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Bioethanol production by reusable *Saccharomyces cerevisiae* immobilized in a macroporous monolithic hydrogel matrices

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HIGHLIGHTS

- *S. cerevisiae* is immobilized inside monolithic macroporous gel.
- The simple procedure is amenable to the industrial production of the supported catalyst.
- The yeasts are efficiently retained in hydrogels monoliths, presenting excellent mechanical properties and allowing high cell viability.
- The hydrogel-yeast complexes achieved similar ethanol yield and productivity than free yeast.
- Immobilized yeast catalyst becomes a reusable heterogeneous catalyst, easily to recover and able to isolate valuable strains with low ecological impact.

Abstract

Performance of yeasts on industrial processes can be dramatically improved by immobilization of the biocatalyst. The immobilization of *Saccharomyces cerevisiae* inside monolithic macroporous hydrogels were produced by *in-situ* polymerization of acrylamide around a live yeast suspension under cryogelation conditions. Preculture of the yeasts was not necessary and this innovative and simple procedure is amenable to scaling-up to industrial production. The yeasts were efficiently retained in monolithic hydrogels, presenting excellent mechanical properties and high cell viability. Macroporous hydrogels showed a fast mass transport allowing the hydrogel-yeast complexes achieved similar ethanol yield and productivity than free yeasts, which is larger than those reached with yeasts immobilized in compact hydrogels. Moreover, the same yeasts were able to maintain its activity by up to five reaction cycles with a cell single batch during fermentation reactions.

Keywords: Bioethanol production, reusable catalyst, heterogeneous
Catalysts

1. Introduction

Each day, millions of people in the entire world use fuel for transportation, heating, cooking and more than a hundred industrial activities. Globally, bioethanol has already been introduced as the direct replacement of fossil oil in Brazil, USA and European countries. Constantly expanding bioethanol production reached 85.2 billion liters in 2012, 95 billion of gallons in 2014 and it is expected to triple this production by 2020 (Cui and

Cheng, 2015). Consequently, the development of new and efficient way to produce eco-friendlier and economically competitive fuels results imperative. Moreover, concerns about global warming and diminished of world fossil-oil reserve has promoted government politics in numerous European and American countries which plan substitute a 20 % gasoline cut of conventional fossil fuels with alternative fuels by the year 2020 (Yüksel and Yüksel, 2004). The fermentation process involves the conversion of sugars to ethanol by yeast. However, the classical fermentation process has several drawbacks, for instance, it could be prone to microorganisms contamination and requires a complex yeast recovery procedure which limiting its reuse.

It has been verified that immobilization yeast systems, in a solid porous matrix, offers numerous advantages over yeast suspension systems in terms of ethanol production, the yeast activity stability, storage and cell viability (Razmovski and Vučurović, 2011; Saraydın et al., 2002). Among them, the possibility of reusable yeast could improve substantially not only the economic field, but the entire global process, providing operational versatility and whole new prospects in terms of instrument and control topics. Likewise, pure *Saccharomyces cerevisiae* strains could be contaminated during reaction with wild yeasts which produces unwanted products and reduces the bioethanol yield (Basílio et al., 2008). In the industrial process, with free yeast suspension, the elimination of contaminant involves complex sterilization or use of unstable antibiotics. However, the isolation of the yeast inside a monolithic hydrogel matrix allows avoiding contamination. Moreover, mutant strains of yeast can produce higher yields (Shiroma et al., 2014), resist high temperature and high alcohol content stress (Abreu-Cavalheiro and Monteiro, 2013). Nevertheless, it is advisable to maintain the strain isolated to avoid cross contamination with wild strains which imply a loss of the expensive strains. The implementation of monolithic immobilized yeast system for alcoholic fermentation is attractive and speedily expanding research area because of their technical and economic advantages compared with the free cell system (Razmovski . and Vučurović, 2011; Razmovski and Vučurović, 2012). Several support materials for cell immobilization have been reported including calcium alginate, κ-carrageenan gel and γ-alumina (Pierre, 2004; Polk et al., 1994). In the best of our knowledge, none of these materials applied in fermentation process could offer such versatility, toughness, and truly possibilities of reutilization as polyacrylamide hydrogels. All the listed properties are mainly due to the polymeric three-dimensional network which absorbs and retains water without dissolving and resists the biological environment. The gels result in a suitable matrix because of they

can immobilize not only chemical compounds but also enzymes and cells (Sheldon, 2007). This aptitude is due to their hydrophilic character, high water content and permeability (Rivero et al., 2015). Moreover, highly cross-linked networks provide, for the biological system, a microenvironment similar to that *in vivo*. Monolithic macroscopic gels can be inserted and removed from the reactor mechanically becoming yeast reuse a simple process. Additionally, to avoid mass transfer limitations, the monolithic gels should contain macropores where mass transport of reactants/products occurs at the same rate than in free solution.

It has been shown that it is possible to fabricate macroporous polyacrylamide gels by cryogelation (Ozmen et al., 2007; Rivero et al., 2015). In the present work, a series of macroporous monolithic polyacrylamide hydrogels with different *S. cerevisiae* load (pAAm-Sc) were prepared via free radical polymerization technique around the yeast suspension (physical entrapment). In order to generate macroporous hydrogels which facilitate not only nutrient diffusion but also reactant-products interchange, the standard polymerization method was modified using a cryopolymerization technique (Zhao et al., 2010). The cryopolymerization was performed at freezing temperature, where ice crystals act as a template and allows the formation of highly reticulated macroporous structure. It is noteworthy that live yeast cells remain active under the chemically aggressive polymerization conditions and the physically aggressive freezing conditions, not requiring the use of cryoprotectors (Coutinho et al., 1988), or polymer barriers as it has been used with similar procedures (Perullini et al., 2005).

The effects of the cells loading, immobilized cell efficiency, cell viability and reusability on the reaction yield on the entrapped *S. cerevisiae* were studied following the kinetic behavior of consecutive cycles of fermentation in batch reactions systems for free and immobilized yeast. Important parameters such as ethanol yield, productivity, sugar consumption and release biomass were reported for each reaction cycle. High ethanol yield and cells viability is observed during consecutive five fermentation cycles. The results suggest that the simple procedure described could be used to prepare reusable isolated biocatalysts for bioethanol production. We believe that, monolithic macroporous hydrogel-yeast catalyst becomes a versatile, biocompatible and reusable heterogeneous catalyst, as well as enable simplified downstream processing, able to isolate valuable strains; achieving a more eco-friendly and economically convenient process, without radical modifications of equipment and devices involve in the classical operation mode.

