Saccharomyces cerevisiae immobilization in polyacrylamide hydrogel obtained at low temperature

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Abstract
Bioethanol of first generation is the world’s leader biofuel and it is produced by fermentation mainly from starch and sugar feedstocks. The use of continuous fermentation processes implies the possibility of making the process automatic while lowering the operating costs. Continuous fermentation can be achieved by using stirred-tank reactors in series (6-8 reactors) or by using, continuous plug-flow reactor in which the yeast is immobilized on a support. There are many types of supports that have already been tested. This paper will focus on the uses of the polyacrylamide supports with different compositions obtained in low temperature conditions (-18°C). The activity of the immobilized yeast was tested in comparison to the free yeast in suspension by fermentation of some molasses solutions in a batch reactor. The influences of the type of support and of fermentation conditions over the parameters for obtaining ethanol (fermentation rates and the yield process) are underlined in this article.

Keywords: immobilization, polyacrylamide, Saccharomyces cerevisiae, molasses, bioethanol

Introduction

Due to rising environmental concerns and to the expected crises in oil industry, alternative energy sources need to be renewable, sustainable, efficient and economically viable. Many countries are interested in developing their markets in the biofuel industry. These interests are motivated by the rising oil prices and the exhausting of oil reserves, the increased emissions, and the requirements of the Kyoto Protocol and the Bali Action Plan on emissions [1].

Bioethanol is an excellent fuel, with very good properties for spark ignition internal combustion engines. Its high octane number and high heat vaporization make ethanol to be more efficient than gasoline [2, 3].

Bioethanol is the world’s leader biofuel and it is produced by fermentation mainly from starch and sugar feedstock. However, the transformation of biological resources such as lignocelluloses biomass and energy-rich crops involves the pretreatment of the feedstock to make possible their fermentation into ethanol [4]. The pretreatment of the feedstock leads to a higher cost ethanol production.

Agricultural wastes can be used also, in particular molasses, a by-product of the sugar industry, to obtain ethanol. Cane molasses is a low-cost source of sugar, and unlike other agricultural by-products, it does not require hydrolysis [5].
For ethanol fermentation, yeasts are the most commonly used microorganisms. Microorganisms can utilize glucose, by Embden–Meyerhof–Parnas pathway, under anaerobic growth conditions [6]. Several microorganisms, including Clostridium sp., the well-known yeast producers of ethanol, Saccharomyces cerevisiae and Zymomonas mobilis are suitable to produce ethanol by fermentation [7].

Under anaerobic cultivation, Saccharomyces cerevisiae produces, beside ethanol, carbon dioxide, glycerol and cell biomass as the most important by-products. Carbon dioxide is a fermentation product, and it can be utilized as a high quality raw material. During osmotic stress, glycerol can be produced [8].

To improve the production of ethanol, different techniques have been evaluated. Among the different cell immobilization techniques, entrapment has been one of the most used methods, because of its simplicity and non-toxic character [9]. Immobilization of yeast cells presents several advantages for industrial fermentation, such as the ease of product separation, improved control of the process and decreased possibility of cells contamination, an increase of yield and stability of cells and a decrease of process costs due to the ease cell recovery and the possibility of their reutilization [5].

Hydrogels have attracted high attention because of their remarkable properties that open horizons in various fields such as biotechnology for cell immobilization, medicine and pharmacy, biochemistry, microbiology as cell support matrix [10]. They can be classified based on several criteria. They can be either natural or synthetic [11]. Depending on the cross-linking nature, hydrogels can be either permanent or physical ones [12]. Hydrogels can also be conventional or stimulus responsive ones. Conventional hydrogels are polymeric networks which absorb water from aqueous medium, but they don’t change their equilibrium swelling with the change of environment, while the stimulus responsive are polymeric networks which change their equilibrium swelling with the change of environment [13, 14].

The process of gel formation at temperature below 0°C, consists in freezing of the main fraction of solvent present in the initial system followed by the thawing of the solvent frozen [10, 15, 16]. During the freezing of a monomer solution, the monomers expelled from the ice concentrate within the channels between the ice crystals, so the polymerization reactions take place only in unfrozen microzones. After polymerization takes place in this circumstances and the ice is melted, a network consisting of uninterrupted macroporous channels is produced, whose structure is a negative response of the ice formed, the so called cryogel [10, 17].

