Contents lists available at ScienceDirect

Food and Bioproducts Processing

journal homepage: www.elsevier.com/locate/fbp

Biodegradation of vegetable residues by polygalacturonase-agar using a trickle-bed bioreactor

Yuly A. Ramírez-Tapias[a,b], Cintia W. Rivero[a,b], Catalina Giraldo-Estrada[c], Claudia N. Britos[a], Jorge A. Trelles[a,b,*]

[a] Laboratory of Sustainable Biotechnology (LIBioS), National University of Quilmes, Roque Sáenz Peña 352, Bernal B1876BXD, Argentina
[b] National Scientific and Technical Research Council (CONICET), Godoy Cruz 2290, CABA C1425FQB, Argentina
[c] Universidad EAFIT, Carrera 49 N° 7 Sur-50, El Poblado 050021, Colombia

ARTICLE INFO

Article history:
Received 28 July 2017
Received in revised form 28 June 2018
Accepted 29 June 2018
Available online 6 July 2018

Keywords:
Enzyme immobilization
Scale-up
Packed bed bioreactor
Pear and cucumber residues
Saccharification
Streptomyces halstedii ATCC 10897

ABSTRACT

Bacterial pectinases degrade the pectic substances present in plant tissues and particularly, polygalacturonases catalyze the hydrolysis of α-(1,4) glycosidic bonds linking D-galacturonic acid units. In this study, polygalacturonase from *Streptomyces halstedii* ATCC 10897 was immobilized by the matrix entrapment technique using different thermogels. Bacteriological agar added with magnesium cation produced beads with a more stabilized microstructure for enzyme retention, monitored by oscillatory measurements of storage and loss modulus. Agar concentration and protein content were optimized to maximize protein entrapment, product conversion, and reaction yield. Results showed that the mixture at 10:90% (v/v) of protein (2 mg/mL) and agar (4% w/v) was the best immobilization condition to retain 91% of protein and hydrolyze 38% of pectin to allow the highest reaction yield (9.279 g/g) and increase stability up to 48 h of successive reactions. Agarose bead biocatalysts were used in a trickle-bed column operated with recirculation, and this bioreactor allowed the degradation of pear and cucumber residues by enzymatic liquefaction to enhance sugar content up to 15.33 and 9.35 mg/mL, respectively, and decrease viscosity by 92.3%. The scale-up of this process adds value to vegetable residues such as fructooligosaccharides or fermentable sugars, which become a sustainable source of fuels and chemicals.

© 2018 Institution of Chemical Engineers. Published by Elsevier B.V. All rights reserved.

1. Introduction

In enzyme technology, the immobilization process has expanded due to the advantages of important applications in biotransformation processes and the advances in technological fields. Protein stabilization by means of entrapment is a physical method to confine enzymes in a network of polymers; this technique prevents damage of the protein structure because it does not involve chemical modifications that may cause detrimental effects or may be stressful to the enzyme (Hiteshi et al., 2013). The main benefits of immobilization are facility in the separation process of the enzymes and products from reaction media, repeated or continuous usage, and simplification of the reactor design (Zhang and Xing, 2011).

Natural or synthetic polymers such as carrageenan, chitosan, alginate, agar or agarose, and also polyacrylamide and polyvinyl alcohol offer a lattice structure suitable for enzyme entrapment. Particularly, agar is a natural thermogel that consists of a mixture of agarose and agaropectin. Agarose is a linear and neutral polysaccharide, and agaropectin is made up of alternating units of galactose and galactose heavily modified with acidic side groups, such as sulfate and pyruvate

* *Corresponding author at*: Laboratory of Sustainable Biotechnology (LIBioS), National University of Quilmes, Roque Sáenz Peña 352, Bernal B1876BXD, Argentina.
E-mail address: jtrelles@unq.edu.ar (J.A. Trelles).
https://doi.org/10.1016/j.fbp.2018.06.006
0960-3085/© 2018 Institution of Chemical Engineers. Published by Elsevier B.V. All rights reserved.

(Mohammed et al., 1998). It is an inert, nontoxic, biocompatible and cost-effective thermogel; it can act as a barrier for enzymes to withstand operating conditions including mechanical shear, solvent, ionic and pH effects (Norziah et al., 2006).

Pectin is an essential component of plant cell walls; it is one of the most complex biomacromolecules in nature composed of different polysaccharides, mainly polygalacturonic acid. Pectinases are a group of enzymes that contribute to the degradation of pectin by various mechanisms. Especially, polygalacturonase (PG) hydrolyzes α-1,4 glycosidic linkages and releases galacturonic acid residues (Jayani et al., 2005). This enzyme is relevant in industrial applications that involve processing of plant tissues and treatment of pectic wastewater (Tapre and Jain, 2014). The production process of concentrated juices generates around 60% of by-products based on raw fruits, pomace and slurry being the main residues of pressing and decantation stages, respectively (Aramberri, 2016). These residues are rich in fiber and sugars but are rarely exploited since they produce a negative impact on the environment, especially on soil and water. So PG immobilization by entrapment using agar and subsequently, an enzyme reactor design has emerged as an ecofriendly strategy to develop an enzymatic system for practical application in pectin biodegradation. It could represent a new approach to solving an environmental problem and add value to these residues, which could be used to produce fructooligosaccharides or fermentable sugars, traditionally made by chemical methods (Binder and Raines, 2010; Hiteshi et al., 2013).