2. Materials and methods

2.1. Materials

All chemicals were of reagent or analytical grade and purchased from Sigma-Aldrich. *Saccharomyces cerevisiae* (industrial quality, Ethanol Red®, Leaf) was provided by Bio4 (Bioetanol Río Cuarto S.A).

2.2. Polymerization

Acrylamide (AAm, 0.5 M) was used as monomer to produce the hydrogel with N,N-methylenebisacrylamide (BIS, molar ratio 0.02:1) as a crosslinking agent. The radical polymerization is initiated by a redox initiator system ammonium persulfate (APS, 10 mg/l) and tetramethylenediamine (TEMED, 10 µl/ml). Phosphate buffer (PBS, 4.0 ml, 0.1 M, pH 7.0) was used as solvent. The vinyl monomers and ammonium persulfate were dissolved in PBS and this solution is called pre-gelling solution (PGS). This PGS was equilibrated at 0 °C for 60 min in a thermostatic ice-water bath. The yeasts were hydrated in PBS for 1 h prior incorporated to PGS. Five different quantities of the yeast were dispersed in PBS (pre-hydrated yeasts). These dispersions were added to the PGS in order to obtain catalyst with 25, 50, 75, 125, 250 mg of immobilized yeast per liter of PGS. After, these dispersions were stabilized at 4° C prior to add the TEMED solution (0.2 ml, 10 % v/v).

2.3. Cryogelation

The polymerization mixture was transferred into test tubes, TEMED was added and, after sealing, the hydrogels were refrigerated at -18 °C for 24 h to form ice crystals that template the macropores of the hydrogel. After polymerization, the hydrogel were left for 4 h, at room temperature to thaw the ice. Then, hydrogels were cut (capsules form) and washed several times with phosphate buffer (0.1 M, pH 7.0) and stored at 4 °C in buffer solution. Hydrogels without yeast were also synthesized, under the same conditions, in order to use it as a standard of comparison in the characterization of these materials.

2.4. Yeast loading studies

To test the effect of cell loading on the ethanol yield, amounts of *S. cerevisiae* were varied between 25 to 250 mg/l. Five different yeast containing hydrogels were made: pAAm-Sc-1, pAAm-Sc-2, pAAm-Sc-3, pAAm-Sc-4 and pAAm-Sc-5 with yeast loadings of 25, 50, 75, 125 and 250 mg Sc/l, respectively. For comparison, a suspension of free *S. cerevisiae* (25 mg/l) stored at 4°C was used.

2.5. Immobilization efficiency of yeast in hydrogel capsules

The amount of cells immobilized was calculated by subtracting the number of cells in the washing solution after 24 h, from the original number of cells added. Cells immobilization

efficiency was defined as the weight percentage of yeast in a hydrogel that has been immobilized (Equation 1).

$$\%\eta_I = \frac{W_o - W_w}{W_0} x\ 100 \qquad \text{Equation 1}$$

Where: W_o, W_w were the mass of pAAm-Sc initial and after 24 h of washing respectively. The data reported is the average of three measurements.

2.6. Cell viability of free and immobilized cells

Cell viability quantifies the overall number of live cells, based on a complete sample of cells. Live cells refer to all living cells susceptible or not to reproduce (Hinshelwood, 1944). Methylene blue stains dead cells cytoplasm, living cells are not colored by the dye (Bonora and Mares, 1982). Methylene blue staining over hydrogel discs was used to determine cell viability immobilized and free yeast. Microbial counts were performed on 50 µm x 50 µm fields and analyzed by optical microscope binocular Arcane X57 107E with a 40X objective. The cell counting was carried out using the software *Motic Image Plus 2.0. L.M*®. Immobilized pAAm-Sc yeasts were previously hydrated in phosphate buffer for 24 h. Statistical cell count was performed using a population of 10 arbitrarily selected disks. Cell viability percentage was calculated as the ratio between living cells population and the total population of cells. For comparison, free yeast viability was quantified by the same method with a Neubauer chamber with 1:100 dilutions (Mills, 1941). Each experiment was made in triplicate.

2.7. Swelling capacity assays

The capacity of hydrogels to absorb a certain amount of water is known as swelling capacity (Baker et al., 1992). The rate of swelling defines the swelling kinetics. The capsules of pAAm hydrogels containing different cell loadings were studied. To analyze the swelling kinetic, dry gels were incorporated and removed from buffer phosphate solution (pH 7.2, 25 °C) at regular time intervals, were dried superficially, weighed and reincorporated in the same solution. The mass and the radii of hydrated capsule hydrogels were measured after each time interval. The same procedure was repeated until each sample achieved a constant weight.

To investigate swelling behavior under different ethanol concentration, dried hydrogels were immersed in water-ethanol mixtures with composition ranging from 10 to 90 % v/v. The mixtures were kept at room temperature for 24 h to allow the hydrogels reach

equilibrium. Afterward, the hydrogels were removed from the solution and blotted with wet filter paper for removal of the excess solvent on hydrogel surface; then they were weighed. The swelling percentage in water was calculated at different times according to Equation 2:

$$\%S_w = \frac{W_{(t)} - W_o}{W_o} x100 \qquad \text{Equation 2}$$

Where: *W(t)* represents the weight of hydrogel swollen state at time t and *Wo* is the weight of the dry hydrogel.

Since ethanol is produced during fermentation, the gel will interact with ethanol/water mixtures. To investigate swelling behavior under different ethanol concentration, dried hydrogels were immersed in water-ethanol mixtures with composition ranging from 10 to 90 % v/v. The mixtures were kept at room temperature for 24 h to allow the hydrogels reach equilibrium. Afterward, the hydrogels were removed from the solution and blotted with wet filter paper for removal of the excess solvent on hydrogel surface; then they were weighed.

The equilibrium swelling percentage in different ethanol solutions was calculated as follow:

$$\%\, S_{EtOH} = \frac{W_{(EtOH)} - W_o}{W_o}\, x\, 100 \qquad \text{Equation 2}$$

Where: $W_{(EtOH)}$ represents the weight of swollen hydrogel in a specified water-ethanol mixture and *Wo* is the weight of the dry hydrogel.

2.8. Mechanical assays: Elastic-viscoelastic behavior by uniaxial compression assay

Uniaxial compression measurements were performed on individual capsule hydrogels in swollen states at room temperature. The homemade instrument used for this measurement was described in preceding studies (Martínez et al., 2015) and allowed to measure the stress (σ) vs. strain (ε) curve which provides important information about the behavior of elastics hydrogels. Furthermore, the elastic module (E) was calculated from the initial slope of this graphic. The measurements were performed three times and the results informed are the average.

