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SHORT REPORTS

Saccharification of citrus wastes by immobilized polygalacturonase in an improved alginate matrix

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Abstract Enzyme immobilization using hydrogels is a low-cost and effective system for the degradation of bulk pectin derived from orange industry residues. Polygalacturonases obtained from four different bacterial strains of *Streptomyces* genus were immobilized in alginate gel and assayed for pectin hydrolysis. The enzyme from *Streptomyces halstedii* ATCC 10897 proved to be superior and more stable within the alginate matrix. Furthermore, a new strategy to improve alginate bead stability using a mixture of calcium and strontium is reported; this technique allowed enhancing the mechanical properties by combining different amounts of these cations for ionotropic gelation. The developed biocatalyst showed maximum hydrolysis at 2 h, generating 1.54 mg/mL of reducing sugars and decreasing the viscosity of polygalacturonic acid by 98.9%. Reusability up to 29 successive reactions (58 h) demonstrated a very stable performance. The heterogeneous biocatalyst was used in the enzymatic saccharification of orange peel albedo (2.23 mg/mL) for adding value to this agro-waste by industrial exploitation.

Keywords Calcium and strontium mixture · Enzymatic stability · Immobilized biocatalyst · Reusability · *Streptomyces halstedii* ATCC 10897

Introduction

Pectins are primarily composed of polygalacturonic acid with branches of molecules such as galacturonan, rhamnogalacturonan, arabinan and methyl residues. They occur in plant cell walls and at middle lamellae in numerous forms and are degraded by different mechanisms. Hydrolysis of α-(1,4) glycosidic bonds of D-galacturonic chain is carried out by polygalacturonases (PG) at mild conditions (Sharma et al. 2013). This enzymatic reaction is attractive for food industries to be applied in the maceration, extraction and filtration of fruit juices. It can also be used for the saccharification of vegetable residues to make the process more sustainable (Garg et al. 2016). Specifically, orange peel is a large fraction of by-products from citrus processing, and the polysaccharide composition of albedo is rich in pectin (homogalacturonan and rhamnogalacturonan), so it has great potential as raw material for the production of oligogalacturonides (Prabasari et al. 2011). Also the specificity, selectivity and activity of PG are important features to prefer enzymatic rather than chemical methods (Sharma et al. 2013). However, due to the high production cost, operational instability, difficulty in product separation and impossibility of reusability, the enzymes in soluble state have limitations for industrial use. Enzyme immobilization is a strategy to overcome these drawbacks because it increases stability and ensures reusability in successive reactions; it makes the process cost-effective for industrial applications.

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Enzyme immobilization can be conducted by three main processes such as binding to a support, cross-linking or entrapment. Inclusion of proteins within a polymeric network is the basis of entrapment techniques that prevent their release into the surrounding medium, while still allowing mass transfer of substrates and products. Entrapment using alginate has shown to be the most effective approach due to its biocompatibility, nontoxicity, low cost and resistance to microbial contamination as compared to other methods (Augst et al. 2006; Cappa et al. 2014). These characteristics are necessary conditions for the application of these techniques in agro-wastes, making this bioprocess eco-sustainable due to its availability as renewable resource. Besides, inexpensive raw materials and easy recovery of biocatalyst for successive reuses reduce energy demand, especially in fermentation process to produce enzymes.

In this work, a new biocatalytic system was developed based on PG from *Streptomyces halstedii* ATCC 10897 immobilized in sodium alginate with both calcium and strontium as cross-linking cations to degrade and saccharify pectic residues from orange albedo through a profitable enzymatic mechanism, which allows easy recovery and high reusability.

Materials and methods

Microorganism and enzyme recovery

Previous studies at the Laboratory of Sustainable Biotechnology (LIBioS) of the National University of Quilmes (Argentina) about polygalacturonase (PG) production showed that *Streptomyces* strains from the laboratory collection were highly active microorganisms (Ramírez-Tapias et al. 2015). The enzymes were produced by liquid cultures with optimized media, the cell-free supernatants were obtained by centrifugation at 12,000×*g* for 20 min and ultrafiltered by 10 kDa MWCO up to 20 × volume concentration factor, and the retentate fraction was used for protein quantification (Bradford 1976), molecular weight PG determination by polyacrylamide gel electrophoresis and for enzyme immobilization.