In this work, a bioprocess using PG produced by *Streptomyces halstedii* ATCC 10897 and immobilized in agar polymer was developed. This biocatalytic system is based on a batch trickle-bed reactor with continuous recirculation of percolate with entrapped PG in Mg-agar for depectinization of effluents from a local manufacturer of fruit and vegetable juices.

2. Materials and methods

2.1. Reagents and microorganism

Culture medium compounds were obtained from Britania S.A. (Argentina), polygalacturonic acid from citrus peel was purchased from Sigma S.A. (Brazil), and agar from Britania Laboratories (Argentina). Bradford reagent was supplied by Bio-Rad Laboratories, and other chemical compounds were purchased from Biopack S.A. (Argentina).

The microorganism *Streptomyces halstedii* ATCC 10897 is from the collection of the Laboratory of Sustainable Biotechnology (LIBioS) of the National University of Quilmes (Argentina).

2.2. Polygalacturonase production and purification

Polygalacturonase from *Streptomyces halstedii* ATCC 10897 was produced by liquid cultures with optimized media for enzyme induction (Ramírez-Tapias et al., 2015) for 12 h at 28 °C. The cell-free crude enzyme was obtained by centrifugation at 12,000 × *g* for 20 min using Beckman J2-MC and ultrafiltered up to 20× volume concentration factor. The Vivaflow Sartorius cross-flow polyethersulfone cassette system of 10 KDa MWCO was used at an operational flow rate of 10 L/h, and the retentate fraction was collected and used for protein quantification by Bradford reagent, PG molecular weight determination using 12% polyacrylamide gel electrophoresis and enzyme entrapment.

2.3. Enzyme entrapment

PG was immobilized by the entrapment technique using thermogel solution at 4% (w/v). Agar–agar was prepared in deionized water by heating to boiling and returning the temperature to 60 °C. Enzyme entrapment was carried out by mixing agar–agar solution with free enzyme at 50% (v/v). This mixture was added dropwise to vegetable oil at 8 °C, which led to the formation of gel beads that were left in the oil for 10 min with gentle stirring. Subsequently, the beads were rinsed with hexane and washed thoroughly with distilled water (Trelles and Rivero, 2013). Finally, the agar–agar beads were size-measured by Vernier caliper (n = 30), weighed, and stored in sterile water for further processing.

2.4. Optimization of entrapment conditions

PG entrapment was optimized regarding type of agar, cationic gel stabilization, and protein loading by sequential experimental tests. First, agars from four different sources were evaluated to identify viscoelastic properties and select one with the highest stiffness and biocatalytic performance. Then, cationic strength was evaluated to determine the effects on matrix stability. Chloride salts of sodium, potassium, aluminum, manganese, calcium, and magnesium were evaluated at a concentration of 200 mM. Agar–agar solutions (4% (w/v)) were prepared using each salt solution, and enzyme entrapment was performed. Then, a *simplex-lattice* binary mixture design was carried out to optimize protein load with the highest enzymatic activity with a constraint of protein load between 10% and 60% (v/v). The response variables were protein entrapment, D-galacturonic acid formation, and reaction yield. The complete experimental design consisted of seven runs including two replications of the central point. Data were fitted to the quadratic polynomial model to identify the effect of each component and interactive effects. This analysis was carried out using Statgraphics Centurion XV.II.

2.5. Rheological testing

Thermogels were rheologically characterized by strain dynamic oscillatory tests using a rheometer AR-G2 (TA Instruments) equipped with 30 mm parallel plate geometry. Mechanical spectra (stress sweep) were recorded over the range 0.1–10.0 Pa at 1.0 Hz frequency. Also temperature ramps were evaluated from 70 °C to 10 °C and from 10 °C to 90 °C setting the ramp rate at 5 °C/min with oscillatory stress at 1.0 Pa and 1.0 Hz. Measurements were conducted in triplicate. After gap adjustment to 1500 μm, 2 mL of sample was placed under the plate, and a cover was used over the geometry to prevent evaporation. The phase angle (δ), storage (G') and loss (G'') modulus were measured.

2.6. Polygalacturonase reaction conditions

The PG assay was performed by reaction of thermogel beads (0.562 ± 0.024 g of biocatalyst per mL of substrate) with 0.5% (w/v) polygalacturonic acid solution as substrate in 0.1 M glycine-NaOH buffer at pH 11. 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 the reaction at 40 °C. Viscosity reduction was measured with a rheometer AR-G2 (TA Instruments) at 24 °C and 300 s^{-1} shear rate. The reaction kinetics was evaluated in order to identify the time of maximum reaction yield, expressed as production of D-galacturonic acid (g) in relation to the protein content (g). The Km and Vmax parameters of Michaelis–Menten kinetics

were determined using substrate solutions at concentrations of 0.25%, 0.50%, 1.00%, 1.50%, 2.00%, and 2.50% (w/v). Periodically, product conversion was measured, and the results were fitted to the Lineweaver–Burk linearization plot. Finally, the effectiveness factor (η) of the heterogeneous biocatalyst was quantified.

2.7. *Effect of temperature and pH on biocatalyst activity*

The effect of temperature on the biocatalyst activity was determined at pH 11 by incubation of beads in reaction medium at different temperatures (20–65 °C). Also, the enzymatic activity was assayed at 40 °C as a function of pH with 0.1 M citrate-phosphate buffer (pH 3–7) and 0.1 M glycine-NaOH buffer (pH 8–11).

