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Use of two different carriers in a packed bed reactor for endopolygalacturonase production by a yeast strain

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Abstract

A packed bed reactor (PBR) design was tested for the purpose of continuous pectinase production with yeasts, as a possible alternative to the traditional batch process using fungal cultures. Two different carriers – a porous glass (Siran) and a cellulosic carrier obtained from spent grains (barley) – were used to immobilize *Kluyveromyces marxianus* CCT 3172, a yeast strain secreting endopolygalacturonase. To improve cell distribution throughout the column, part of the outflow was recycled. Cell loads of 0.204 and 0.247 $g_{biomass}/g_{carrier}$ were obtained at the top and bottom of the PBR with spent grains, respectively. Using the PBR with Siran as the immobilization support, 0.071 $g_{biomass}/g_{carrier}$ was the biomass load at the top of the column while at the bottom a value of 0.147 $g_{biomass}/g_{carrier}$ was found. The highest value for pectinase volumetric productivity (P_V = 1.68 U/ml h) was achieved in the PBR with Siran for a D = 0.260 h^{-1} and a glucose concentration on the inlet of S_{in} = 40 g/l. Both carriers were suitable for pectinase production. The best results were obtained with a high and uniform biomass concentration in the column, together with high dilution rates and total glucose consumption.

Keywords: Continuous reactor; Immobilized yeast cells; *Kluyveromyces marxianus*; Pectinase; Siran; Spent grains

1. Introduction

Pectinases are a group of enzymes acting on pectin and other pectic substances found in vegetable tissues. Pectin consists of a α-1,4 polymer of D-galacturonic acid; the main chain is 60–90% methylated and includes rhamnose units and side chains of arabinan, galactan and arabinogalactan. This complex polymer has a structural, scaffolding function in the primary cell wall and in the middle lamella of plant tissues [1]. In natural habitats, several microorganisms secrete different pectolytic enzymes to invade the cell walls and thus grow on the rich substrates found in plants. In the industrial world, pectinases have found use in any process that deals with extracting juices from fruits and vegetables and in the processing of plant tissues. Some examples are: in fruit juice clarification and viscosity reduction, in preliminary grape treatment in wine industries, in tomato pulp extraction, in chocolate and tea fermentation, vegetable waste treatment, fibre degguming in textile and paper industries [2]. *Aspergillus niger* is currently the only microorganism used for pectinase industrial production. It secretes a mixture of pectolytic enzymes (polygalacturonases, polymethylgalacturonases, pectin lyases and pectin esterases) together with other degrading enzymes such as arabinofuranosidases and amyloglucosidases [3]. In fact, commercial pectinase is a blend of enzymes. This can be useful due to the complexity of plant tissues and all the different chemical bonds to hydrolyse, but in some industrial cases a specific type of pectinase or a specific blend is needed [4]. The yeast strain *Kluyveromyces marxianus* CCT 3172 was selected from a cocoa fermentation as a good endopolygalacturonase producer [5,6].

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Continuous production of an endopolygalacturonase from yeast would be an interesting alternative to the current fungal batch production. To increase the productivity of continuous processes, high cell density systems can be used. Some carriers have been successfully tested for yeasts and bacteria immobilization, namely porous materials such as porous glass and ceramic, synthetic polymers, cellulosic fibres and cellulose derivatives, activated charcoal, artificial polymers and gel matrixes as k-carrageenan, Ca and Ba alginate, and pectate [7–13].

In this work, a packed bed reactor (PBR) was chosen for pectinase production. Two carriers were tested for cell immobilization: a commercial porous silicate glass (Siran) and a recently tested cellulosic support, prepared from spent grains, a by-product of the brewing industry. Although Siran has been used with satisfying results for cell and enzyme immobilization [14–18], its high cost can be a drawback for industrial productions. The cellulosic carrier has already been tested with a brewing strain (*Saccharomyces uvarum*) and was found to be very efficient, having a high yeast loading capacity, together with an easy preparation, reusability, availability and an inert, non-toxic nature [19,20].

2. Materials and methods

2.1. Media and strain

Wild-type *K. marxianus* CCT 3172 used to inoculate both continuous reactors was pre-grown in 200 ml (in 500 ml Erlenmeyer flasks) semi-synthetic media at 30 °C, 120 rpm for 24 h.

The semi-synthetic media (SS) for yeast growth included (g/l): 5 K_2HPO_4, 2 $(NH_4)_2SO_4$, 0.4 $MgSO_4{\cdot}7H_2O$, 1 yeast extract, and different glucose concentrations (20, 40, 80).