Polyacrylamide is a synthetic polymer and it is the most representative and frequently used cryogel, with lots of applications, often obtained by redox initiating systems [18].

Entrapment of cells in polyacrylamide gel matrix requires the polymerization of acrylamide (AAm) monomers in an aqueous solution. The polymerization of acrylamide leads to the formation of linear polyacrylamide chains by a free radical process [19].

The purpose of the present study was to immobilize yeast Saccharomyces cerevisiae in a polyacrylamide gel support under specific conditions of low temperature. The compositions for the obtained support were modified to highlight the effect of: yeast concentration put into the support, the acrylamide concentration, the molar ratio acrylamide: N,N’-methylenebisacrylamide (BAAm) and the gel preparation temperature.

The immobilized yeast was tested in a batch reactor for the fermentation of some molasses solutions. The effect of different concentrations of molasses on the production of ethanol was evaluated. The fermentation rates and the yield process were examined to describe the consumption of sugars and the production of ethanol. Finally, the inner and the outer surfaces of the fresh and immobilized beads of yeast cells were scrutinized by means of scanning electronic microscope (SEM).
Materials and methods

The immobilization of yeast using polyacrylamide gel. Acrylamide (AAm, Merck), N,N’-methylenbisacrylamide (BAAm, Merck), ammonium persulfate (APS, Merck), and N,N,N’,N’- tetramethylethylenediamine (TEMED, Merck) were used. Three stock solutions of APS, TEMED and BAAm were prepared by dissolving 0.16 g of APS, 0.5 mL of TEMED and 0.264 g of BAAm in 20 mL of distilled water.

The redox initiator system was the APS and TEMED. Eight solutions with different compositions were prepared in graduated flasks (see table 1). Each of them contains AAm (3 different concentrations), BAAm (3 different molar ratios AAm:BAAm), TEMED (function of the amount of BAAm) and distilled water to obtain quite the same volume. The solutions from the graduated flasks were mixed very well.

Different quantities of dry yeast (Ethanol Red, Fermentis) were introduced in the graduated flasks. The solutions from the flasks were cooled to 0°C in ice-water bath, and then, argon gas was purged into the tightly closed graduated flasks for 20 min. After that, APS stock solution was added.

The polymerization was carried out for 24 h, by the immersion of the graduated flasks in a thermostated bath at -18°C (exception of sample 0 that polymerized at 0°C). After polymerization, the gels were cut into specimens of approximately 5 mm in length. The cut beads were immersed in a large excess of water to wash out any soluble polymers, unreacted monomers and the initiator.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AAm (g)</th>
<th>BAAm solution (mL)</th>
<th>TEMED solution (mL)</th>
<th>Water (mL)</th>
<th>APS solution (mL)</th>
<th>Yeast (g)</th>
<th>Yeast conc. in support (g/mL)</th>
<th>ACM conc. (% gr.)</th>
<th>Molar ratio AAm: BAAm</th>
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</thead>
<tbody>
<tr>
<td>N0</td>
<td>0.97</td>
<td>2</td>
<td>2</td>
<td>14</td>
<td>2</td>
<td>4</td>
<td>0.16</td>
<td>3.9</td>
<td>80</td>
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<tr>
<td>N1</td>
<td>0.97</td>
<td>2</td>
<td>2</td>
<td>14</td>
<td>2</td>
<td>4</td>
<td>0.16</td>
<td>3.9</td>
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<td>14</td>
<td>2</td>
<td>0.8</td>
<td>0.037</td>
<td>3.9</td>
<td>80</td>
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<tr>
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<td>14</td>
<td>2</td>
<td>0.4</td>
<td>0.019</td>
<td>3.9</td>
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</tr>
<tr>
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<td>4</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
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<td>7.5</td>
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<tr>
<td>N5</td>
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<td>3</td>
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<td>3</td>
<td>4</td>
<td>0.157</td>
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<td>3.9</td>
<td>160</td>
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<tr>
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<td>0.5</td>
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<td>15.5</td>
<td>2</td>
<td>4</td>
<td>0.16</td>
<td>3.9</td>
<td>320</td>
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</table>