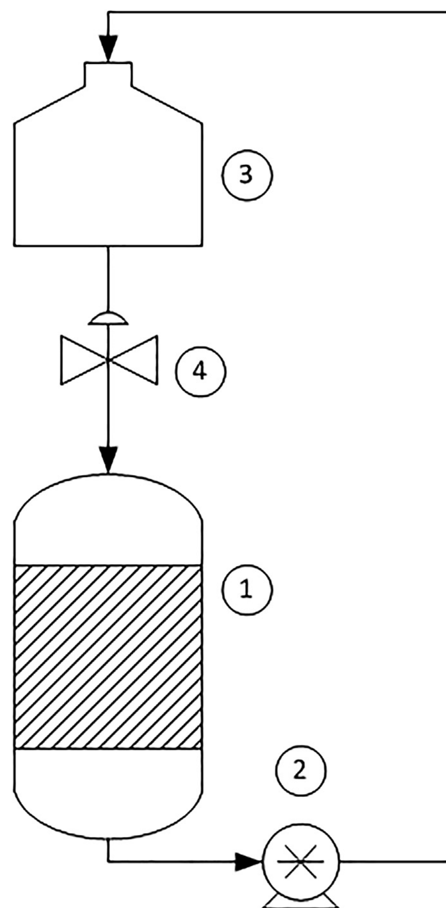
2.8. *Reusability and storage stability*

The reusability of immobilized PG on agar–agar beads was evaluated by polygalacturonic acid 0.5% (w/v) hydrolysis reaction. After each cycle of 1 h, the used biocatalyst was recovered from the reaction medium and washed with distilled water. Consecutive reactions with the biocatalyst were performed until enzyme inactivation.

The immobilized biocatalyst was stored at 4 °C in distilled water, samples were taken periodically, and enzymatic activity was evaluated. Storage stability was defined as the relative activity between the first and the successive reactions.

2.9. *Trickle-bed column for degradation of vegetable residues*

The trickle-bed reactor consisted of a polypropylene column (H/D = 4) filled with 20 mL of biocatalyst beads. The reactor was flow rate controlled with a peristaltic pump and a feed valve, experiments were conducted at flow rates of 1 mL/min and 8 mL/min. Specific liquid holdup was calculated as volume of liquid feed per hour per volume of packed reactor (Martins Dos Santos et al., 1997). Reaction performance was evaluated at each configuration, and the one that gave the best hydrolysis was selected for the degradation of pear and cucumber residues from a local producer of fruit and vegetable juices. Scheme 1 depicts the experimental setup for column operation. Feed streams of residues were filtered to remove coarse solids and avoid packed column clogging, and then were characterized in terms of viscosity, sugar content, and soluble solids.



Scheme 1 – Diagram of the experimental setup of trickle bed bioreactor. 1: Packed bed column (H = 80 mm, D = 20 mm), 2: peristaltic pump, 3: feed vessel (25 mL), 4: stopcock valve.

3. Results and discussion

3.1. *Selection of thermogel matrix*

Entrapment immobilization processes were carried out with four types of agar named: *Gracilaria*, *Pterocladia*, *Gelidium* and bacteriological, and they were evaluated for reaction performance and mechanical stability (Table 1). Protein entrapment varied between 67% and 80% using each thermogel at a final concentration of 2.0% (w/v), and bacteriological agar was the matrix that provided the highest product conversion and reaction yield and it remained active up to 42 h. Additionally, oscillatory sweeps were tested to infer thermogel microstructure, so stress forces were applied to evaluate the biocatalyst response expressed by phase angle ($\tan \delta = G''/G'$), and the results showed that four agar types behaved as an elastic solid, bacteriological agar being stronger ($\delta = 11.63°$). Though the phase angle (δ) of an ideal solid is 0°, some of the mechanical energy was dissipated (Mohammed et al., 1998; Osswald and Rudolph, 2014), and bacteriological agar presented the lowest value probably due to a greater content of agarobiose than agaropectin in its composition in comparison to other agars. Also the swelling ratio was measured in relation to diameter changes after biocatalyst reusability, and it was less than 3.15%, suggesting minimum destabilization of matrices by the effect of osmotic swelling, meaning preservation of bead pore size. Based on the described properties, bacteriological agar showed the best performance to develop a highly PG active immobilized biocatalyst.

3.2. *Stabilization of biocatalyst matrix by the addition of cations*

Agar is composed of agaropectin (sulfated charged) and agarose (neutral). Although agarose is the polysaccharide fraction having the highest gelling potential, charged groups of agaropectin could promote interaction with proteins, and the addition of cations differing in radius or charge densities may stabilize the matrix (Gulrez et al., 2003). Then, chloride salts were evaluated during PG immobilization to identify the effects on both enzymatic activity and gel strength. Results showed that protein entrapment was reasonably promoted by the addition of cations (Fig. 1); aluminum chloride allowed the

Table 1 – Catalytic and stability properties of polygalacturonase immobilized by entrapment in different agar-based matrices.

