Saccharomyces Cerevisiae Immobilization in Polyacrylamide Cryogel – activation and Testing in Continuous Reactor

IOAN CALINESCU[1], PETRE CHIPURICI[1], ADRIAN TRIFAN[1], ELVIRA ALEXANDRESCU[2], ROMULUS DIMA[1], CORINA TEIU[1]*

[1]Politehnica University of Bucharest, Faculty of Applied Chemistry and Material Science, 149 Calea Victoriei, Bucharest, 010072, Romania

[2]Romanian Research and Development Institute for Gas Turbines, COMOTI, 220 D, Iuliu Maniu, Bucharest, 061126, Romania

Immobilized yeast on polyacrylamide cryogel was evaluated for the continuously production of ethanol using molasses as carbon source in filled bed reactor. This paper will focus on the polyacrylamide gel supports obtained at low temperature, a condition that allows obtaining a structure with large pores and specific surface. The activation of the supported yeast before use is of great importance. A polyacrylamide support containing 2% dry yeast was tested to investigate the growth and proliferation of immobilized yeast cells in the time of activation. For this study gravimetric and scanning electron microscopy was used. The activity of the immobilized yeast was tested in a continuous fermentation reactor to underline the influence of the molasses concentration, the molasses solution flow, the quantity of support filled into the reactor and the fermentation temperature over the ethanol concentration, productivity and yield process.

Keywords: bioethanol, continuous reactor, yeast immobilization, polyacrylamide cryogel

The interest in alternative sources of energy is increasing worldwide because the world energy is in depletion, and the atmospheric pollution derived from the combustion of fossil fuels must be reduced [1, 2]. Converting the biomass into biofuels solves both the exploitation of the alternative sources of energy and the decreasing of polluting gases emissions [3].

The processing of biomass in order to obtain biofuels and the conversion into secondary energy products does not require high capital investments [4].

Ethanol is one of the most important and studied biofuel, with very good properties for spark ignition internal combustion engines, more efficient than gasoline due to its high octane number and heat vaporization [5, 6].

Ethanol can be produced from various raw materials, classified into three main types: sugars, starches and cellulose materials. Unlike starches and cellulose materials that require conditioning or pretreatment steps, sugars can be converted directly into ethanol [4, 7].

About 95% of ethanol produced in the world is obtained by processing agricultural products [8]. For ethanol production from sugars, molasses, a by-product of the sugar industry, is the most used raw material. It contains about 50 wt % of sugar and about 50 wt % organic and inorganic compounds, including water [4].

Ethanol production is usually carried out in three main steps: the preparation of the solution containing fermentable sugars, the fermentation of sugars into ethanol (using a fermentation agent) and the separation and purification processes of ethanol, usually made by distillation–rectification–dehydration [9].

Saccharomyces Cerevisiae, known as an ethanologenic yeast, is the predominant microorganism used in the alcoholic fermentations [10].

Different aspects meant to increase ethanol productivity and to reduce labor intensity have been extensively studied in the past years. These issues were determined to be: the bioreactor volume, the energy consumption and the cell immobilization [11-13].

The reduction of costs derived from ethanol production employing bioprocesses that involves immobilized cells systems come from several aspects, such as: the cost of raw materials, the simplicity and low cost of immobilization techniques used, the stability of the immobilized biocatalyst, the high cell concentration in the bioreactors and the design of a suitable bioreactor system [12, 14].

During the last few years, ethanol production using immobilized yeast cells have been extensively studied due to some technical and economical advantages, over the free cell systems [15].

The immobilization of cells can be carried out by different methods such as: adsorption, covalent bounding, cross-linking, entrapment and encapsulation. Cell immobilization by entrapment is the most used method due to its simplicity and it has been used more often for the continuous production of ethanol. The principle of this method is the inclusion of cells within a rigid polymeric matrix [16].

Polyacrylamide was the first support used for the entrapment of cells. It has the advantage that the cells immobilized can retain their viability. The cell viability depends on several factors as: the temperature used in the polymerization process, the contact time between the cells and the monomers, and the time required for gel to form. The porosity of the gel depends on the amounts of the acrylamide monomer and the bi-functional cross-linking agent used [17-19].