2.2. Carriers

The carriers used were porous silicate glass beads (Siran, QVF Engineering, Mainz, Germany, with bead diameters 2–3 mm and porosity 50–65%) and a cellulosic support prepared from spent grains, a by-product of the brewing industry. Siran beads were washed in distilled water and autoclaved twice before the first use. The steps followed to obtain the carrier from spent grains are described in Brányik et al. [19]. Hundred grams of dry spent grains was submitted to an acid treatment (1500 ml of a 3% (v/v) HCl solution, 60 °C, 2.5 h) in order to hydrolyse the starchy endosperm and embryo of the barley kernel. After cooling, it was washed with water and dried. The remaining solids (ca. 30 g) were partially delignified with NaOH (500 ml of a 2% (w/v) solution, 30 °C, 24 h, 120 rpm). Several washing steps with water were needed to reach a neutral pH. After drying, the carrier (ca. 10 g) was ready to be used.

2.3. Enzyme assays

Endopolygalacturonase activity in the reactor effluent was assessed using the method described by Honda et al. [21]. One unit (U) is defined as 1 μmol of galacturonic acid released after 1 min of hydrolysis of polygalacturonic acid in the presence of the enzyme at 40 °C, pH 4.1.

2.4. Analytical methods

Glucose concentration was determined by the DNS method for reducing sugars quantification [22]. For pectinase activity determination, the samples from reactors were centrifuged, filtered and then dialyzed with a 14,000 MWCO membrane (Medicell International, London, UK) against cold distilled water for 16 h. Lactose was used as a tracer for hydrodynamic studies. Its concentration was determined using the specific enzymatic kit for detection of lactose and D-galactose from Boehringer Manheim/Roche.

2.5. Cells contact angle measurements

A solution of 20 g/l of agar and 10% (v/v) glycerol was cast on a microscope slide. Cell samples were taken from a continuous reactor outflow and washed with a solution with increasing ethanol concentration (10, 20 and 50% (w/v)). One millilitres of a cell suspension in 50% ethanol with an Abs 600 nm = 2.0 was spread on the solidified agar and glycerol and allowed to dry. This step was repeated four times [23]. Contact angles were measured at room temperature using water, formamide and α-bromonaphthalene in a contact angle apparatus (Kruss GmgH, Germany) by the sessile drop technique. The total surface tension (γ^{tot}) and its components (γ^{LW}, γ^{+}, γ^{-}, γ^{AB}), the values of the free energy of interaction between cells and water ΔG^{tot}_{sws} and the components (ΔG^{LW}_{sws}, ΔG^{AB}_{sws}) were calculated according to van Oss et al. [24].

2.6. Scanning electron microscopy (SEM)

A sample of biocatalyst was taken from the CSTR reactor, washed with water and with a solution with increasing ethanol concentration (10, 25, 50, 75, 90, 100%). It was allowed to dry for 5 days in an exicator and covered with a thin gold layer to allow for SEM observation.

2.7. Biomass quantification

The free biomass concentration at the reactor's outlet was measured by reading the absorbance of samples at 600 nm and then converting this value to dry weight per volume using an appropriate calibration curve.

At the end of the reactor operation, samples of the biocatalyst were withdrawn from different heights of the fixed bed. For the spent grains, the carrier with adsorbed yeast cells was gently washed with 200 ml of distilled water. The

resulting suspension was filtered and washed with water; the filter paper with carrier and immobilized cells was dried at 105 °C for 16 h. A washing step (during 24 h at 120 rpm) with a 3% (w/v) NaOH solution released the attached biomass. After washing with distilled water and filtering, the biomass free carrier was dried at 105 °C for 5 h. The biomass weight was calculated as being the weight difference of the dry carrier before and after the NaOH washing [20]. Corrections of the biomass weight for the losses of carrier itself were carried out by blank experiments with clean carrier.

For the Siran carrier, the biocatalyst samples were dried for 48 h at 105 °C and weighed. The biomass was then combusted at 550 °C for 2 h and the residual material was weighted. The biomass load (X_i) was calculated as the weight difference between the dry biocatalyst and the clean carrier. Biomass loads were expressed in $g_{biomass}/g_{carrier}$.

2.8. Bioreactor start-up and operation

Before operation, the reactor was sterilized with a hypochlorite solution (3 days). After this period of time, 10 reactor volumes of sterile distilled water were used to wash the column. The packed bed reactor (PBR) was a ''Perspex'' column, with height to internal diameter ratio (H/D_i) of 12 and an operation volume of 310 ml.