Polygalacturonase immobilization in alginate beads

The immobilization process was adapted from the method described by Trelles and Rivero (2013). In brief, PG entrapment was carried out by mixing 5% (w/v) sodium alginate solution with free enzyme in a volume ratio of 1:1. This mixture was added dropwise to 0.2 M $CaCl_2$ solution, which led to calcium alginate bead formation. These beads were kept in cationic solution for 10 min with gentle stirring and then were washed with distilled water. The percentage of protein entrapment was calculated by the quantification of protein released in washing water in relation to the initial total protein.

Once selected, *S. halstedii* ATCC 10897 was evaluated by sequential experiments. First, the effects of sodium alginate concentration and cross-linking ion on polygalacturonic acid hydrolysis were identified by enzyme immobilization within the hydrogel matrix at 2 and 3% (w/v) of sodium alginate and divalent cations (Ca^{2+} and Sr^{2+}) at 0.2 M. Then, the different mixtures of calcium and strontium were evaluated for higher biocatalyst stability. Swelling effects were estimated by bead diameter measurement using a Vernier caliper ($n = 25$); the relation of bead diameter before the first and after successive reactions indicated the swelling ratio.

Enzyme assay

The PG assay was performed by reaction of alginate beads with 0.5 mg of protein and 0.5% (w/v) polygalacturonic acid solution as substrate in buffer 25 mM Tris–HCl pH 7. PG activity was measured by the colorimetric method with 3′,5′-dinitrosalicylic (DNS) acid reagent using D-galacturonic acid as standard for the quantification of reducing sugars released during reaction at 40 °C. Complete hydrolysis involved the release of 5 mg/mL of D-galacturonic acid, so the relation of this value to the reducing sugars produced by enzymatic reactions indicated substrate conversion.

Viscosity reduction was measured with a rheometer (AR-G2/TA Instruments) at 24 °C and 300/s shear rate. The Km and Vmax parameters of Michaelis–Menten kinetics were determined using substrate solutions at concentrations of 0.1, 0.25, 0.50, 1.00 and 1.50% (w/v). Periodically, product conversion was measured, and the results were fitted to the Lineweaver–Burk linearization plot.

Orange albedo hydrolysis

Orange peel residues were collected, and the albedo fraction was separated and boiled for 10 min for inactivation of endogenous enzymes. The dried material was ground and passed through mesh 50 (0.325 mm), resuspended at 2% (w/v) in buffer 25 mM Tris–HCl pH 7, and incubated with the biocatalyst at 50 °C with 200 rpm orbital agitation.

Results and discussion

Gel formation of sodium alginate is driven by the interactions between carboxylate groups of alginate and divalent cations; this hydrogel forms a mechanically

stable network that can be used to immobilize cells and proteins (Pawar and Edgar 2012). So, four different PG were evaluated as heterogeneous biocatalyst based on their entrapment using sodium alginate (2.5%) and $CaCl_2$ (0.2 M) for ionotropic gelation and bead formation. The average bead size was 2.73 mm, the percentage of protein entrapment resulted in 87% and was not strain dependent ($p > 0.05$). The biocatalyst from different bacterial sources evidenced different enzymatic activity, and the substrate hydrolysis varied from 14.5 to 23.5% (Table 1). Differences are probably due to variability in the location of amino acids in protein, variance in substrate uptake or their steric conformation within the hydrogel network affecting mass transfer (Hiteshi et al. 2013). Negative effects due to calcium were discarded because the presence of this cation in free enzyme did not influence enzyme activity ($p = 0.002$). The results indicated that immobilized PG from *S. halstedii* ATCC 10897 was the best biocatalyst, which released 1.17 mg/mL of D-galacturonic acid.

Therefore, different immobilization conditions were evaluated to achieve a heterogeneous biocatalyst with the highest enzymatic activity and stability. The concentrated protein solution used for the immobilization process was obtained after a purification procedure that allowed a recovery of 2.559 mg/mL that showed a single band in both native and SDS-PAGE, indicative of a high pure monomeric enzyme with approximate molecular weight of 48 kDa.