Batch fermentation. The batch fermentation experiments in order to obtain ethanol were carried out using molasses solution as sole carbon source for *Saccharomyces cerevisiae* (50.8% fermentable sugars), and some nutrients, such as: (NH₄)₂SO₄ 1 g/L, KH₂PO₄ 1g/L and K₂HPO₄ × 3 H₂O 1 g/L. The pH was adjusted at 5.5, using HCl 0.5N. A volume of 50 mL of fermentation mixture was added over 5 g of the immobilized yeast on polyacrylamide. The fermentation conditions are described in the table no. 2. The fermentation was carried out in a thermo shaker. The samples for analysis were taken after 2, 4, 6, 8, and 24 h.
Table 2. Reaction conditions for fermentation experiments

<table>
<thead>
<tr>
<th>Exp. no</th>
<th>Type of support</th>
<th>Yeast, g/L</th>
<th>Molasses, g/L</th>
<th>Temperature, °C</th>
<th>The fermentation day</th>
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<td>35</td>
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<td>16</td>
<td>242</td>
<td>40</td>
<td>1</td>
</tr>
</tbody>
</table>

**Bioethanol and sugars detection.** Bioethanol concentration in the fermentation mixture was determined with gas chromatography, Buck Scientific 910 Environmental & BTX, equipped with flame ionization detector (FID) and a capillary column Stabilwax (MXT-1 0.53 x 60m, I.D. 5.0μ, DB1). Helium was used as a carrier gas. Isopropanol was used as an internal standard. The samples from the fermentation medium were taken with a syringe with a Nylon filter of 0.45μm.

The sugars concentrations were determined by HPLC (JASCO 24XX). The sugars were separated using a Prevail (Alltech) amino column. The sugars eluted from the column using an isocratic flow of 1ml/min of water/acetonitrile (25/75) eluent. After separation, the sugars were quantified using a refractive index differential detector (Waters 410). The detector was operated at 35°C.

**Electron microscopic scanning.** The inner and the outer surfaces of the fresh and immobilized beads of yeast cells were scrutinized by means of a High Resolution Scanning Electron Microscope (HREM), FEI Inspect F 50 (field emission gun). A 5Kv voltage was used and fracture surfaces were examined, after gold sputter coating.

**Results and discussions**

To analyze and compare the experimental results obtained, the average fermentation rates and the specific fermentation rates for 2 periods of time, 0-4 h and 4-8 h were determined (eq. 1 and 2). The conversion and the transformation yield of sugars into ethanol were determined (eq.3-4).

\[
Fermentation\ rate = \frac{[\text{ethanol}]_j - [\text{ethanol}]_0}{t_j}
\]  \[1\]
Fermentation rate_2 = \frac{Fermentation rate_1}{[yeast]_0} \quad [2]

Conversion = \frac{([sugars]_0 - [sugars]_1)}{[sugars]_0} \times 100 \quad [3]

Yield = \frac{[ethanol]_1}{([sugars]_0 - [sugars]_1)} \times 0.5111 \quad [4]

The objective of the experimental program was the modification of the yeast supported concentration, the modification of the support (the concentration of AAm, the molar ratio AAm: BAAlm, and the gel preparation temperature), and in a second stage, the modification of the fermentation conditions (the molasses concentration, the temperature, and the uses of the immobilized yeast in first and second day of fermentation). The free dry yeast, that was hydrated and used in the same conditions as the immobilized yeast, was also tested.

The influence of the immobilized yeast concentration (experiments 1, 2, 3)
Samples N1, N2, and N3 (table 1) were used. The yeast concentrations in the three experiments were 16, 3.6 and 1.8 g/L. The experiments were conducted in the first and the second day. The results of these experiments are presented in figure 1.

![Figure 1](image-url)

**Figure 1.** The influence of the yeast concentration (free (F) and immobilized (Im)) on ethanol production; (a) ethanol concentration in time; (b) the transformation yield of sugars into ethanol.

From the results presented in figure 1(a), it can be seen that the differences are quite small between experiments made in the first day with immobilized yeast, although the yeast concentrations are different. This happened because at high concentrations of yeast in the support, a very small part of the yeast cells are on the surface of the support, the others are being entrapped in it, and they don’t have activity from the beginning (see figure 2).
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Regarding the transformation yield of sugars into ethanol, the results presented in figure 1(b) show that this yield is higher at the end of the fermentation. For the immobilized yeast, the results obtained in the second day of fermentation are higher than the ones of the first day, and they are being superior to those obtained in the fermentation with the free yeast, at comparable values of sugar conversion.