When using the spent grains as cells support, 25 g of sterile dry carrier was aseptically inserted in the column and then inoculated with a pre-grown yeast culture. After 24 h of batch growth in the reactor, continuous operation started by feeding SS medium with 40 g/l glucose at the bottom of the column. A recycle rate of 40 ml/h was used during the entire operation time, by re-introducing a part of the outflow to the bottom of the column.

When Siran was used as carrier, approximately 150 g of sterilized dried beads was introduced into an Erlenmeyer flask with SS medium (20 g/l glucose) and then inoculated with a pre-grown 100 ml culture. After 24 h of incubation at 30 °C, 120 rpm, the Siran beads were transferred into the reactor which was then filled with fresh SS media (40 g/l). Continuous operation started 24 h after the transfer. In this experiment, the recycle rate was 200 ml/h. For all the operating conditions, a minimum of five residence times was allowed before changing to another set of conditions.

The dilution rate was considered to be D (h^{-1}) = volumetric feed rate/total working volume of the reactor. All assays were performed at 25 °C.

3. Results and discussion

3.1. Cell immobilization

The cellulosic carrier from spent grains is both irregular in shape and non-homogeneous in chemical composition, originating ''active sites'' preferably colonized by yeasts [19]. Siran beads are composed of silicate glass with an open pore structure, relatively uniform in size but with an irregular orientation and shape (Fig. 1).

Contact angles measured by the sessile drop technique [23] were used to calculate the yeast surface properties according to van Oss et al. [24,25]. The values of total surface tension and free energy of interaction for *K. marxianus* CCT 3172 cells, for base-treated spent grains carrier (T. Bányik, unpublished results) and for Siran [26] are presented in Table 1. The high positive ΔG^{tot}_{sws} value found for the Siran carrier is associated with its surface

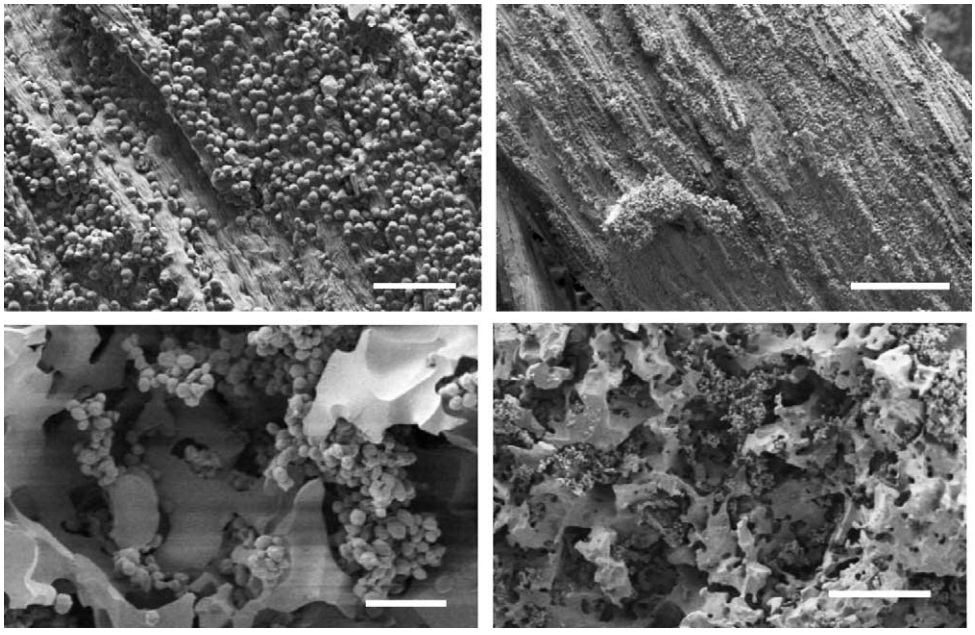

Fig. 1. SEM photos. Top images – cells on spent grains. Bottom images – cells on Siran beads. Left images – bar corresponds to 20 μm; Right images – bar corresponds to 100 μm.