The aim of this study was to evaluate the suitability of the acrylamide-based cryogel, for utilization in yeast immobilization when applied in ethanol fermentation of sugar molasses, with regard to operational stability, and ethanol productivity of the proposed process. Validated by the scanning electron microscopy (SEM) images and associated fermentation experiments, the possible mechanism underlying the higher biocatalytic function was also studied.

* *email : anca_corina22@yahoo.com Tel.: 0040722618059*

Immobilized yeast, type	AAm, (g)	BAAm solution, (mL) (0.66 g / 50 mL)	TEMED solution, (mL) (1.25 mL / 50 mL)	Water, (mL)	APS solution, (mL) (0.4 g / 50mL)	Yeast, (g)
For continuous reactor test	14.6	30	30	210	30	60
For growth of yeast cells on support test	0.485	1	1	7	1	0.2

Table 1
COMPOSITIONS OF THE MIXTURE USED TO OBTAIN POLYACRYLAMIDE SUPPORT FOR THE IMMOBILIZATION OF YEAST

Experimental part

Methods

The immobilization of yeast using polyacrylamide cryogel

Acrylamide (AAm, Merck), N,N'-methylene bisacrylamide (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 0.40 g of APS, 1.25 mL of TEMED and 0.66 g of BAAm in 50 mL of distilled water. The redox initiator system was the APS and TEMED. Two type of immobilized yeast were obtained, one for continuous reactor tests and one for growth tests (table 1).

AAm, solutions of BAAm, TEMED and distilled water were introduced into a graduated flask. The graduated flask was mixed very well. The dry yeast (Ethanol Red, Fermentis) was then introduced in the graduated flask. The solution from the flask was cooled to 0^0C in ice-water bath, and then, argon gas was purged into the tightly closed graduated flasks for 20 min. After that, the APS solution was added.

The polymerization was carried out for 24 h, by the immersion of the graduated flasks in a thermostated bath at -18^0C. 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.

Microorganism and media

The dry alcohol yeast used was *Saccharomyces Cerevisiae* from Fermentis (94-96% dry weight; $>20*10^9$ living cells / g).

Different media compositions were prepared:

For activation of immobilized yeast used in a continuous reactor: glucose 10 g/L, KH_2PO_4 3 g/L, Na_2PO_4 2.25 g/L, and yeast extract 1 g/L [16].

For continuous reactor tests, three type of solutions with different concentrations of 167 g/L, 242 g/L, respectively 286 g/L molasses (50.8% fermentable sugars); $(NH_4)_2SO_4$ 1 g/L, KH_2PO_4 1 g/L and $K_2HPO_4 \times 3\ H_2O$ 1 g/L. The *p*H was adjusted at 5.5, using HCl 0.5N.

For growth of yeast cells on support test: molasses (120 g/L), $(NH_4)_2SO_4$ (5 g/L), KH_2PO_4 (3 g/L) and $MgSO_4*7H_2O$ (0.5 g/L). The medium was buffered at *p*H 5 by adding 5.4 g/L of citric acid and 6.9 g/L of Na_2HPO_4 [18].

All media were sterilized by autoclaving at 116^0C for 20 min. prior to use.

The activation of immobilized yeast used in continuous reactor

120 mL nutritive solution (type 1) were dropped over 25 g of supported yeast. The samples were kept for 24 h at temperature of 36^0C.

Continuous reactor

Continuous fermentation was performed in a water-jacketed bioreactor with a working volume of 0.612 L (including the inoculum), and a total volume of 0.8164 L. The bioreactor comprised of a plug flow Plexiglass tubular column (40 mm i.d., 650 mm length). The reactor was filled 75% and contained 400/ 200 g immobilized yeast on polyacrylamide and 130 - 190 mL of fermentation medium.

The above medium was continuously fed into the lower unit of the bioreactor and fermented at three temperatures: 32.5, 35, respectively 37^0C. The exhaust gas and effluent solution were removed via an outlet located at the top of the bioreactor.

The ethanol productivity was calculated as the grams of ethanol per liter of liquid volume produced per hour (g/ L x h).

Analytical methods

Bioethanol and sugars detection. Bioethanol concentration in the fermentation mixture was determined by 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. 5.0u.i., 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.