2.9. Thermal analysis of immobilized yeast by differential scanning calorimetric (DSC) and thermogravimetric assays (TGA)

A differential scanning calorimeter equipped with a cooling device (Netzsch DSC-204-F1-Phoenix), was used to measure the phase transition of water adsorbed on the polymer samples: pAAm-Sc-4 and pAAm, under high purity N_2 flow. All the samples were previously swollen in distilled water until swelling equilibrium state was reached. The DSC curves were obtained by sample cooling (10 °C/min) from room temperature to –30 °C using liquid N_2, followed by a reheating at a scanning rate of 10 °C/min until reach 25 °C. The measurement was repeated five times with the same protocol to check the reproducibility. The experimental error of these measurements was ±150 J/g and ±2°C in temperature measurement. The thermogravimetric measurements were carried out in a thermogravimetric device, Netzsch TG-209-F1-Libra. The equipment was use to analyze temperature tolerance of polymer samples: pAAm-Sc-4 and pAAm as a comparative system. The following operating conditions were chosen: 10 mg of the dry hydrogel, heated from 50 ºC to 300 °C, at 5 °C/min rate under flowing (200 ml/min) high purity nitrogen. Each sample was analyzed three times, and the average value was informed. The experimental error of these measurements was for all studied samples of ± 0.5 % in weight loss measurement and ± 2 °C in temperature measurement.

2.10. Fermentation process and reusability of immobilized *S. cerevisiae*

Fermentation was performing in 250 ml erlenmeyer stirred flasks which were used as a batch reactor under anaerobic conditions. Free yeast and immobilized pAAm-Sc-4 capsules, were pre-treated in aqueous phosphate buffer solution (0.1 M, pH 7.2) containing 3 % glucose, 0.1 % urea, 0.3 % yeast extract, 0.3 % malt extract. Soluble starch (3 % w/w) was used as feedstock. Free yeast and a similar quantity of immobilized yeast were immersed in two batch system with fresh nutrient liquid medium and fermented by incubation at 42 °C under gentle rotary shaking for 90 h. As is commonly reported for bio-reactions the variables which were the object of study during fermentation were glucose consumption, ethanol yield and ethanol productivity (g/(l. min)). Besides, cell viability and biomass formation were measured at the beginning and at the end of each fermentation process to analyze possible leakage or cell growth outside of the capsules. After each reaction cycle, the pAAm-Sc capsules were extracted from the batch reactor, washed three times with phosphate buffer and reincorporated into the batch reactor with fresh medium. For monitoring reactant (glucose) consumption, a commercial glucose enzymatic kit (Glucose enzymatic, Wiener Lab®) was used. The concentration of ethanol produced in the solution was determined by refractometry, using a calibration curve of

refractive index vs ethanol concentration. The theoretical yield of ethanol is defined as the stoichiometric amount of ethanol which can be produced from 1 mol of glucose according to the reaction:

$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

glucose → ethanol + carbon dioxide

Under ideal conditions, neither biomass nor by-products are generated. The reaction is controlled only by the carbon source (Jacob and Monod, 1961; Patzek, 2006).

3. Results and discussions

3.1. Characterization of pAAm-*Sc* capsules

A series of polymeric hydrogels based on polyacrylamide matrix were used for entrapment of *S. cerevisiae*. Polyacrylamide hydrogel without yeast was used as a control system. The SEM micrograph of entrapped *S. cerevisiae* pAAm-*Sc* and the pAAm hydrogel are presented in Figure 1.

Figure 1: SEM micrograph of a, b, c) the hydrogel pAAm and d, e, f) entrapped *S. cerevisiae* pAAm-*Sc*.

Figures 1 a, b, c) show that the micrographs of pAAm are in agreement with previously reported results (Tan et al., 2010), the surfaces are rough and porous, and present pores with a size around 20 μm. It can be seen in Figure 1 d) yeasts are homogeneously and randomly distributed on the overall surface, moreover, the diameter of the cells varies from 2 to 10 μm (Figure 1 d)). In Figure 1e and f), pores with a size of 2 μm can be observed, and in the inner walls of the polymer, small pores are present ranging from microns to nanometers. This pore size distribution could allow the inflow of nutrients (glucose and urea) from the external environment and outflow fermentation products (mostly ethanol) back into the bulk solution. Furthermore, it is possible to observe in the pAAm-*Sc* images (Figure 1 d, e) that holes with a similar size of the yeast are present. This free space seems to be produced when some yeasts are released. Simultaneously, the small size of the pores seems to avoid the leakage of cells from the hydrogel network, constituting a cage structure which allows partial confinement in a defined space without complete loss of movement, but restricting their metabolic activity.

3.2. Swelling capacity assays

The Figure 2a shows the swelling profile of the pAAm-*Sc* hydrogels formulations. As it can be seen, the different cell loading ratios result in significantly different kinetic swelling profiles. In all the cases, the plots of swelling ratio versus time exhibited logarithmic curves, which is characteristic of hydrogels (Caykara et al., 2006). The yeast incorporation reduces the interporous space of the crosslinking hydrogel network, consequently, the initial swelling rate and equilibrium swelling fall down in comparison to the bare hydrogel. As it is shown in Figure 2a, the equilibrium swelling value in pure water of a pAAm hydrogel is 1500 %. The equilibrium swelling values, presented in Table 1, show an exponential decrease when the cell loading increases. This behavior could indicate that strong attractive interactions between polymeric chains and yeasts are present (Wu et al., 2005).

Table 1: Immobilization efficiency and swelling equilibrium of the pure pAAm hydrogels and pAAm hydrogels with different *S. cerevisiae* loading.

In a second analysis, hydrogels were exposed to the different water-ethanol solution to simulate the behavior of hydrogel-yeast complexes with ethanol in an environment similar to that achieving during fermentation reaction with free yeasts. The exposure of hydrogels capsule to ethanol causes a noteworthy decrease in swelling capacities (Figure 2b). When the ethanol concentration achieves a critical value, the hydrogel collapses into a more compact state. At ethanol concentrations of 15 % v/v pure hydrogel has lost the 50 % of its initial swelling capacity and pAA-Sc-4 approximately 30 %. For ethanol concentration larger than 50 % v/v, the hydrogels with and without cells are not able to swell. Therefore, ethanol behaves as a non-solvent for polyacrylamide chains. When non-solvent and water are mixed, the molecules of the solvents present attractive interactions increasing the free energy of chain polymer. The upper affinity among polymer segments would induce the collapse of the polymer network. The pAAm hydrogel -without ionizable units- exhibits a continuous change in the swelling ratio over the entire range of ethanol concentrations. On the other hand, it is well known that *S. cerevisiae* exhibits alcoholic inhibition due to osmotic stress under ethanol concentrations higher than 20 % w/v (Ding et al., 2009). Therefore, the collapse will not occur during a real fermentation run.