Table 1
Surface tension γ^{tot} and free energy of interaction for cells *K. marxianus* CCT 3172 grown in continuous culture (ΔG^{tot}_{cwc}), for the base-treated spent grains carrier and for Siran (ΔG^{tot}_{cwc})

	γ^{tot}	ΔG^{tot}_{sws}	ΔG^{tot}_{cwc}
Cells from continuous culture	61.0	–	22.9
Base-treated carrier	41.5	−57.7	–
Siran [26]	56.1	119.8	–

All values in mJ/m^2.

hydrophilic character, and the negative value determined for the cellulosic carrier shows the presence of highly hydrophobic areas. From these values, the free energy of interaction between *K. marxianus* cells and the two different carriers was calculated [24]. The values of $\Delta G^{tot}_{cws} = 1.86 \pm 8.70\,mJ/m^2$ (the wide error bar is associated with the non-uniform surface composition of the cellulosic carrier) for the interaction cells–water–spent grains and $\Delta G^{tot}_{cws} = 55.5 \pm 5.0\,mJ/m^2$ for cells–water–Siran showed an energetically less favourable adhesion between yeast cells and the surface of Siran beads. These results are in agreement with the observed behaviour of cells in the two different biocatalyst beds (Fig. 1). The Siran carrier seems to immobilize cells only by spatial retention on its porous structure.

It has an open pore matrix with an important presence of void spaces, allowing liquid motion and cell percolation through the fixed bed. This originates a difference in the biomass load at different heights in the column (for distances from the top of 0, 8, 18 and 31 cm biomass loads (X_i) of 0.072, 0.054, 0.065 and 0.143 $g_{biomass}/g_{carrier}$, respectively, were found at the end of the reactor operation). As it was not possible to withdraw biocatalyst samples during reactor operation, four separate assays were performed using a smaller column and 1/10 of the Siran weight (approximately 15 g). A fixed dilution rate of $D = 0.33\,h^{-1}$ was used and the four assays were stopped at 120, 240, 408 and 672 h for immobilized biomass quantification. After 240 h of continuous operation, biomass load reached a stationary value (approximately 0.070 $g_{biomass}/g_{carrier}$) (Fig. 2). From these experiments, it can be assumed that the immobilized biomass inside the PBR was nearly constant after the first 240 h of reactor's operation.

When the cellulosic carrier was used, cells were attached to the irregular surface not only by retention inside fibres, threads and crevices, but also by cell–surface adhesion due to different interaction forces. Moreover, the spent grains packing worked as a "filter layer" giving rise to zones of local accumulation of yeasts. The CO_2 bubbles formed during the experiment were to account for a sponge-like bed structure. The gas was periodically liberated through the top of the column, hence mixing the packed bed and releasing parts of the biomass deposits. At the end of the operation time, the PBR reactor had a biomass load of 0.247 $g_{biomass}/g_{carrier}$ at the bottom and 0.204 $g_{biomass}/g_{carrier}$ at the top showing a higher homogeneity in the bed colonization than for the PBR with Siran.

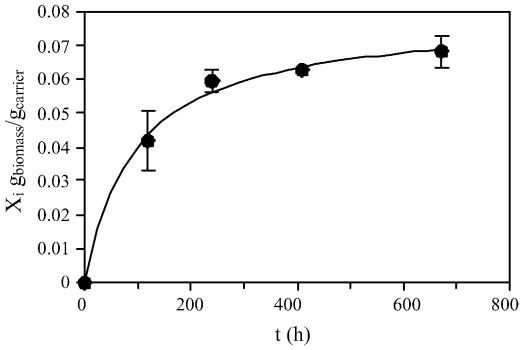

Fig. 2. Immobilized biomass load (X_i) in the Siran packed bed (assays with the smaller column reactors).

3.2. Hydrodynamic studies (residence time distribution)

Experiments were carried out to study the hydrodynamic behaviour inside the PBR for both tested carriers. Lactose was used as a tracer since this particular strain of *K. marxianus* is unable to metabolize it efficiently. Besides, glucose is not totally consumed during the experiments with the tracer, which acts as a catabolic repressor to lactose consumption.

A lactose concentration step was imposed at steady-state conditions for both reactors. The residence time distribution is presented in Fig. 3a and b. When the Siran carrier was used, the best fit for experimental tracer response seems to be an ideal CSTR. For the axial dispersion model, Peclet number is the fitting parameter, defined as uL/D (u being the linear velocity, L the height of the biocatalyst bed and D the axial dispersion coefficient); $Pe = \infty$ corresponds to ideal plug flow, and $Pe = 0$ to ideal mixed flow. The low Peclet (uL/D) value and the poor correlation obtained ($Pe = 1.07 \pm 1.01$, $r^2 = 0.87$) for the fitting with the axial dispersion model also suggest a nearly perfect mixing inside this biocatalyst bed. In fact, as already discussed above, this packed bed has an open pore matrix with a high void volume, which is likely to have a low resistance to mass transfer and fluid motion (Fig. 1).