Characterization of materials

Scanning electron microscopy: The lyophilization step is necessary before the scanning Electron microscopy analysis to be performed. The immobilized beads of yeast cells were frozen in a freezer at -20°C for 24 h, and then freeze-dried in a Labconco FreeZone freeze-dryer, at 0.470 mBar for 48 h. The temperature of the drying chamber was -50°C.

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

Sample no.	Treatment stages						Weight samples after lyophilization, g	
								Newly appeared yeast, g
1	A	D					0.6038	-
2	A	B	D				0.622	-
3	A	C	A	D			0.7453	0.1415
4	A	B	B	D			0.5736	-
5	A	C	C	A	D		0.9669	0.3631
6	A	B	B	B	D		0.582	-
7	A	C	C	C	A	D	1.1369	0.5331

Table 2
DESCRIPTION OF THE TREATMENT STAGES AND THE WEIGHT SAMPLES AFTER LYOPHILIZATION

Treatment stages:

A - Washed 3 times with water (3*100mL);

B - Kept in ultrapure water that contained only nutrients (100 mL) at 30 ^{0}C, 24 h

C - Kept in molasses solution (120 g/L) that contained nutrients (100 mL) at 30 ^{0}C, 24 h

D - Lyophilization

Results and discussions

Selection of suitable support for yeast immobilization

As support, it was utilized a polyacrylamide cryogel. The preparation of gel at low temperatures allows obtaining a structure with large pores and a good specific surface (~ 70 m^2/g of lyophilized support). The obtained gel was partially reticulated with BAAm (molar ratio AAm : BAAm = 80 : 1), the chemical initiators being the APS and TEMED solutions.

Initially it was obtained a polyacrylamide support with high concentration of immobilized yeast (16 % dry yeast) that was used in the continuous reactor. Subsequently it was obtained a polyacrylamide support with a lower concentration of yeast (2% dry yeast). This support was tested to further investigate the growth and proliferation of yeast cells during fermentation. Gravimetric analysis and Electron microscopic scanning (SEM) characterization also confirmed that a large numbers of yeast cells were formed on the outside surface of the support.

Yeast cell development on the support

According to the preparation of the support described at point 2.1., the dry yeast is dispersed in the aqueous solution of AAm, BAAm and TEMED, and the polymerization is initialized by adding APS. For this reason, the yeast cells will be dispersed throughout the gel mass, both in the walls and on the surfaces. Consequently, a lot of cells introduced into the system won't be useful because they are not in contact with fermentation solution. The cells positioned on the walls surface have favorable conditions to develop and will form new cells that will adhere to the gel surface.

To demonstrate the cells capacity to develop and the support ability to retain the yeast cells, 7 identical samples of gel were obtained, with a mass of 10 g and an initial content of dry yeast 0.2 g (table 1). After the curing time (24 h, at -18 °C), the samples were washed with ultrapure water (3*100 mL), and were distributed in 7 flasks thermostated at 30 °C. Some of the samples were kept in ultrapure water that contained only nutrients (100 mL), while others were kept in aqueous solutions of molasses (120 g / L) with nutrients (100 mL).

After 24 h , samples no. 2 and 3 were removed from the solutions, washed with water, and lyophilized. The solutions were repalced (water or molasses) with fresh ones for samples no. 4 – 7, and they were kept for another 24 h on thermostat. After that, the samples no. 4 and 5 were washed with water, and lyophilized, and for samples no. 6 and 7 the solutions were replaced again and they were kept for another 24 h on thermostat. Then, those were washed with water, and lyophilized too. The lyophilized samples were weighed and analyzed by scanning electron microscopy (SEM). The treatment stages and the weight results are presented in table 2.