Agar type	Activity performance		Mechanical stability		
	Product conversion[a] (mg/mL)	Reaction yield[a] (g/g)	Relative activity at 42 h[b] (%)	Swelling ratio (%)	δ[c] (°)
Gracilaria	1.657	1.070	NA	3.12	13.39
Pterocladia	1.672	1.002	NA	2.95	14.06
Gelidium	1.674	1.046	NA	3.15	14.42
Bacteriological	1.749	1.265	51.1	2.35	11.63

[a] Enzymatic reaction was performed by hydrolysis of polygalacturonic acid. Product conversion was expressed as D-galacturonic acid release (mg/mL) and reaction yield as product formation (g) relative to protein content (g).
[b] Biocatalyst was reused successively for 42 h. Activity values below 50% compared to the initial activity are considered as deactivated (NA: inactive biocatalyst).
[c] Phase angle (δ) was calculated by the viscoelastic procedure in between 0.1 and 1.0 Pa of oscillatory stress, at 30 °C and 1.0 Hz.

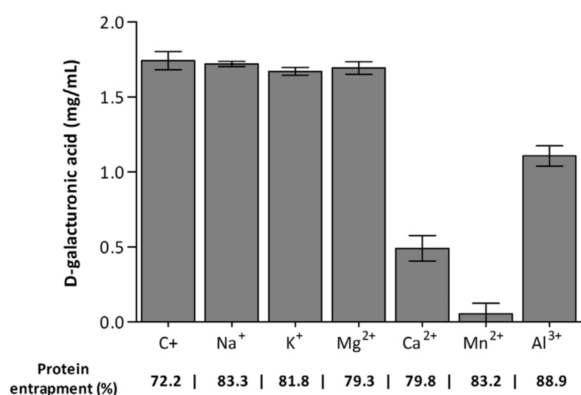


Fig. 1 – Effect of chloride salts at 200 mM on protein entrapment within bacteriological agar 2% (w/v) and hydrolytic conversion of polygalacturonic acid 0.5% (w/v) at 40 °C and pH 11. Treatment C⁺ refers to the control with deionized water.

maximum fraction of entrapped proteins (88.9%), but enzymatic activity was adversely affected because of the partial denaturalization observed by aggregation of soluble enzymes. However, Na^+, K^+ and Mg^{2+} evidenced the same catalytic performance compared to the biocatalyst without cations (C^+). So, these treatments were subjected to oscillatory procedures and reusability tests to measure the effects of cations on mechanical resistance (Table 2). The results of the dynamic test showed changes in the solidifying temperature due to the effect of the cations, which increased by 3 °C with Mg^{2+} compared to the control treatment. The destabilization of beads by melting effects started at 65 °C but at 83 °C agar reversed to the sol state, so agar exhibited its characteristic hysteresis solidifying from 32 to 35 °C and melting at 83 °C. Since the phase angle is higher at the reaction temperature (40 °C), it was markedly diminished by cation addition at both evaluated temperatures. The biocatalyst with Mg^{2+} was stiffer because the storage modulus (G′) was enhanced and the loss modulus (G″) was smaller, representing a more elastic than viscous portion when Mg^{2+} was used. The nature of Mg^{2+} also allowed hydrogen bonding between water molecules by kosmotropic effect (Illanes et al., 2012). Reusability was also an important result, relative activity after 48 h of continuous reactions was 63% using $MgCl_2$, being 1.4-fold more active than the control treatment probably due to the stability of molecular interactions between agar, cations and protein. Although Mg^{2+} at 200 mM showed positive results, lower concentrations were evaluated, and 20 mM was determined as the minimum magnesium content that allowed similar results of matrix stability (p-value > 0.05).

3.3. *Binary mixture design for protein loading optimization*

Bacteriological agar (4% (w/v)) with $MgCl_2$ 20 mM and protein solution (2.062 mg/mL) were mixed at different volume fractions according to the *simplex-lattice* quadratic model design (Table 3). Based on the correlation factors (R^2), the adjusted models explain more than 93% of the variations observed in response variables. Both components in the mixtures affected the response variables, since the porosity of agar beads is governed by the concentration of the matrix, so the pore size influences protein retention and mass transfer. On the other hand, the protein fraction makes the reaction take place.

Protein entrapment was significantly affected by the mixture ratio, and as protein loading is a correlated variable, it was also affected (p-value < 0.005). The percentage of entrapment varied in the range 75.6% (run 3)–91.1% (average value of runs 1 and 7) and further protein leaching at reaction conditions dur-

Table 2 – Effect of adding cations to bacteriological agar gel for PG entrapment. Matrix viscoelastic behavior by dynamic oscillatory procedure (1 Hz) at ramp temperature (5 °C/min).

Chloride salt (200 mM)	Solidifying temperature (°C)	δ (°) at 30 °C	δ (°) at 40 °C	G′ (Pa)	Relative activity at 48 h[a] (%)
C^+	32.0	10.41	36.99	4340	NA
NaCl	33.0	2.22	22.83	6325	NA
KCl	34.0	2.26	20.56	7615	60.5
$MgCl_2$	35.0	2.49	18.85	9526	63.0

C^+: Control without addition of chloride salts.
[a]Biocatalyst was reused successively for 48 h. Activity values below 50% compared to the initial activity are considered as deactivated (NA: inactive biocatalyst). Values are the mean of 2 replicates. Standard error was less than 8%.

Table 3 – Matrix of binary mixture experimental design for PG biocatalyst optimization varying the volumetric fraction of protein and bacteriological agar in $MgCl_2$ (Mg-agar).