Using spent grains as cell carrier, a good agreement was found both for the axial dispersion model with a *Pe* number of 5.51 ± 1.01 ($r^2 = 0.98$) and for a series of three CSTRs. From this, it can be implied that the reactor mixing is not negligible, however it has a lower extent than in the packed bed with Siran. The fibrous structure of the spent grains bed, working like a thick filter media, justifies the lower mixing found for the experiment with this cellulosic carrier.

A recycle was used in both situations but in the case of the spent grains bed its volumetric flow was five times lower than the one used with Siran to avoid fluidization of the lighter spent grains carrier. This fact can also account for the differences in the mixing characteristics.

3.3. Pectinase production

During the operation of both packed bed reactors, the free biomass concentration, glucose concentration and pectinase

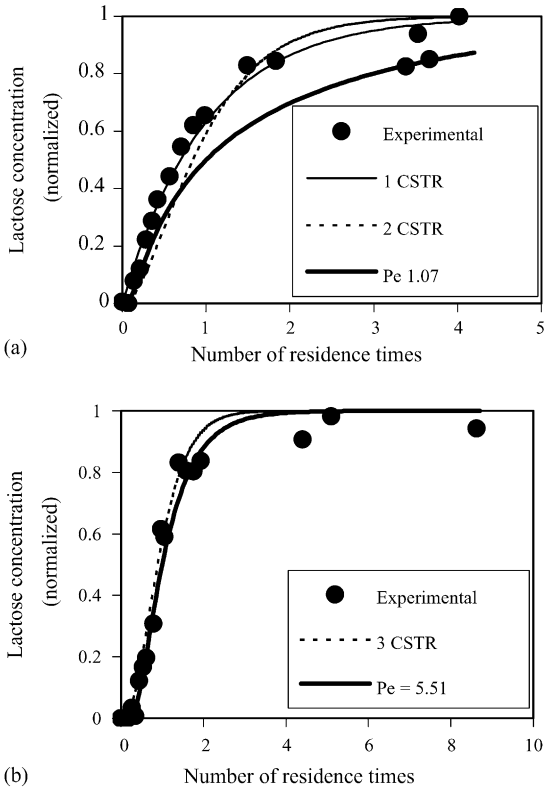

Fig. 3. Tracer response and model fitting for the packed bed with Siran (a) and for the packed bed with spent grains (b).

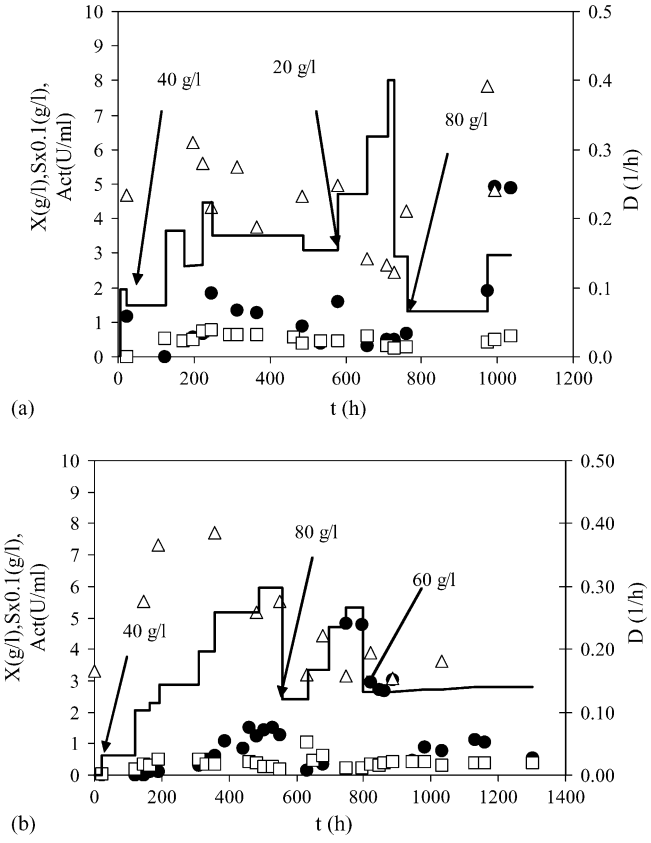

Fig. 4. Evolution of glucose concentration S (circles), free biomass concentration X (squares), pectinase activity Act (triangles), and dilution rate D (—) during PBR operation with spent grains (a) and Siran (b). The arrows mark the changes in the inlet glucose concentration.

activity were measured at the reactor's outlet (Fig. 4). Similar values were found for pectinase activities: in the PBR with spent grains the values oscillated between 2.45 and 7.82 U/ml, while in the PBR with Siran the pectinase activities ranged from 3.08 to 7.72 U/ml.