The dry substance mass into a sample is:

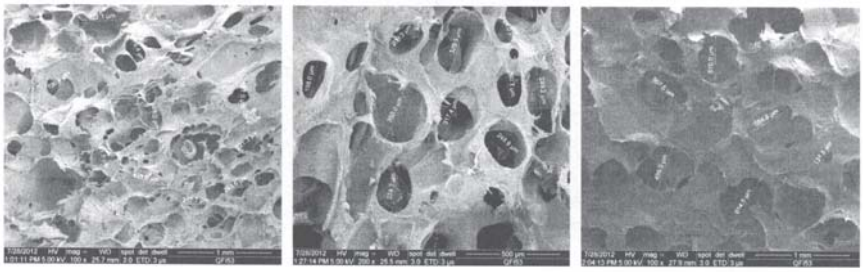
0.485 (AAm) + 0.0132 (BAAm) + 0.025 (TEMED) + 0.008 (APS) + 0.2 (yeast) = 0.7312 g

After the first wash, the sample looses 0.7312-0.6038=0.1274 g, which represents 26.3% from the AAm quantity, if we assume that only the AAm was dissolved, not the yeast.

After 24 or 48 h, the sample kept in water has quite the same weight; the quantity of AAm dissolved, and of the dry yeast newly formed in this time, is minimum. The support is keeping the same shape, and contains both macropores (with diameters of 400-500 mm) and micropores (fig. 1). The yeast cells from the walls surface do not develop, it can be observed at most how the superficial layer of gel that covers the cells is dissolving (fig. 2).

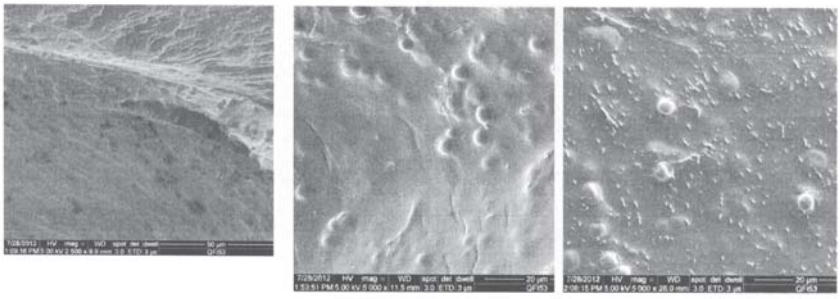
After lyophilization, the support samples kept in molasses solution have an increased weight in the first day: 0.7453-0.6038=0.1415 g. This increase in weight may represent only the fresh yeast, formed on the support surface. In the second day, the increase of weight is bigger, respectively: 0.9669-0.7453=0.2216 g. In the third day, the increase of weight is: 1.1369-0.9669=0.17 g

After the second day, it can be observed that the molasses solution separated from the support contains yeast cells in suspension, that can determine the fermentation process. This can be explained as the support



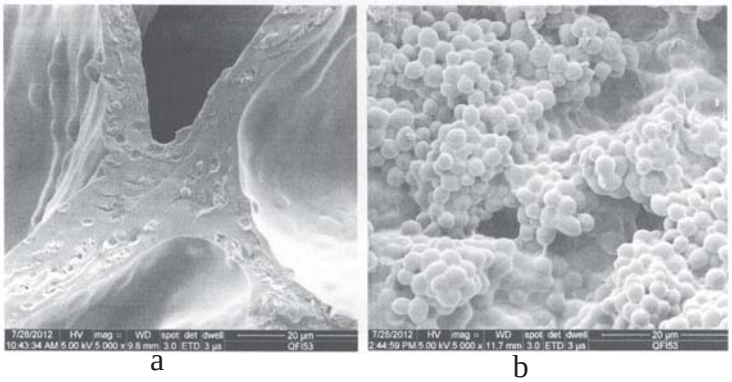
Fresh sample (sample 1) After 24 h in water (sample 2) After 72 h in water (sample 6)

Fig. 1. Electron microscopic scanning images of the samples kept only in water (large scale 0.5-1 mm)



Fresh sample (sample 1) After 44 h in water (sample 4) After 72 h in water (sample 6)

Fig. 2. Electron microscopic scanning images of the samples kept only in water (detailed images of the walls surface)



a b

Fig. 3. Sample 5 after 48 h of incubation into the molasses solution, section view (a) and outer surface (b)