Figure 2: a) Swelling kinetics for gel series pAAm (X), pAAm-Sc-1(•), pAAm-Sc-2( ) pAAm-Sc-3( ) pAAm-Sc-4( ) pAAm-Sc-5( ) in aqueous solution. **b)** Hydrogels swelling capacity under different water-ethanol solutions exposition, pAAm (•) and pAAm-Sc-4 (∇).

In addition, the hydrogel behavior could contribute to protect the immobilized yeasts from the high concentration of ethanol. In this way, the polymer acts as a protecting barrier which allows improving viability and stability of cells for longer periods of time.

3.3. Cell viability

Table 2 shows the cell viability obtained for free and immobilized yeast systems and total initial cells for each yeast loading respectively.

Table 2: Total initial (prior fermentation) cell number and cell viability for free and immobilized hydrogels (HG) yeast systems for different *S. cerevisiae* loading.

The cells viability results to be 77 % in all the experiences. It is noteworthy that the polymerization conditions seems not to affect the immobilized yeast cells viability, even that a strong oxidant (persulfate ion) and free radicals are present. On the other hand, this behavior simplifies greatly the immobilization of the yeast cells, compared with alternative procedures (Perullini et al., 2005). Once obtained the immobilized yeast, the maintenance of high levels of living cells could be attributed to the protective effect of the hydrogel matrix. It could be observed by optical microscopy, that cells are randomly disposed over the gel surface and mainly forming colonies.

3.4. Mechanical properties

The incorporation of yeast into the hydrogel changes the swelling capacity. Thus, if the structure of hydrogels capsules suffers an alteration because of the increases in the cell loading, the mechanical properties could also be modified. Elastic modulus (E) was determined by uniaxial compression from stress and strain plot (Figure 3). The E data for the different pAAm-Sc gels are described in Table 3.

Table 3: Elasticity module the pure pAAm hydrogels and pAAm hydrogels with for different *S. cerevisiae* loading

The elastic modulus (E) of pAAm-Sc hydrogels are higher than bare hydrogel, which is in agreement with the decreasing swelling capacity previously observed. Moreover, successive increments of cell loading produce a gradual transition elastic-to-viscoelastic behavior as it shown in Figure 3.

Figure 3: Stress vs. Strain plots for of immobilized *S. cerevisiae* over polyacrylamide hydrogels with different cell loading (mg Sc/l pre-gel solution). Gel series pAAm (X), pAAm-Sc-1(•), pAAm-Sc-2(▲), pAAm-Sc-3 (▼), pAAm-Sc-4 (◄) and pAAm-Sc-5 (►) in aqueous solution.

A cell loading beyond 150 g/ml decreases the immobilization efficiency, obstructs the interporous space and accelerates the cell death. In order to select the immobilization matrix the following criteria has been employed: appropriate mechanical properties, high immobilization efficiency (97%) and cell viability (up to 80%). Taking into account these standards, the pAAm-Sc-4 is the selected matrix to use in the fermentation experiments.

3.5. Thermal analysis by differential scanning calorimetric and thermogravimetric non-isothermal assays

DSC and TGA are studied to analyze the yeasts effect inside hydrogel matrix on physical interaction and thermal decomposition of the hydrogel-yeast complex. Hydrogels of pAAm-Sc-4 and pAAm were analyzed. The Figure 4a shows the typical DSC thermograms for polyacrylamide hydrogels with the characteristic freezing (downwards) and melting (upwards) characteristic peaks associated with water containing into the matrix (Nesrinne and Djamel, 2013). Generally, these states of water represent among 90-97 % of the total water of hydrogels. As can be seen, cell loading seems to influence on the temperature difference between the two peaks, probably due to the different interaction of water present in each sample. This peak shifting could be evidence a specific yeast-matrix interaction, which could not follow the same behavior of pure matrix due to the presence of the quantities of yeast.

The pAAm-Sc-4 and pAAm TGA data are presented in Figure 4b, the first derivate of weight loss are also represented. The TGA profiles were built as a function of temperature at a heating rate of 10 °C/min.

TGA assays report weight loss in the characteristic three decomposition stages of polyacrylamide hydrogel for both cases under study (Al-Sabagh et al., 2013; Madorsky, 1964). The initial stage of weight loss occurred between 50 °C to 200 °C. This is due to

the evaporation of intra and intermolecular moisture, other volatile compounds, protein and carbohydrates of yeast (weight loss about 10 % of the total weight) (Al-Sabagh et al., 2013). Polyacrylamide hydrogels contain hydrophilic groups which could induce the sample to interact with water molecules. The second stage occurred in the range of 210-330 °C, resulting a weight loss of 22 % and corresponding to the imine reaction of a small number of the amide groups and the thermal decomposition of hydrophobic side chains (Al-Sabagh et al., 2013). The third stage happens in the range of 350-500 °C and represents a weight loss of approximately 55 %. This one corresponds to the decomposition of amide groups and the degradation of the polymer main chains. Besides, the thermogram shows that this weightlessness is accompanied by some deformation of the hydrogel backbone skeleton structure (Kim et al., 2004). Beyond this temperature, the polymer decomposes completely. This study suggests that thermal stability of the hydrogel was slightly improved with cell addition; due to there are not substantial differences between the pyrolysis behavior of hydrogel with and without yeast.

Figure 4: a) Differential scanning calorimetric assays of immobilized *S. cerevisiae* over acrylamide hydrogels with different cell loading [g Sc/ml pre-gel solution]. Rate 10 °C/min. pAAm (dash line), pAAm-Sc-4 (Solid line). **b)** Thermogravimetric analysis of pAAm-Sc-4 (black solid line) and pAAm bare (black dash line). Loss weight versus temperature of pAAm-Sc-4 (gray solid line) and pAAm bare (gray dash line).

3.6. Analysis of fermentation process: glucose consumption and ethanol yield assay

The ethanol fermentation process depends on many factors, such as initial glucose and inoculums concentrations, the time required for efficient fermentation, reactor temperature, etc. (Nikolić et al., 2009). The first set of experiments (first cycle) was conducted in order to compare the behavior of free and immobilized yeast. Cell count of pre-hydrated yeast, before polymerization tramped yielded a result of 3.6 x 10^5 cell/ml. The initial glucose concentration was 30 g/l, the temperature was fixed at 42 °C and rotary shaker was set-up at 250 rpm. The fermentation was carried out during 90 h. Data of ethanol and glucose concentrations achieved at different operational time are reported in Table 4 and corresponding with the kinetics show in Figures 5 a and b.