Run	Components		Immobilization		Enzymatic reaction		Stability
	Protein	Mg-agar	Entrapment	Protein loading	Product conversion	Reaction yield	Relative activity at 48 h
	% (v/v)		(%)	(mg/g)	(mg/mL)	(g/g)	(%)
1	0.100	0.900	92.1	0.198	1.837	9.796	57.5
2	0.350	0.650	77.2	0.582	2.218	4.348	64.2
3	0.600	0.400	75.6	0.976	2.343	2.664	65.5
4	0.350	0.650	79.8	0.602	2.311	4.862	66.6
5	0.225	0.775	81.6	0.395	2.153	6.357	61.9
6	0.475	0.525	77.6	0.794	2.303	3.686	68.6
7	0.100	0.900	90.1	0.194	1.926	8.761	59.6
p-Value			0.0045		0.0011	0.0003	
R^2			93.46%		96.71%	98.36%	

Values are the mean of 2 replicates. Standard error was less than 12%.
Protein loading: milligrams of target protein bound per gram of matrix.

Table 4 – Reusability cycles of different immobilized related enzymes in Mg-agar.

Enzyme	Source	Cycles	Reference
β-1,4-Xylanase	*Geobacillus stearothermophilus*	3	Bibi et al. (2015)
α-Glucosidase	*Bacillus licheniformis*	6	Nawaz et al. (2014)
Pectinase	*Bacillus licheniformis*	4	Rehman et al. (2014)
Polygalacturonase	*Streptomyces halstedii*	48	This study[a]

[a] In this study a mixture of protein and gel was used at the ratio 10:90% (v/v).

ing 24 h was not significant. Besides, product conversion was favored by a larger fraction of protein, and the maximum product concentration was 2.343 mg/mL (run 3). However, the effect on reaction yield was markedly the opposite, being almost 3.48 times lower than in runs 1 and 7.

The integrative analysis of response variables indicated that using 5 times more protein content in the matrix, product conversion increased by 1.25-fold. Thus runs 1 and 7 were the most efficient biocatalyst since using the lowest protein loading it was capable of hydrolyzing pectin up to 38% (based on 5 mg/mL) to produce D-galacturonic acid. Finally, the optimum concentration of bacteriological agar was 3.6% (w/v) and protein loading was 0.196 mg/g, which suggests that the greater the agar concentration, the smaller the pore size to favor enzyme retention (Bibi et al., 2015).

The optimization of immobilization conditions by sequential experiments allowed the development of a biocatalyst that increased the reaction yield by 7.33-fold, minimizing protein loading in the agar network with similar stability properties. The recycling efficiency of PG biocatalyst showed outstanding operational stability, retaining more than 80% of relative activity at 42 h with final reusability up to 48 h. Variations of the active site of the enzyme, probably due to repeated usage or the leakage of enzyme from the matrix during several washing steps, could be the reason for decreasing catalytic activity. However, this optimized biocatalyst was exceptionally more stable than other related enzymes immobilized in similar thermogels (Table 4).

Protein immobilization techniques improve the stability of enzymes, but they generally change the kinetic behavior and decrease the reaction yield. In this study, the optimized biocatalyst allowed the production of 1.881 mg/mL D-galacturonic acid, and using free enzyme values around 2 mg/mL were achieved (Fig. 2A), being a noteworthy result because the final reaction yield just varied from 9.279 to 11.15 g/g for both systems. Also, there were minor differences based on the reaction kinetics; while the reaction with free PG took about 20 min to reach the maximum yield, with entrapped PG it took 30 min. The effectiveness factor indicates the relative importance of diffusion and reaction limitations; this value resulted in 0.714 ($0 \leq n \leq 1$) due to slight diffusional barriers in the hydrogel; however, a large content of water occluded in the gel network promoted mass transfer of substrates and products (Varzakas et al., 2005). Finally, larger limitations found in previous investigations about this enzyme immobilized in agarose supports were overcome (Ramírez-Tapias et al., 2016). Michaelis–Menten parameters for immobilized enzyme were determined by Lineweaver–Burk graph (Fig. 2B), and the results showed a maximum reaction rate (Vmax) of 0.421 μmol/min, and the Km constant was 0.03 M.

3.4. *Effect of temperature and pH on agar-PG biocatalyst activity*

The effect of pH and temperature on biocatalyst activity is shown in Fig. 3. Entrapped enzyme displayed good activity in a broad pH range from 7 to 11; the average product conversion was 1.987 mg/mL of D-galacturonic acid (Fig. 3A) and 39.7% of pectin hydrolysis. This finding demonstrated an improvement of enzyme stability because from neutral to extreme basic pH the activity remained complete; the same enzyme in soluble state showed maximum activity at pH 11, which declined to 35% at pH 7 (Ramírez-Tapias et al., 2015). Fig. 3B depicts the effect of temperature on the hydrolytic reaction; at 20 and 30 °C the biocatalyst reduced its product conversion to 43% and 80% of maximum activity, respectively. The optimum range of temperature for biocatalytic hydrolysis was 40–50 °C, and product conversion was 1.855 mg/mL of D-galacturonic acid. At 60 °C a slight decline occurred, and also thermogel beads were softer because close to 60 °C the melting process of weaker junction zones of molecules in agar starts (Norziah et al., 2006). It is worth noting that low product conversion at