Table 3
EXPERIMENTAL CONDITIONS USED FOR TESTING THE IMMOBILIZED YEAST IN THE CONTINUOUS REACTOR

Exp. no.	V_s, mL	$D_{molasses}$, mL/h	t_s, h	Support quantity, g	Temp., ^0C	Conc. of molasses solution, g/L	$D_{effluent}$, mL/h	CO_2 flow, ml/s
1	185	30	6.2	400	32.5	242	28	0.2
2	180	57	3.2	400	32.5	242	54	0.482
3	190	22.7	8.4	400	32.5	242	21	0.225
4	155	58.4	2.7	400	32.5	167	56	0.388
5	147	59.4	2.5	400	32.5	286	55	0.608
6	130	31.76	4.1	200	32.5	242	30	0.31
7	130	18.9	6.9	200	32.5	242	18	0.18
8	130	32.1	4.0	200	35	242	30	0.293
9	130	31.8	4.1	200	37	242	30	0.35

surface is almost saturated with yeast cells, and from the newly formed cells, some adhere on the support, while the others fall off and disperses in the nutritive solution.

Knowing that the dry yeast, Ethanol Red contains 20 x 10^9 viable cells/ g, it can be appreciated the number of viable cells grown on the support after 24, 48 and 72 h: 2.8 x 10^9, 7.3 x 10^9 and respectively 1.07 x 10^{10}. The capacity of water retain was determined as 2450% from the dry mass of gel, the inflated gel had 24.5 x 0.5321 = 13 g. Accordingly, the number of newly appeared cells yeast per gel gram will be: 2.15 x 10^8, 5.43 x 10^8, respectively 7.9 x 10^8 These values are comparable with the ones from literature [Zheng et all 2011] (26 - 49 x 10^8 cells/mL), and can be even higher if a support with opened pores will be obtained, or if particles support with smaller diameter will be used.

The newly yeast cells are formed only on the external surface of gel support. The internal surface contains closed

pores on which the yeast cells do not develop, because they are not in contact with the growth medium (fig. 3).

From the initial number, relatively few yeast cells are on the external surface of the support, where they can develop, most of them being in the gel mass or on the internal surface, where they can not have activity.

It can be considered that the activation of the yeast cells is done after at least 24 h of incubation in the growth medium which contains also nutrients.

Continuous ethanol fermentation in a filled-bed reactor with immobilized yeast on polyacrylamide

The influence of the molasses solution flow rate, the molasses solution concentration, the amount of support filled into the reactor, and the fermentation temperature were investigated. The operating data are presented in table 3.

The reactor contains support, liquid phase and gases. For an accurate determination of the stationary time, at the end of each experiment, the feed with molasses solution was stopped. The reactor was cleared to determine the solution volume (V_s) which exists in the active zone of operation during the fermentation process.

Knowing the flow rate of the molasses solution ($D_{molasses}$) used in the process, the hydraulic stationary time (t_s) is determined:

$$t_s = \frac{V_s}{D_{molasses}} \quad (1)$$

The reactant and product mass flows were calculated with equations (2-4):

$$Q_{molasses}[g/h] = D_{molasses}[mL/h] * Conc_{molasses}[g/L]/1000 \quad (2)$$

$$Q_{ethanol}[g/h] = D_{effluent}[mL/h] * Conc_{ethanol}[g/L]/1000 \quad (3)$$

$$Q_{CO2}[g/h] = D_{CO2}[mL/s] * (3600/1000) * (44/22.4) \quad (4)$$

With these values the theoretical yield for the transformation of fermentable sugars into ethanol (fermentable sugars concentration in molasses = 50.4%) is determined:

$$Yield_{theoretical}[\%] = \frac{Q_{ethanol}}{Q_{molasses} * 0.504 * 0.5111} * 100 \quad (5)$$

The ethanol productivity is determined function of the volume of the continuous reactor (V_s):

$$Productivity[g_{ethanol}/(L*h)] = \frac{Q_{ethanol}}{V_s} \quad (6)$$

There were analyzed also the unreacted sugars to determinate the real yield of sugars converted into ethanol:

$$Q_{sugars}[g/h] = D_{effluent}[ml/h] * Conc_{sugars}[g/L]/1000 \quad (7)$$

$$Yield_{real}[\%] = \frac{Q_{ethanol}}{(Q_{molasses} * 0.504 - Q_{sugars}) * 0.5111} * 100 \quad (8)$$

The operational parameters during the continuous process are presented in table 4.

