase from *Aspergillus ustus* on silica gel. *Biotechnology Letters* 22.
28. Guzik, U., Hupert-Kocurek, K., & Wojcieszyńska, D. (2014). Immobilization as a strategy for improving enzyme properties-Application to oxidoreductases. *Molecules, 19*(7), 8995–9018. https://doi.org/10.3390/MOLECULES19078995
29. Sheldon, R. A., & van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews, 42*(15), 6223–6235. https://doi.org/10.1039/c3cs60075k
30. Wahab, R. A., Elias, N., Abdullah, F., & Ghoshal, S. K. (2020, July 1). On the taught new tricks of enzymes immobilization: An all-inclusive overview. *Reactive and Functional Polymers*. Elsevier B.V. https://doi.org/10.1016/j.reactfunctpolym.2020.104613
31. Ramirez, H. L., Gómez Brizuela, L., ÚbedaIranzo, J., Arevalo-Villena, M., & Briones Pérez, A. I. (2016). Pectinase immobilization on a chitosan-coated chitin support. *Journal of Food Process Engineering, 39*(1), 97–104. https://doi.org/10.1111/jfpe.12203
32. dos Santos, J. C. S., Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R. C., & Fernandez-Lafuente, R. (2015). Importance of the support properties for immobilization or purification of enzymes. *ChemCatChem, 7*(16), 2413–2432. https://doi.org/10.1002/CCTC.201500310
33. Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R. C., & Fernandez-Lafuente, R. (2013). Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization. *RSC Advances, 4*(4), 1583–1600. https://doi.org/10.1039/C3RA45991H
34. Melo, R. R. de, Alnoch, R. C., Vilela, A. F. L., Souza, E. M. de, Krieger, N., Ruller, R., … Mateo, C. (2017). New heterofunctional supports based on glutaraldehyde-activation: A tool for enzyme immobilization at neutral pH. *Molecules, 22*(7), 1088. https://doi.org/10.3390/MOLECULES22071088
35. Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology and Biotechnological Equipment*. Diagnosis Press Limited. https://doi.org/10.1080/13102818.2015.1008192
36. Hritcu, D., Popa, M. I., Popa, N., Badescu, V., & Balan, V. (2009). Preparation and characterization of magnetic chitosan nanospheres. *Turkish Journal of Chemistry, 33*, 785–796. https://doi.org/10.3906/kim-0812-42
37. de Andrade, B. C., Gennari, A., Renard, G., Nervis, B. D. R., Benvenutti, E. V., Costa, T. M. H., … Volken de Souza, C. F. (2021). Synthesis of magnetic nanoparticles functionalized with histidine and nickel to immobilize His-tagged enzymes using β-galactosidase as a model. *International Journal of Biological Macromolecules*, *184*, 159–169. https://doi.org/10.1016/J.IJBIOMAC.2021.06.060
38. Podrepšek, G. H., Knez, Ž, & Leitgeb, M. (2020). Development of chitosan functionalized magnetic nanoparticles with bioactive compounds. *Nanomaterials, 10*(10), 1913. https://doi.org/10.3390/NANO10101913
39. Gennari, A., Mobayed, F. H., Nervis, B. D. R., Benvenutti, E. v., Nicolodi, S., Silveira, N. P. da, … Souza, C. F. V. de. (2019). Immobilization of β-galactosidases on magnetic nanocellulose: Textural, morphological, magnetic, and catalytic properties. *Biomacromolecules*, *20*(6), 2315–2326. https://doi.org/10.1021/ACS.BIOMAC.9B00285.