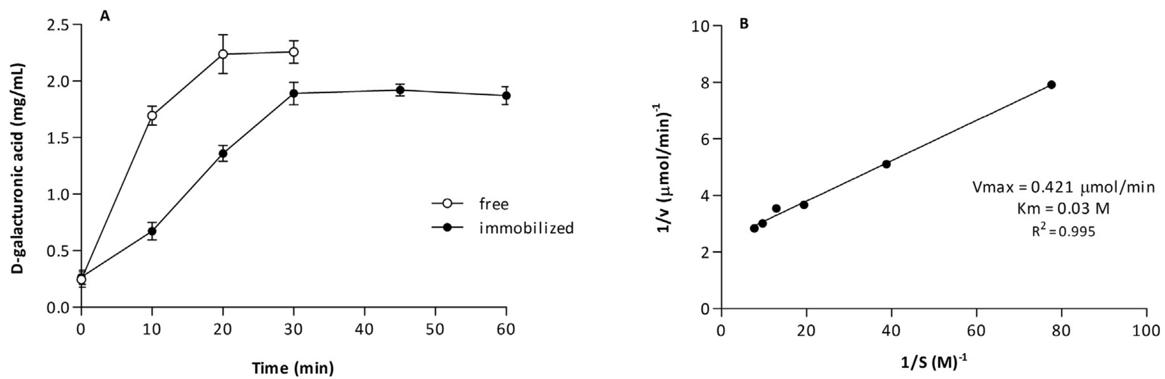


Fig. 2 – Kinetics of polygalacturonic acid hydrolysis at 0.5% (w/v). (A) Reaction time course with soluble and entrapped PG (0.2 mg of protein per mL of reaction mixture) and (B) Lineweaver–Burk fitting of Michaelis–Menten expression for enzyme kinetics.

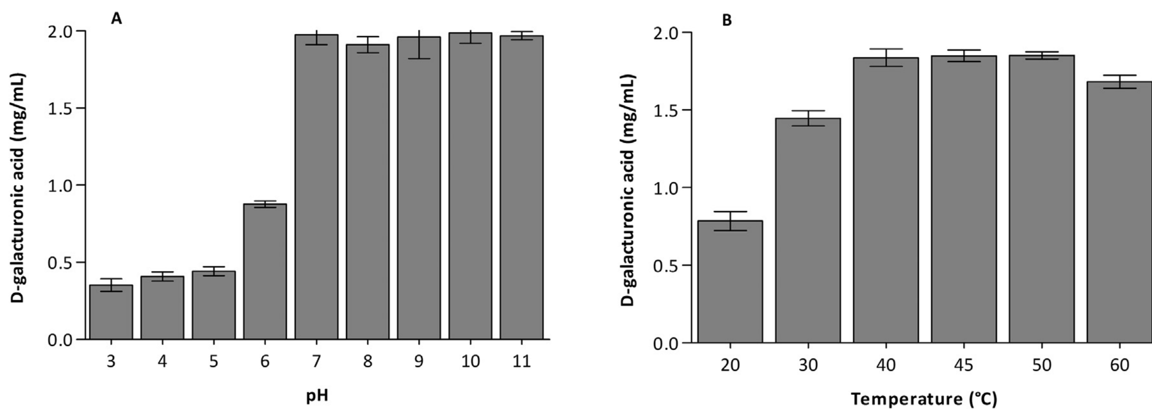


Fig. 3 – Characterization of the developed biocatalyst with PG activity. (A) Effect of pH at 40 °C, and (B) effect of temperature at pH 11.

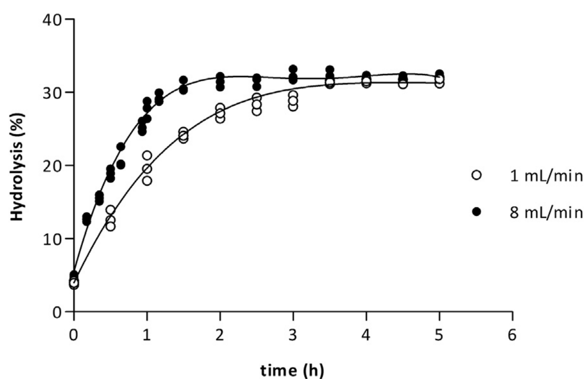


Fig. 4 – Effect of flow rate percolate recirculation of polygalacturonic acid at 0.5% (w/v) in hydrolysis reaction using a trickle-bed bioreactor.

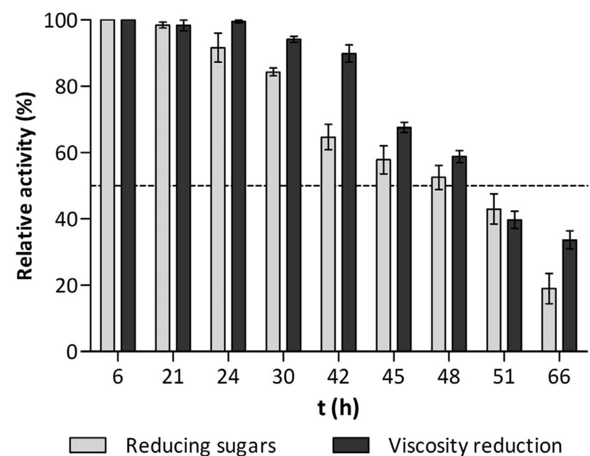


Fig. 5 – Reusability assay by consecutive batches operating percolated column with packed bed of immobilized polygalacturonase within agar beads at 10:90% (volume ratio of enzyme and agar) added with 20 mM of $MgCl_2$.

20 °C and 30 °C was probably due to hindering effects caused by tortuosity. Moreover, the storage stability at 4 °C was analyzed to ensure hydrolytic activity; the results demonstrated that the biocatalyst retained 93% of its initial activity for 225 days.