Table 4: First fermentation cycle of free and immobilized (pAAm-Sc-4) yeast. Ethanol and glucose concentration, ethanol yield and percentage of ethanol theoretical yield and

volumetric productivity at different operational times. Initial conditions: 1gr *S. cerevisiae* (~100 caps), 30 g/l glucose, 42 °C, 250 rpm.

During the first fermentation cycle, for free and immobilized yeast, the curves show the typical fermentation timeline for batch bioreactors characterized by three main phases: lag phase, exponential growing phase and stationary phase (Figure 5 a and b) (Fan et al., 2015). Regarding first fermentation cycle for free yeasts, as shown in Figure 5 a, sugar consumption was almost complete within 60 h and ethanol yield reached the maximum level of 9.85 % w/w, after 50 h of fermentation. For immobilized yeast, the maximum yield of ethanol (8.39 % w/w) is reached at similar time and complete glucose consumption takes a longer time (See Figure 5 b). Perhaps, this is due to a lower substrate and product diffusion. After 60 h of fermentation-free yeast experiments a reduction of alcohol yield is observed. Thatipamala *et al*. reported that during ethanol batch fermentation the substrate and product inhibition considerably affect ethanol and biomass yield (Thatipamala et al., 1992). Similarly, Siqueira *et al*. reported that high substrate concentrations inhibited growth and fermentation of yeast during ethanol production from soybean molasses in a batch system (Siqueira et al., 2008).

Based on this finding, this behavior could be attributed to a very well-known inhibition effect caused by an exposition of cells at high ethanol concentration. The data obtained are broadly consistent with the fact that after the first reaction cycle, free cells viability decreased by 5 %. These drawbacks (yeast stress, mortality and ethanol yield declining) seem to be less important for immobilized yeasts, which do not show a decrease of ethanol yield or cell viability. An explanation of these results could be related to the already mentioned porous polymer matrix, which allows the confinement of yeast and lowers the effective alcohol concentration. Thus, a lower concentration of ethanol is in close contact with the yeast, reducing the stress factor which causes osmotic inhibition.

On the other hand, mass transport limitations hinder chemical reactions and consequently the initial reaction rate was lower for immobilized than for free yeasts. After the first fermentation cycle, the free yeasts were extracted from the reaction medium, centrifuged and incorporated into a fresh reaction medium to start a second reaction cycle. The immobilized yeast was simply removed mechanically from the reactor, gently washed and incorporated into a fresh reaction medium. The results are shown in Figure 5 c and d for free and immobilized yeast respectively (also see Table 5). Throughout, the second reaction cycle a different behavior is observed for both kinds of biocatalysts. After 80 h

of operation, free yeasts consumed only 8 % of the initial glucose and the concentration of ethanol product is lower than 3 %. Meanwhile, immobilized yeasts (pAAm-Sc-4) have consumed all the initial glucose and the ethanol concentration reaches 11 % of yield. This trend becomes more pronounced during the third operation cycle, where free yeast cells show total inactivity to produce ethanol or consume glucose (Figure 5 e and f).

On the other hand, reutilized immobilized yeasts show similar activity to previous cycles. Due to the partial reduction of ethanol concentration observed in the third and fourth cycle and with the aim to improve it until normal values, an extra glucose injection of 10 g/l was added to the fourth immobilized yeast fermentation batch, respectively.

Besides the better performance, the monolithic nature of the hydrogel with immobilized yeast cells makes removal of the biocatalyst from the spent solution for reutilization a simple procedure, especially when it is compared with a free yeast suspension. Additionally, the external surface of the hydrogel capsule could be sterilized to remove bacterial, viral or fungal contamination allowing to reuse costly (ex. genetically engineered) yeasts, without killing the yeast cells immobilized inside.

The immobilized yeast responds favorably to the high initial glucose concentration showing full catalytic activity. Yeasts are microorganisms which need carbon and nitrogen to growth, breath and reproduce. Probably, the shortage of carbon source (glucose), the continuous fermentations cycles and the constant ethanol exposition acts negatively and consequently ethanol yield partially decrease. Several authors reported that in batch fermentation system for immobilized *S. cerevisiae,* the ethanol yield depended strongly on initial glucose concentration (King and Hossain, 1982; Solis-Pereyra et al., 1996). However, substrate inhibition was noticed at very high initial glucose concentrations (above 30 %). By using fed-batch fermentation, it is possible to overcome substrate inhibition at high sugar concentrations in both free and immobilized system (Ozmihci and Kargi, 2007; Roukas, 1996). Studies of continuous and semicontinuous systems are being conducted regarding this. For the cycles 4 and 5 of immobilized yeast (Figure 5 g and h) the conditions were modified. The reaction was carried out at the same temperature but under a higher initial glucose concentration (10 %). When the quantity of sugar is 10 % presents the same profile as when the sugar quantity is 3 %, however, it is possible to reach higher productivities and ethanol yield (see Table 5). The immobilized yeasts seem to be more protected from the sugar exposition, this is an important finding that could contribute favorably to the process design.

Conditions for fermentation process and finals results of the ethanol and glucose concentration achieved for free and immobilized yeast are presented in Table 5.

Table 5: Fermentation cycles of free and immobilized (pAAm-Sc-4) yeast. Maximum values of produced ethanol and consumed glucose concentration, ethanol yield, and percentage of ethanol theoretical yield and volumetric productivity. Initial conditions cycles 1, 2, 3: 1gr *S. cerevisiae* (100 caps), 30 g/l glucose, 42 °C, 250 rpm. Cycles 4: 100 g/l glucose, Cycles 5: 150 g/l glucose.

Figure 5: Cycles of fermentation batch process **a)** First cycle, free yeast **b)** First cycle, immobilized yeast **c)** Second cycle, free yeast **d)** Second cycle, immobilized yeast **e)** Third cycle, free yeast **f)** Third cycle, immobilized yeast **g)** Fourth cycle, immobilized yeast **h)** Fifth cycle, immobilized yeast. For immobilized yeast, cells are tramped over polyacrylamide hydrogels with 125 cell loading [mg Sc/l pre-gel solution]. Black line represents glucose consumption (left axes) and gray line represents ethanol production (right axes).

4. Conclusions

An innovative procedure to immobilize yeast inside macroporous monolithic polyacrylamide was described. The *in-situ* radical polymerization of acrylamide in aqueous solution under cryogelation conditions shown that live yeasts tolerate the polymerization chemical stresses and the cryogelation physical stresses without the use of cryo-protectors or polymer barriers. It is probable that highly hydrophilic hydrogels act as bioprotector system of yeasts. An adequate swelling capacity and good mechanical properties were observed for yeast/hydrogel system with pre-gelling solution ratio of 125 mg/l. In addition, high immobilization efficiency and cell viability are achieved.