40. Seenuvasan, M., Malar, C. G., Preethi, S., Balaji, N., Iyyappan, J., Kumar, M. A., & Kumar, K. S. (2013). Fabrication, characterization and application of pectin degrading Fe 3O4-SiO2 nanobiocatalyst. *Materials Science and Engineering C, 33*(4), 2273–2279. https://doi.org/10.1016/j.msec.2013.01.050

41. Singh, R. K., Tiwari, M. K., Singh, R., & Lee, J. K. (2013, January). From protein engineering to immobilization: Promising strategies for the upgrade of industrial enzymes. *International Journal of Molecular Sciences*. Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/ijms14011232
42. Dai, X. Y., Kong, L. M., Wang, X. L., Zhu, Q., Chen, K., & Zhou, T. (2018). Preparation, characterization and catalytic behavior of pectinase covalently immobilized onto sodium alginate/graphene oxide composite beads. *Food Chemistry, 253*, 185–193. https://doi.org/10.1016/J.FOODCHEM.2018.01.157
43. Li, T., Wang, N., Li, S., Zhao, Q., Guo, M., & Zhang, C. (2007). Optimization of covalent immobilization of pectinase on sodium alginate support. *Biotechnology Letters, 29*(9), 1413–1416. https://doi.org/10.1007/S10529-007-9409-3
44. Mohammadi, M., RezaeiMokarram, R., Shahvalizadeh, R., Sarabandi, K., Lim, L. T., & Hamishehkar, H. (2020). Immobilization and stabilization of pectinase on an activated montmorillonite support and its application in pineapple juice clarification. *Food Bioscience, 36*, 100625. https://doi.org/10.1016/j.fbio.2020.100625
45. Shukla, S., Saxena, S., Thakur, J., & Gupta, R. (2010). Immobilization of polygalacturonase from *Aspergilusniger* onto glutaraldehyde activated Nylon-6 and its application in apple juice clarification. *Acta Alimentaria, 39*(3), 277–292. https://doi.org/10.1556/AAlim.39.2010.3.4
46. Rehman, H. U., Aman, A., Silipo, A., Qader, S. A. U., Molinaro, A., & Ansari, A. (2013). Degradation of complex carbohydrate: Immobilization of pectinase from Bacillus licheniformis KIBGE-IB21 using calcium alginate as a support. *Food Chemistry, 139*(1–4), 1081–1086. https://doi.org/10.1016/J.FOODCHEM.2013.01.069
47. Rehman, H. U., Aman, A., Nawaz, M. A., Karim, A., Ghani, M., Baloch, A. H., & Qader, S. A. U. (2016). Immobilization of pectin depolymerising polygalacturonase using different polymers. *International Journal of Biological Macromolecules, 82*, 127–133. https://doi.org/10.1016/J.IJBIOMAC.2015.10.012
48. Caserta, R., Teixeira-Silva, N. S., Granato, L. M., Dorta, S. O., Rodrigues, C. M., Mitre, L. K., … de Souza, A. A. (2019). Citrus biotechnology: What has been done to improve disease resistance in such an important crop? *Biotechnology Research and Innovation*, *3*, 95–109. https://doi.org/10.1016/J.BIORI.2019.12.004
49. Martins, H. F., Carvalho, S. S. R. de A., Bispo, J. A. C., Souza, S. M. A. de, & Martinez, E. A. (2019). Maracujá-amarelo (*Passiflora edulis* f. Flavicarpa): cinética da secagem artificial e natural da casca / Yellow passion fruit (*Passiflora edulis* f. Flavicarpa): kinetics of artificial and natural drying of the peel. *Brazilian Journal of Development*, *5*(11), 23234–23245. https://doi.org/10.34117/BJDV5N11-044
50. van Dyk, J. S., Gama, R., Morrison, D., Swart, S., & Pletschke, B. I. (2013). Food processing waste: Problems, current management and prospects for utilisation of the lignocellulose component through enzyme synergistic degradation. *Renewable and Sustainable Energy Reviews, 26*, 521–531. https://doi.org/10.1016/J.RSER.2013.06.016
51. de Moura, F. A., Macagnan, F. T., dos Santos, L. R., Bizzani, M., de Oliveira Petkowicz, C. L., & da Silva, L. P. (2017). Characterization and physicochemical properties of pectins extracted from agroindustrial by-products. *Journal of Food Science and Technology, 54*(10), 31113117. https://doi.org/10.1007/S13197-017-2747-9
52. Edwards, M. C., & Doran-Peterson, J. (2012, August). Pectin-rich biomass as feedstock for fuel ethanol production. *Applied Microbiology and Biotechnology*. Springer. https://doi.org/10.1007/s00253-012-4173-2
53. de Souza, A. P., C Leite, D. C., Pattathil, S., Hahn, M. G., & Buckeridge, M. S. (n.d.). Composition and structure of sugarcane cell wall polysaccharides: Implications for second-generation bioethanol production. https://doi.org/10.1007/s12155-012-9268-1
54. Fang, G., Chen, H., Zhang, Y., & Chen, A. (2016). Immobilization of pectinase onto Fe3O4-SiO2-NH2 and its activity and stability. *International Journal of Biological Macromolecules, 88*, 189–195. https://doi.org/10.1016/j.ijbiomac.2016.03.059
55. Bilal, M., Zhao, Y., Rasheed, T., & Iqbal, H. M. N. (2018, December 1). Magnetic nanoparticles as versatile carriers for enzymes immobilization: A review. *International Journal of Biological Macromolecules*. Elsevier B.V. https://doi.org/10.1016/j.ijbiomac.2018.09.025