From figure 4 it can be noticed that the stationary time has an important influence on the fermentation process. Although on longer reaction time the ethanol concentration and the transformation yield of sugars into ethanol are slightly increased, the process productivity decreases dramatically. On such continuous reactor shorter stationary time is favorable, eventually can be used two continuous reactors in series.

The initial concentration of molasses has an important role in the fermentation process. Its influence can be noticed from figure 5. At smaller concentrations of molasses, the yields transformation of sugars into ethanol obtained are very good, but the ethanol concentration and the productivity are low. The last two parameters are increasing with the molasses concentration, but only till limited values (~240 g/L). On higher concentrations the inhibition phenomenon of alcoholic fermentation occurs.

As shown in figure 6, the process runs in good conditions, even if the quantity of support utilized is decreased. The influence of the support amount is even greater as the flow rate is greater. The optimal quantity of the support, in these conditions, is 30-40 g/100 mL fermentation medium.

From figure 7 it can be noticed the importance of temperature parameter in the fermentation process using a continuous reactor. On the interval 32.5-35^{0}C, the temperature has a good effect. The rise of temperature leads to the decrease of ethanol concentration and thus of the productivity.

Exp. no.	Ethanol conc., g/L	Mass flows			Yield		Productivity, $g_{ethanol}/(L*h)$	Molar ratio ethanol/CO_2
		molasses, g/h	ethanol, g/h	CO_2 g/h	$Yield_{teoretical}$ %	$Yield_{real}$ %		
1	60	7.26	1.69	1.70	90.42	96.36	2.76	0.95
2	56.0	13.79	3.00	3.04	84.40	94.60	4.90	0.94
3	64.0	5.49	1.36	1.34	96.45	97.20	2.23	0.97
4	44.2	9.75	2.47	2.40	98.46	98.68	4.04	0.98
5	50.3	16.99	2.77	3.18	63.39	71.71	4.53	0.83
6	56	7.69	1.67	1.63	84.40	91.59	2.73	0.98
7	62.5	4.57	1.11	1.10	94.19	96.42	1.81	0.97
8	60	7.77	1.81	1.98	90.42	94.81	2.96	0.87
9	58	7.70	1.73	2.12	87.41	88.78	2.83	0.78

Table 4
RESULTS OBTAINED IN THE FERMENTATION EXPERIMENTS, CONTINUOUS REACTOR, SECOND DAY OF FERMENTATION

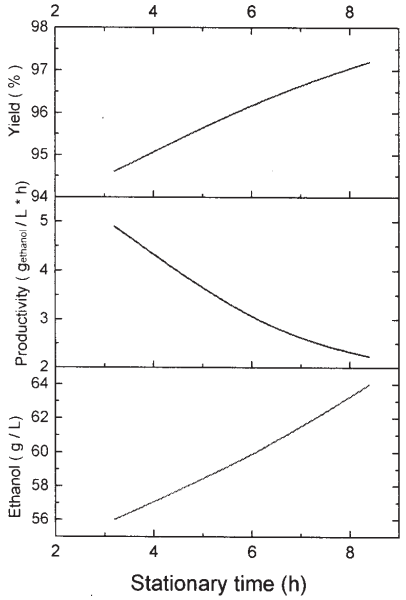


Fig. 4. The influence of the stationary time on the ethanol concentration, productivity and on the real yield of sugars transformation into ethanol, initial molasses concentration 242 g/L, temperature 32.5 °C.

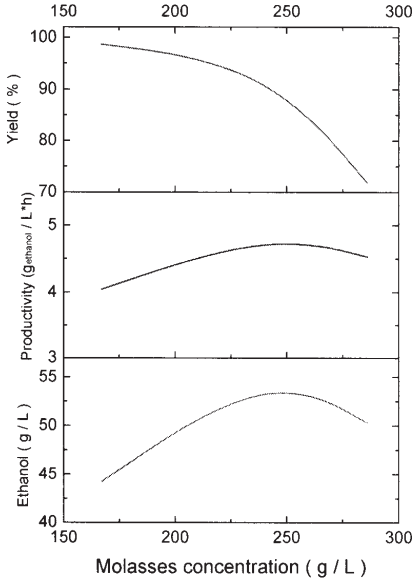


Fig. 5. The influence of the initial molasses concentration on the ethanol concentration, productivity and the real yield of sugars transformation into ethanol, stationary time 3 h, temperature 32.5°C