It was found that bioethanol production by fermentation is possible using immobilized cells in the macroporous monolithic polyacrylamide matrix. While free and immobilized yeast cells are active for fermentation during the first reaction cycle, ethanol yield in free yeast experiments decreases after 60 h of operation, presumably due to osmotic inhibition or cell death.

Yeast cells immobilized inside the macroporous hydrogels were able to maintain its catalytic activity during five consecutive reaction cycles. In contrast, free yeasts showed

a drastic loose of catalytic activity after only one fermentation cycle and became completely inactive after a second fermentation cycle. Moreover, immobilized yeasts seem to be better to withstand exposure to high sugar concentrations and showed less susceptible to contamination than free yeast.

The advantages of a monolithic macroporous hydrogel as matrix compared with free yeast suspensions are: first an easy separation of the reaction mixture and its reutilization possibility due to their monolithic characteristics; second the improved mass transport due to the macroporosity of the matrix; and third the protective effect of the hydrogel matrix which avoids contamination. Moreover, the fabrication procedure involves a single step of *in-situ* radical polymerization under cryogelation conditions without need of protecting additives or encapsulation.

In conclusion, the monolithic macroporous hydrogel-yeast complexes present advantages compared to the free yeasts respect to overall cost and ethanol yield improvement. The ability to reuse the same biocatalyst could be especially important when mutant or genetically engineered cells are used to increase the maximum ethanol yield. Since the separation of the ethanol from the aqueous solution is an energy consuming step, increasing the ethanol/water ratio is an important goal in bioethanol production.

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Figures:

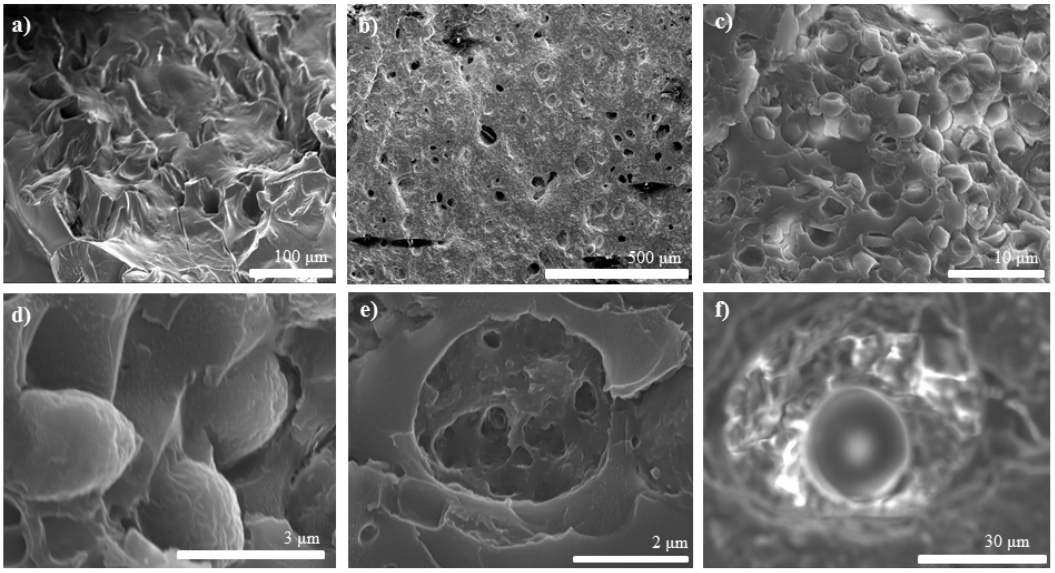

Figure 1: SEM micrograph of a, b, c) the hydrogel pAAm and d, e, f) entrapped *S. cerevisiae* pAAm-*Sc*.

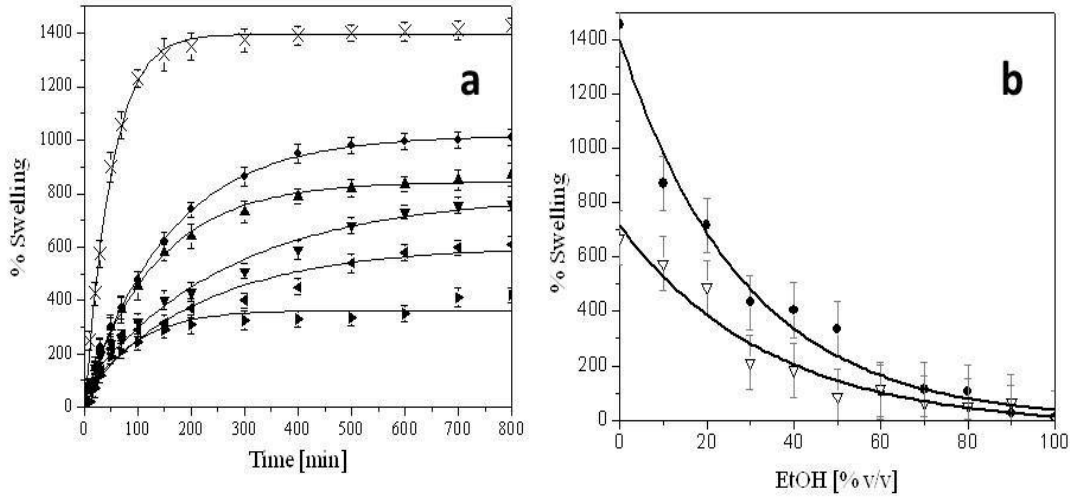

Figure 2: a) Swelling kinetics for gel series pAAm (X), pAAm-Sc-1(•), pAAm-Sc-2(▲) pAAm-Sc-3(▼pAAm-Sc-4(◄) pAAm-Sc-5(►) in aqueous solution. **b)** Hydrogels swelling capacity under different water-ethanol solutions exposition, pAAm (•) and pAAm-Sc-4 (▽).The values presented are the result of three measurements