Enzymes immobilized using calcium and strontium produced biocatalyst beads with an average size of 2.75 ± 0.21 and 2.60 ± 0.22 mm ($n = 25$), respectively. Alginate concentration did not affect the initial bead diameter. Table 2a shows that using 3% (w/v) of sodium alginate, the percentage of entrapped protein was significantly higher, retaining 1.139 ± 0.007 mg/mL ($p < 0.05$); this variable was not dependent on the cross-linking cation type. The alginate matrix entrapped PG with suitable stiffness that was dependent on polymer concentration; at 3% (w/v) stronger beads were produced, retaining a greater fraction of proteins.

After the immobilization procedure, the hydrolysis reaction was carried out, and the matrix provided a proper exchange of substrate and products across the network. The highest product conversion (29.6% of hydrolysis) was obtained with the biocatalyst made with alginate at 3% (w/v) and Ca^{2+} 0.2 M. Certainly, calcium addition gave better biotransformation efficiency, but the drawback to immobilize the enzymes with calcium was the instability of beads, evidenced by their mechanical performance, because they showed more osmotic swelling leading to increased pore size, destabilization and rupture of the matrix. Figure 1 displays how calcium adversely affected bead size during sequential enzymatic reactions (63.5% swelling ratio) and produced a less stable biocatalyst decreasing its reusability to 44 h. These effects of divalent cations on alginate gels has been reported (Mørch et al. 2006; Davidovich-Pinhas and Bianco-Peled 2010), suggesting that the swelling behavior and affinity of the polymer to cations depend on alginate copolymer structure (mannuronic and guluronic acid). The strontium cation probably contributed to tighter egg-box junctions resulting in a more stable biocatalyst, but the hydrolytic action was less efficient. Surface abrasion was evidenced in 2% (w/v) sodium alginate beads, and this condition also showed worse operational stability. On the other hand, enhanced stability results were afforded by strontium and 3% (w/v) alginate concentration, but enzymatic reactions did not exceed 23.91% of product conversion. In this sense, a novel methodology was carried out using mixtures of divalent cations to modulate mechanical properties and develop an active biocatalyst. Table 2b lists the enzymatic activity, including viscosity reduction and stability features that showed differences attributed to the effect of the cationic mixtures. Using a solution with calcium and strontium at a ratio of 4:1 (v/v), the substrate hydrolysis was over 30%; also the time of reusability was higher and

Table 1 Entrapment of polygalacturonase from *Streptomyces* strains in Ca-alginate beads (2.5% sodium alginate and 0.2 M $CaCl_2$) and hydrolytic activity of the free and immobilized enzyme

Strain	Free enzyme[a]	Protein entrapment (%)	Immobilized enzyme[b]	
	D-Galacturonic acid (mg/mL)		D-Galacturonic acid (mg/mL)	Conversion (%)
S. griseus	1.12	87.2	0.87	17.4
S. halstedii	1.28	87.7	1.17	23.4*
S. netropsis	1.11	88.1	0.91	18.2
S. thermocoprophilus	0.98	86.7	0.73	14.5

Average bead size: 2.65 mm. Values are mean of 3 replicates, standard error was < 2%

* Conversion values differ significantly in enzymatic activity (LSD test $p < 0.05$)

Total protein content per milliliter of reaction mixture was [a] 0.35 mg and [b] 0.5 mg within alginate matrix

Table 2 Effects of sodium alginate concentration and divalent cations on protein entrapment, hydrolysis of polygalacturonic acid and stability properties of heterogeneous biocatalyst during reuses (a) and effects of mixture of cross-linking cations for immobilized biocatalyst using alginate 3% (w/v) (b)

Alginate % (w/v)	Cation (−)	Protein entrapment[a] (%)	D-Galacturonic acid[a] (mg/mL)	Conversion[a] (%)	Operational stability[b]* (h)	Swelling[b] (%)
(a)						
2	Ca^{2+}	81.5	1.30	26.05	36	59.5
	Sr^{2+}	78.5	1.07	21.33	40	43.4
3	Ca^{2+}	89.5	1.48	29.60	44	63.5
	Sr^{2+}	88.6	1.20	23.91	60	37.3

Ratio Ca^{2+}:Sr^{2+} (v/v)	D-Galacturonic acid[a] (mg/mL)	Conversion[a] (%)	Viscosity reduction[a] (%)	Operational stability[b]* (h)	Swelling[b] (%)
(b)					
4:1	1.53	30.68	98.9	58	42.6
1:1	1.50	29.94	97.8	50	47.1
1:4	1.28	25.69	96.5	54	41.5