3.5. *Trickle-bed column for degradation of vegetable residues*

Packed bed columns at 25% bed voidage with biocatalyst particles operating at different flow rates of substrate were evaluated to determine parameters for practical applications in fruit and vegetable residues. Fig. 4 shows the hydrolysis ratio of polygalacturonic acid (0.5% (w/v)) during 5 h of feed recirculation at 1 and 8 mL/min; these results exhibited a significant difference with the initial reaction rates, which were 9.70 ± 0.92 and 16.90 ± 0.65 h^{-1}, respectively. At 1 mL/min 31.8 ± 0.4% hydrolysis was reached after 3 h, while operating at 8 mL/min the highest value was achieved in 1.5 h. It has been reported that the conversion rate in packed bed configurations depends on diverse factors related to biocatalyst particle features, reactant hydrodynamic behavior, interfacial interactions among gel beads, liquid and air phases, and reactor geometry (Satterfield, 1975; Zhang and Xing,

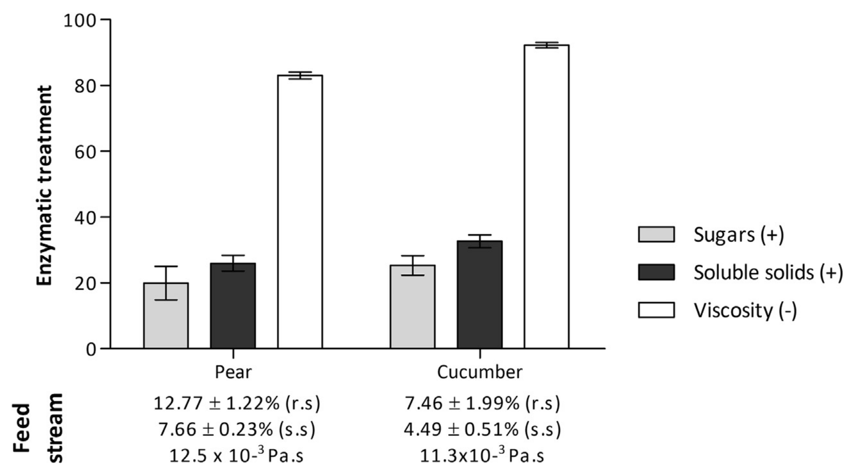


Fig. 6 – Characterization of feed streams (reducing sugars (r.s), soluble solids (s.s) and viscosity) and use of immobilized PG biocatalyst in the degradation of pear and cucumber residues. Assays were carried out at 30 °C and 3 h in a trickle-bed bioreactor operating at 8 mL/min.

2011). In this study, biocatalyst particles were uniform in size (3.52 ± 0.09 mm average diameter) and shape (sphericity factor 0.941); the hydrogel nature of agar makes beads highly wettable on the surface, so internal particle porosity, tortuosity, and the mass transfer coefficient might favor product conversion. Moreover, hydrodynamics was affected by the flow rate, which changed the liquid pattern, and liquid may gather into rivulets or the laminar film surrounding the path among particles, thus influencing liquid film thickness and contacting efficiency. However, at higher flow rate and continuous feed recirculation, more contacting events may occur between the biocatalyst and substrate promoting the reaction. Specific liquid holdup was increased by high flow rates and based on maximum hydrolysis time for both treatments; this factor was 4-fold higher for assays at 8 mL/min. So by operating the bioreactor at 8 mL/min, it could provide better performance using vegetable tissues. In addition, reusability assays at this operation condition were similar to those obtained at microscale, being 32 consecutive batches (48 h) up to 60% of relative activity (Fig. 5). Total volume processed was 0.8 L with an overall production of 1100 mg of reducing sugars. Residues derived from juice manufacture have many structural polysaccharides mainly composed of pectic substances. In this regard, the catalytic performance of entrapped PG in enzymatic liquefaction was evaluated using two different sources, pear and cucumber (Fig. 6). The degradation of these residues produced effluents with less suspended solids, a higher content of soluble solids and reducing sugars, and reduced viscosity. After enzymatic treatment, the concentration of reducing sugars was higher due to the conversion of pectin into soluble oligogalacturonides, reaching values of 15.33 mg/mL and 9.35 mg/mL for pear and cucumber, respectively. The viscosity decreased up to 92.3% due to saccharification of polymers in both assays.

According to these satisfactory results, it is possible to conclude that the trickle-bed configuration is efficient for the degradation of pectinaceous compounds and presents a potential for scale-up by addressing specific transport phenomena of substrates and products for reactor engineering and direct applications in agro-industries. This technology could add value to vegetable residues by biotransformation into fructooligosaccharides or fermentable sugars for biofuels.

4. Conclusion

Biocatalyst development by polygalacturonase entrapment in bacteriological agar was optimized in terms of mechanical stability and reaction performance. The biocatalyst was used in a batch bioprocess for the degradation of pectic compounds of pear and cucumber residues using a trickle-bed column with recirculation. This bioreactor configuration showed promising results for scale-up and reactor engineering for applications in alternative process to add value to residues generated by the manufacturing of concentrated juices. The findings reported herein demonstrated biocatalyst stability under storage conditions for a long period and outstanding biocatalyst reusability.