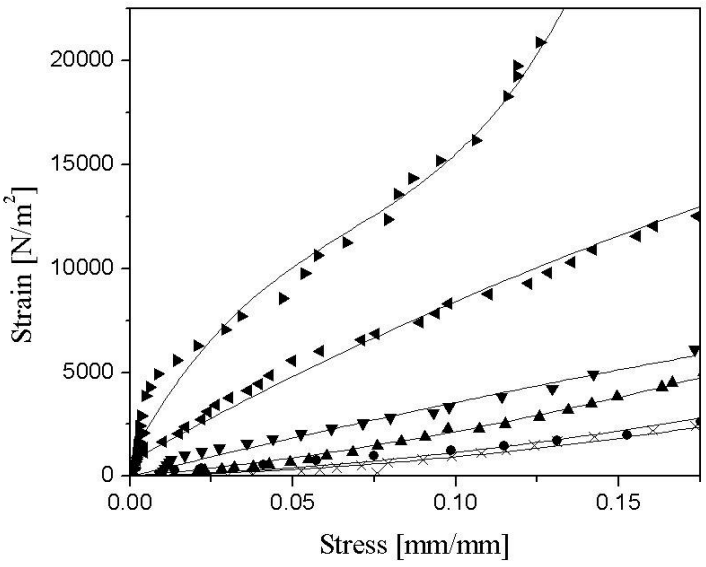

Figure 3: Stress vs. Strain plots for of immobilized *S. cerevisiae* over acrylamide hydrogels with different cell loading [g Sc/ml pre-gel solution]. Gel series pAAm (X), pAAm-Sc-1(•), pAAm-Sc-2(▲), pAAm-Sc-3 (▼), pAAm-Sc-4 (◄) and pAAm-Sc-5 (►) in aqueous solution.

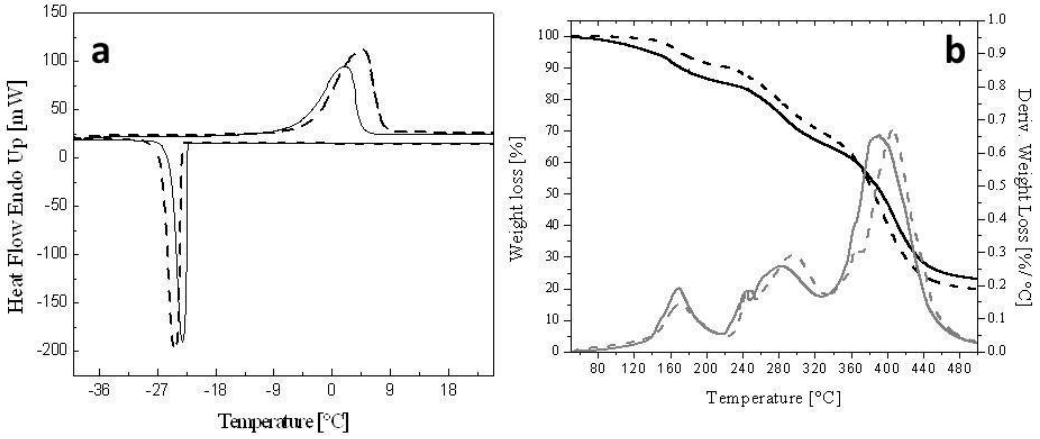

Figure 4: a) Differential scanning calorimetric assays of immobilized *S. cerevisiae* over polyacrylamide hydrogels with different cell loading [g Sc/ml pre-gel solution]. Rate 10 °C/min. pAAm (dash line), pAAm-Sc-4 (Solid line). **b)** Thermogravimetric analysis of pAAm-Sc-4 (black solid line) and pAAm bare (black dash line). Loss weight versus temperature of pAAm-Sc-4 (gray solid line) and pAAm bare (gray dash line).

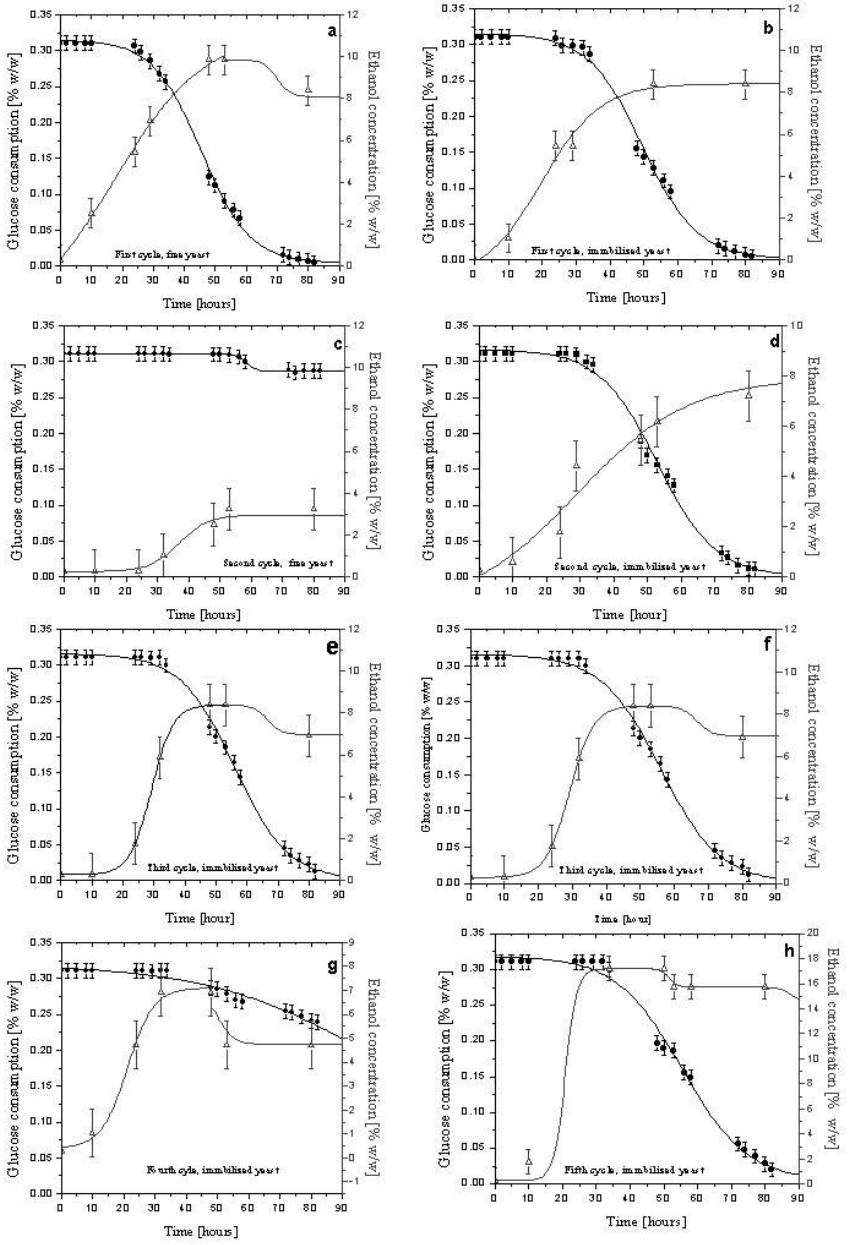

Figure 5: Cycles of fermentation batch process **a)** First cycle, free yeast **b)** First cycle, immobilized yeast **c)** Second cycle, free yeast **d)** Second cycle, immobilized yeast **e)** Third cycle, free yeast **f)** Third cycle, immobilized yeast **g)** Fourth cycle, immobilized yeast **h)** Fifth cycle, immobilized yeast. For immobilized yeast, cells are tramped over polyacrylamide hydrogels with 125 cell loading (mg Sc/l pre-gel solution). Black line represents glucose consumption (left axes) and gray line represents ethanol production (right axes).The values presented are the result of three measurements

Tables:

Table 1: Immobilization efficiency and swelling equilibrium of the pure pAAm hydrogels and pAAm hydrogels with different *S. cerevisiae* loading.