Protein loading was fixed at 0.5 mg/mL

* Operational stability regards to time of reusability up to 50% of initial activity (relative activity)

Values are mean of 3 replicates, [a] standard error was < 2% and [b] 6%

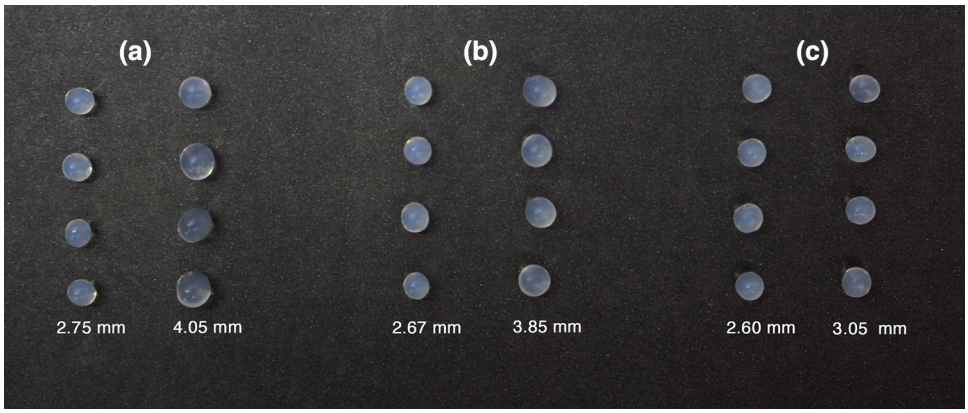

Fig. 1 Enzyme entrapment within alginate beads made by ionotropic gelation with divalent cations at 0.2 M and their bead size changes by swelling effect after 44 h of reusability. Cross-linking agents were $CaCl_2$ (**a**), a mixture of $CaCl_2$ and $SrCl_2$ at a volume ratio of 4:1 (**b**) and $SrCl_2$ (**c**). Average diameters of particles are described, and the maximum standard error was 4.3% ($n = 25$)

osmotic swelling was lower than using calcium only. So using the cross-linking solution with a higher calcium content and a reduced fraction of strontium, product conversion was as high as when using only calcium, and the mechanical behavior during successive reactions was significantly enhanced compared to calcium and very close to that obtained with strontium. Finally, the volume ratio of 4:1 Ca^{2+}:Sr^{2+} proved to be the best ionotropic gelation agent to balance the biotransformation performance and stability properties of the biocatalyst. As indicator of the stiffness of alginate beads, the oscillatory stress sweep test was performed on the hydrogel, and the results of storage modulus (G') in the plateau zone (oscillatory stress between 0.01 and 0.2 Pa at 1 Hz) were 5.37, 6.19, and 5.68 kPa for calcium, strontium and the 4:1 (v/v) mixture, respectively, proving that strontium conferred more stability and the mixture provided an intermediate value.

The specific reaction kinetics was studied and after 2 h of enzymatic reaction, 1.54 mg/mL D-galacturonic acid was released and 98.9% viscosity reduction was achieved, being the maximum values of hydrolysis. Similar results were obtained with free enzyme at 0.5 h (data not shown); slight differences compared to free enzyme were due to the diffusion barrier that the substrate has to overcome through the matrix, requiring longer time to reach the binding site of entrapped enzyme (Andriani et al. 2012). Besides, this result showed a better kinetic behavior compared to a previous report about the same PG immobilized by covalent binding to an agarose (Ramírez-Tapias et al. 2016). Michaelis–Menten parameters for immobilized enzyme were determined, and the result of maximum reaction rate (Vmax) was 0.539 µmol/min and the Km constant value was 0.062 M (supplementary data).

The reusability assay indicated that there was no significant loss of enzymatic activity for 28 h and the biocatalyst remained stable up to 58 h of successive reactions. Storage stability at 4 °C was also evaluated, and the immobilized biocatalyst retained 65% of the initial enzymatic activity after 21 days. Since sodium alginate gel has been widely used as a vehicle for drug delivery and to entrap whole cells, enzyme entrapment remains a subject of study because each biocatalytic system studied has shown specific behavior depending on the protein structure and substrates. Nevertheless, the profitability of enzyme immobilization in conversion processes is related to the biocatalyst reuses, which is a key factor for cost-effectiveness in industrial applications (Bolivar et al. 2015). In this regard, the results revealed herein are markedly higher than those of other glycolytic enzymes for which 7 reuses were reported (Ertan et al. 2007; Gangadharan et al. 2009; Bogra et al. 2013). Andriani et al. (2012) could recycle a cellulase just for three cycles, and the maximum reusability reported was 11 batches (Li et al. 2007).