Acknowledgments

This research was supported by National Agency for Scientific and Technological Promotion (PICT 2013-2658 and PICT 2014-3438), National Council of Scientific and Technical Research (PIP 2014-KA5-00805) and National University of Quilmes (PUNQ 1409/15).

References

Aramberri, M., 2016. Análisis ambiental de los residuos de las industrias jugueras. El caso del Alto Valle de Rio Negro y Neuquen, Argentina. Universidad Nacional de Quilmes.

Bibi, Z., Shahid, F., Ul Qader, S.A., Aman, A., 2015. Agar–agar entrapment increases the stability of endo-β-1,4-xylanase for repeated biodegradation of xylan. Int. J. Biol. Macromol. 75, 121–127, http://dx.doi.org/10.1016/j.ijbiomac.2014.12.051.

Binder, J.B., Raines, R.T., 2010. Fermentable sugars by chemical hydrolysis of biomass. Proc. Natl. Acad. Sci. U. S. A. 107, 4516–4521, http://dx.doi.org/10.1073/pnas.0912073107.

Gulrez, S.K.H., Al-Assaf, S., Phillips, G.O., 2003. Hydrogels: methods of preparation characterisation and applications. Prog. Mol. Environ. Bioeng. 51, 117–150, http://dx.doi.org/10.1016/j.jvs.2009.12.028.

Hiteshi, K., Chauhan, S., Gupta, R., 2013. Immobilization of microbial pectinases: a review. CIBTech J. Biotechnol. 2, 37–52.

Illanes, A., Cauerhff, A., Wilson, L., Castro, G.R., 2012. Recent trends in biocatalysis engineering. Bioresour. Technol. 115, 48–57, http://dx.doi.org/10.1016/j.biortech.2011.12.050.

Jayani, R.S., Saxena, S., Gupta, R., 2005. Microbial pectinolytic enzymes: a review. Process Biochem. 40, 2931–2944, http://dx.doi.org/10.1016/j.procbio.2005.03.026.

Martins Dos Santos, V.A.P., Leenen, E.J.T.M., Rippoll, M.M., Van Der Sluis, C., Van Vliet, T., Tramper, J., Wijffels, R.H., 1997. Relevance of rheological properties of gel beads for their mechanical stability in bioreactors. Biotechnol. Bioeng. 56, 517–529, http://dx.doi.org/10.1002/(SICI)1097-0290(19971205)56:5<517:AID-BIT5>3.0.CO;2-L.

Mohammed, Z.H., Hember, M.W.N., Richardson, R.K., Morris, E.R., 1998. Kinetic and equilibrium processes in the formation and melting of agarose gels. Carbohydr. Polym. 36, 15–26, http://dx.doi.org/10.1016/S0144-8617(98)00011-3.

Nawaz, M.A., Karim, A., Aman, A., Marchetti, R., Qader, S.A.U., Molinaro, A., 2014. Continuous degradation of maltose: improvement in stability and catalytic properties of maltase (α-glucosidase) through immobilization using agar–agar gel as a support. Bioprocess Biosyst. Eng., 631–638, http://dx.doi.org/10.1007/s00449-014-1302-6.

Norziah, M.H., Foo, S.L., Karim, A.A., 2006. Rheological studies on mixtures of agar (*Gracilaria changii*) and k-carrageenan. Food Hydrocoll. 20, 204–217, http://dx.doi.org/10.1016/j.foodhyd.2005.03.020.

Osswald, T., Rudolph, N., 2014. Polymer Rheology., pp. 237, http://dx.doi.org/10.3139/9781569905234.

Ramírez-Tapias, Y.A., Rivero, C.W., Britos, C.N., Trelles, J.A., 2015. Alkaline and thermostable polygalacturonase from *Streptomyces halstedii* ATCC 10897 with applications in waste waters. Biocatal. Agric. Biotechnol., 1–8, http://dx.doi.org/10.1016/j.bcab.2014.12.004.

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 Chem. 208, 252–257, http://dx.doi.org/10.1016/j.foodchem.2016.03.086.

Rehman, H.U., Aman, A., Zohra, R.R., Qader, S.A.U., 2014. Immobilization of pectin degrading enzyme from *Bacillus licheniformis* KIBGE IB-21 using agar–agar as a support. Carbohydr. Polym. 102, 622–626, http://dx.doi.org/10.1016/j.carbpol.2013.11.073.

Satterfield, C.N., 1975. Trickle-bed reactors. AIChE J. 21, 209–228, http://dx.doi.org/10.1002/aic.690210202.

Tapre, A.R., Jain, R.K., 2014. Pectinases: enzymes for fruit processing industry. Int. Food Res. J. 21, 447–453.

Trelles, J.A., Rivero, C.W., 2013. Whole cell entrapment techniques. In: Guisa, J.M. (Ed.), Immobilization of Enzymes and Cells. Humana Press, Madrid, Spain, pp. 365–374, http://dx.doi.org/10.1007/978-1-62703-55-7.

Varzakas, T.H., Leach, G.C., Israilides, C.J., Arapoglou, D., 2005. Theoretical and experimental approaches towards the determination of solute effective diffusivities in foods. Enzyme Microb. Technol. 37, 29–41, http://dx.doi.org/10.1016/j.enzmictec.2004.06.015.

Zhang, C., Xing, X., 2011. 2.23 — Enzyme bioreactors. In: Comprehensive Biotechnology, second ed. Elsevier B.V, http://dx.doi.org/10.1016/B978-0-08-088504-9.00099-4.