In the second day of fermentation, the activity of the immobilized yeast is comparable with the one in suspension. This fact is due to the appearance of some micropores obtained by the partial dissolution of polyacrylamide, which allow the access of biomass to some of the yeast cells entrapped in the walls (see fig. 3). However, the dissolution process of polyacrylamide is limited, as the structure of the support after 2 days of fermentation is not changed as compared to the structure which was in place at the end of the first day.

The fermentation rates and the specific fermentation rates are higher for the immobilized yeast used in the second day than for the immobilized yeast used in the first day and is comparable with the values obtained for the free yeast in suspension (see fig. 4).

The results presented in figure 4 show that with the decreasing of the yeast concentration, the average rates fermentation are decreasing too, while the specific rates are
increasing. At 4 g/L yeast concentration, the specific rates obtained using the immobilized yeast are higher in the second day then those obtained using the yeast in suspension.

Figure 4. The rates of fermentation obtained for the first and second day of fermentation, for the immobilized and free yeast; [molasses]=242 g/L.

The influence of the molar ratio AAm: BAAm (experiments 1, 6, 7)

To obtain supports 1, 6 and 7, different ratios of AAm: BAAm (80, 160 and 320:1) were used, the other features remaining the same. These supports have been tested in the molasses fermentation processes, and the results are presented in figures 5 and 6.

Figure 5. The influence of molar ratio AAm: BAAm on ethanol production;
(a) ethanol concentration in time;
(b) (b) the transformation yield of sugars into ethanol
The results presented in figures 5 and 6 show that the activity of immobilized yeast on polyacrylamide increases slightly with the decreasing of the cross-linking agent quantity, but this leads to a lower strength of gel in time and lower mechanical properties. In conclusion, the choice of a suitable concentration of the cross-linking agent is very important for the support properties, but also for the yeast activity.

**The influence of AAm concentration in the support**

In the synthesis of the polyacrylamide supports 1, 4 and 5 different quantities of AAm (3.9, 5.7, and 7.5%) were used, the other features remaining the same. The tests done with these supports conducted to the results from figures 7 and 8.

**Figure 6.** The influence of the molar ratio AAm: BAAM on fermentation rate

**Figure 7.** The influence of AAm concentration in support on ethanol production; (a) ethanol concentration in time; (b) the transformation yield of sugars into ethanol.
The results presented in figures 7 and 8 show the influence of the AAm concentration. In the range 3.9-7.5%, the concentration of AAm has a quite small influence on ethanol concentrations and on fermentation rates obtained. The AAm concentration is also responsible for the mechanical properties and stability of the support. The results obtained indicate an optimal value of 7.5% in the concentration of AAm.

**The influence of the molasses concentration using immobilized and free yeast**

Supported yeast (type N1) and free yeast in suspension with different concentrations of molasses have been tested. The results are presented in figure 9.

The results presented in figure 9 show that the immobilized yeast has a lower activity than the free yeast in suspension (in the first day of fermentation). The increase of the molasses concentration should conduct to higher rates of ethanol, but this didn’t happen in the first hours of the fermentation process due to the inhibitory effect of the sugars at high concentrations.

Regarding the transformation yield of sugars into ethanol, these are higher when the molasses concentration is lower. To minimize the costs of the ethanol separation from the reaction medium, the molasses concentration cannot be too low, because, ultimately we want to obtain higher ethanol concentrations.
Conclusions

The purpose of the present study was to immobilize yeast *Saccharomyces cerevisiae* in a polyacrylamide gel support obtained under specific conditions at low temperature. The compositions for the obtained support were modified to highlight the effect of yeast concentration put into the support, as the AAm concentration, the molar ratio AAm: BAAm and the gel preparation temperature. The fermentation rates and the yield process were examined to describe the consumption of sugars and the production of ethanol.

The immobilized and free yeast were tested in a batch reactor for the fermentation of some molasses solutions for several days. It was noticed that the immobilized yeast activity depends on the support characteristics (molar ratio AAm: BAAm), the yeast concentration immobilized in the support and mostly on the fermentation period, since during the second day the immobilized yeast has an activity almost as good as the one of the free yeast in suspension.

Regarding the transformation yield of sugars into ethanol it can be stated that this value changes in time, being higher at the end of the process. The molasses and yeast concentrations also have an important influence.

Using an optimal choice for the support composition, for the immobilized yeast concentration and for the fermentation conditions, the obtained immobilized yeast can be used for a significantly longer period of time with results at least as good as the ones obtained for yeast in suspension.

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