This biocatalyst was used for the biodegradation of albedo from orange peels. Figure 2 depicts the enzymatic saccharification and decrease in viscosity of albedo waste, which yielded 2.23 mg/mL of reducing sugars and 91.29% viscosity reduction at 2 h of reaction. This immobilized biocatalyst with PG activity allowed obtaining 9% (w/w) of valuable sugars on dry basis, which could be used in industrial processes as nutraceutical foods and fermentable sugars.

Finally, the PG active biocatalyst developed in this work showed enhanced results of stability and reusability, which could be attributed to the use of a mixture of different amounts of calcium and strontium as cross-linking agents. This strategy is a meaningful finding for biotransformation systems applied to pectin polymer degradation either in food processing or agro-industrial waste treatment, which also has the advantage of using raw materials available commercially at low cost; sodium alginate as natural polymeric matrix from renewable resources and cationic salts produced by Argentine, a leader in these mineral production. So, these results are very competitive compared to commercial pectinases hydrolyzing citrus polygalacturonic acid (Combo et al. 2012) with the added advantage that they enable catalytic system immobilization and the prospect of adding value to some agro-wastes.

Conclusion

Polygalacturonase from *S. halstedii* ATCC 10897, immobilized in sodium alginate with calcium and strontium at a 4:1 (v/v) ratio, is a novel biocatalyst based on the use of this eco-compatible polymer. This is the first report on matrix improvement using a mixture of calcium and strontium as cross-linking agents; the developed biocatalyst was successfully used to degrade pectic substances that could add value to organic wastes. The reusability of immobilized enzyme enlarges the potential of a biocatalyst to be used in the saccharification of plant tissues such as orange peel albedo. Finally, the implementation of this biocatalytic process to reduce the use and generation of substances hazardous to human health and the environment

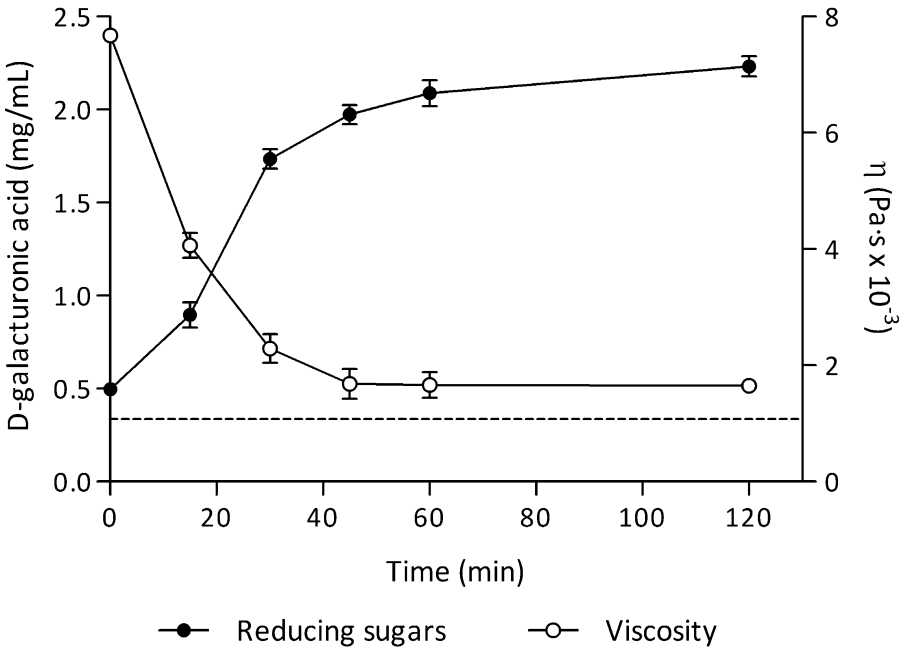

Fig. 2 Saccharification of orange peel albedo by hydrolysis with immobilized PG biocatalyst. The dashed line indicates the viscosity of buffer 25 mM Tris–HCl pH 7, which denoted 100% viscosity reduction

could be considered as a strategy framed in Green Chemistry.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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