Using the PBR with spent grains, the volumetric productivity (P_V) values range from 0.505 to 0.97 U/ml h and increase with the dilution rate (Fig. 5). For the Siran packed reactor, productivity also increased with the dilution rate and ranged from 0.39 to 1.68 U/ml h (Fig. 5). Comparing all the tested situations, the highest value of P_V (1.68 U/ml h) was found for the Siran bed working with a $D = 0.260\ h^{-1}$ and an inlet sugar concentration of 40 g/l.

It must be pointed out that, although higher enzyme activities are obtained for higher inlet substrate concentration, this only remains true when total substrate consumption occurs. In fact, increasing D with high glucose concentrations in the inlet is useless, since it results in a higher sugar concentration at the outlet. This was not noticeable for S_{in} = 20 g/l with the tested dilution rates. In the Siran PBR glucose total consumption was achieved only until $D = 0.196\ h^{-1}$ at S_{in} = 40 g/l.

As no sample was taken from the packed bed throughout the operation time, the values of the immobilized biomass were estimated using the biomass accumulation trend from Fig. 2. In the case of the Siran bed after the first 240 h of continuous operation, the average biomass concentration was considered constant and equal to 34 g/l of reactor volume. A similar behaviour of the bed colonization was assumed for the spent grains, and therefore the biomass concentration was considered also constant (18.5 g/l) after 240 h of the reactor's operation.

Although the packed bed with Siran had a higher biomass concentration, it was unevenly distributed throughout the column. In fact, the bottom of the reactor (about 15–20% in

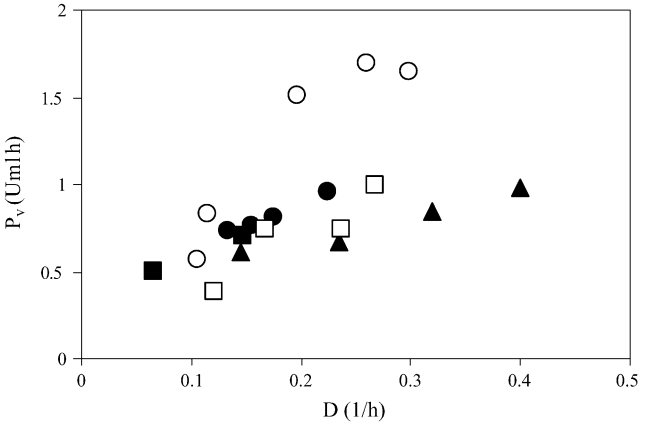

Fig. 5. Pectinase volumetric productivity (P_V) for different inlet concentrations of glucose. For PBR with spent grains the data are shown as filled symbols and for PBR with Siran data as empty symbols. Triangles correspond to S_{in} = 20 g/l, circles to S_{in} = 40 g/l, squares to S_i = 80 g/l.

terms of reactor volume) had a large and, in some places, compact yeast accumulation (estimated as 25–30% of the total biomass in the column) that probably imposed a high mass transfer resistance for glucose and pectinase. The values found for P_V at S_{in} = 80 g/l are lower than for S_{in} = 40 g/l. These contradictory results can also be a consequence of the uneven biomass distribution in the Siran column. As the assays with S_{in} = 80 g/l were performed after the ones with S_{in} = 40 g/l, the biomass accumulation at the bottom of the column was increased and these clustered cells are probably less efficient in converting sugar to pectinase due to nutrient restrictions imposed by the high mass transfer resistance.

Both carriers can be successfully used for cell immobilization although, to increase the biomass loads and improve its distribution throughout the column, the surface of the Siran carrier should be activated. In fact, a pre-treatment with trimethylchlorosilane favourably changes its surface properties for yeast attachment, as a six-fold decrease is found for the free energy of interaction (ΔG^{tot}_{sws}) [25].

From the performed assays, it can be concluded that the best results for pectinase production can be achieved using a high X_i in the column and a high D, together with total glucose consumption.

The better biomass distribution throughout the column obtained in the spent grains PBR and the advantages related to this available by-product from brewing industries make it a suitable option as a cell carrier.

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