Hydrogel system	Yeast loading [mg Sc/l]	Immobilization efficiency % (*)	% Swelling equilibrium (*)
pAAm	-	-	1469 ± 31.5
pAAm-Sc-1	25	99.90 ± 0.05	1166 ± 30.0
pAAm-Sc-2	50	99.50 ± 0.15	1111 ± 45.0
pAAm-Sc-3	75	99.30 ± 0.30	869 ± 25.5
pAAm-Sc-4	125	97.80 ± 0.10	658 ± 28.5
pAAm-Sc-5	250	97.25 ± 0.60	510 ± 30.5

(*) the values presented are the result of three measurements

Table 2: Total initial (prior fermentation) cell number and cell viability for free and immobilized hydrogels (HG) yeast systems for different *S. cerevisiae* loading.

Cell system	Yeast loading (**)	Total initial cells (*)	% Cell viability (**)
Free Sc	25 mg Sc/l buffer sn.	3.60 x 10^5 cells/ml	77.78 %
pAAm-Sc-1	25 mg Sc/l pre-gel sn.	5.06 x 10^5 cells/ml HG	82.21 %
pAAm-Sc-2	50 mg Sc/l pre-gel sn	6.28 x 10^5 cells/ml HG	85.51 %
pAAm-Sc-3	75 mg Sc/l pre-gel sn	4.57 x 10^5 cells/ml HG	83.80 %
pAAm-Sc-4	125 mg Sc/l pre-gel sn	5.65 x 10^5 cells/ml HG	82.20 %
pAAm-Sc-5	250 mg Sc/l pre-gel sn	2.83 x 10^5 cells/ml HG	86.13 %

(*) For free-Sc the volume quantified was the volume of the fermentation vessel, meanwhile the volume taken into account for immobilized systems was the volume of each capsule weighted by the number of capsules required in every fermentation process.

(**) the values presented are the result of three measurements.

Table 3: Elasticity module the pure pAAm hydrogels and pAAm hydrogels with for different *S. cerevisiae* loading

Hydrogel system	Elasticity module [N/m^2] (*)	R-Square	Elasticity range stress
pAAm	13628.70	0.967	0.00-0.200
pAAm-Sc-1	21868.99	0.967	0.00-0.200
pAAm-Sc-2	30813.87	0.963	0.00-0.200
pAAm-Sc-3	34917.08	0.991	0.00-0.200
pAAm-Sc-4	75453.89	0.985	0.00-0.015
pAAm-Sc-5	166256.34	0.989	0.00-0.010

(*) the values presented are the result of three measurements

Table 4: First fermentation cycle of free and immobilized (pAAm-Sc-4) yeast. Ethanol and glucose concentration, ethanol yield and percentage of ethanol theoretical yield and volumetric productivity at different operational times. Initial conditions: 1gr *S. cerevisiae* (100 caps), 30 g/l glucose, 42 °C, 250 rpm.

Operation time, h	Ethanol concentration, % (w/w)	Glucose concentration, mg/dl	Ethanol yield, g/g	Percentage of ethanol theoretical yield, %	Volumetric productivity, g/(l.h)
Free yeast					
26	6.17 ± 0.50	0.300 ± 0.01	24.68 ± 0.50	40.45	2.37
38	7.86 ± 0.50	0.280 ± 0.01	31.44 ± 0.50	51.54	2.07
54	8.36 ± 0.25	0.156 ± 0.02	33.44 ± 0.50	54.82	1.49
76	6.99 ± 0.50	0.026 ± 0.01	27.96 ± 0.50	45.83	0.95
Immobilized yeast					
26	5.38 ± 0.30	0.300 ± 0.01	21.52 ± 0.50	35.27	2.07
38	6.93 ± 0.50	0.255 ± 0.01	27.72 ± 0.50	45.44	1.82
54	8.31 ± 1.50	0.100 ± 0.01	33.24 ± 0.50	54.49	1.48
76	8.39 ± 1.50	0.016 ± 0.01	33.56 ± 0.50	55.01	1.13

Table 5: Fermentation cycles of free and immobilized (pAAm-Sc-4) and yeast. Maximum values of produced ethanol and consumed glucose concentration, ethanol yield, and percentage of ethanol theoretical yield and volumetric productivity. Initial conditions cycles 1, 2, 3: 1gr *S. cerevisiae* (100 caps), 30 g/l glucose, 42 °C, 250 rpm. Cycles 4: 100 g/l glucose, Cycles 5: 150 g/l.

Cycle	Material	Initial glucose	% Ethanol (w/w)	Glucose, mg/dl	Ethanol yield, g/g	Ethanol theoretical yield, %	Volumetric productivity, g/(l.h)
Immobilized yeast							
1st	pAAm-Sc-4/ 100 caps	3%	8.34± 1.50	0.036± 0.01	33.36	50.55	1.26
2nd	pAAm-Sc-4/100 caps	3%	7.69± 1.50	0.000± 0.01	30.76	46.61	0.85
3rd	pAAm-Sc-4/100 caps	3%	8.31± 1.50	0.185± 0.02	33.24	50.36	1.54
4th	pAAm-Sc-4/100 caps	10%	7.12± 2.00	1.010± 0.05	28.48	43.15	1.51
5th	pAAm-Sc-4/100 caps	15%	15.66± 5.50	0.490± 0.05	62.64	94.91	2.45
Free yeast							
1st	1g *S. cerevisiae*	3%	9.94± 2.5	0.069± 0.05	39.76	65.18	1.84
2nd	1g *S. cerevisiae*	3%	2.95± 2.5	0.286± 0.05	11.80	19.34	0.45
3rd	1g *S. cerevisiae*	3%	0.00± 2.5	0.000± 0.01	0.00± 0.50	0.00	0.00