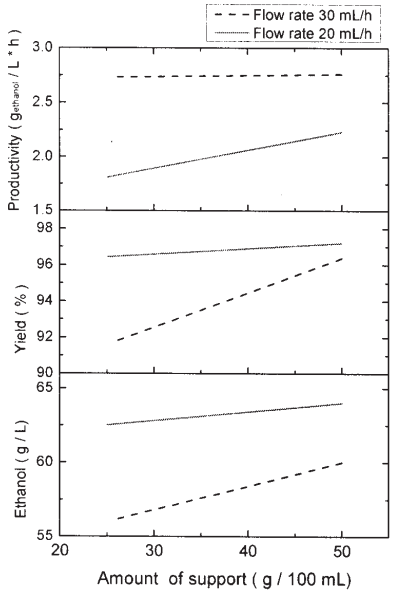


Fig. 6. The influence of the quantity of support on the ethanol concentration, productivity, and the yield of sugars transformation into ethanol temperature 32.5 °C, for two flow rates of the molasses solution

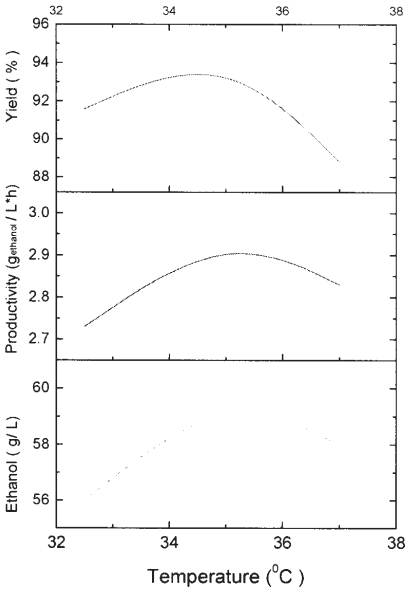


Fig. 7. The influence of fermentation temperature on the ethanol concentration, productivity, and the real yield of sugars transformation into ethanol, stationary time 4 h, molasses concentration 242 g/L, support quantity 25 g/100 mL fermentation medium

Conclusions

The aim of this study was to evaluate the suitability of the acrylamide-based cryogel, for uses in yeast immobilization when applied in ethanol fermentation of sugar molasses.

A method to obtain cryogel of polyacrylamide with entrapping of yeast cell was established. The support has a good polydispersity of pores and maintains the viability of the yeast cells.

The activation of the yeast before uses is of great importance. In the process of obtaining immobilized yeast much of these is embedded in the gel walls or in the closed pores and has no contact with fermentation media. During activation, the yeast that come in contact with the nutrient medium growths and develops new yeast cells which adhere very well to the support. A good density of viable cell can be obtained. Activation time require at least 24 h. The activation process was studied by gravimetric and scanning electron microscopy.

The obtained support was tested in a continuous reactor for fermentation of molasses solutions. The influence of various reaction parameters (concentration of molasses solutions, residence time, temperature of fermentation, the amount of support) were studied.

The values obtained for productivity and yield of sugars conversion into ethanol are comparable to those cited in the literature.

Acknowledgements: The work has been funded by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Romanian Ministry of Labour, Family and Social Protection through the Financial Agreement POSDRU/88/1.5/S/60203 and POSDRU/89/1.5/S/52432.

References

1.ARISTIDOU, A., PENTTILA, M., Curr. Opin. Biotechnol., 11, 2000, p.187.
2.ZALDIVAR, J., NIELSEN, J., OLSSON, L., Appl. Microbiol.Biotechnol., 56, 2001, p.17.
3.BELGIORNO, V., DE FEO, G., DELLA ROCCA, C., Waste Manag., 23, nr. 1, 2003, p. 1.
4.YAN L., SHUZO T., Appl. Microbiol. Biotechnol., 69, 2006, p. 627.
5.BAILEY, B.K., Performance of ethanol as a transportation fuel, in Handbook on Bioethanol: Production and Utilization, Applied Energy Technology Series, C.E. Wyman (Ed.), Taylor and Francis, 1996, p. 37
6.STANISZEWSKI, M., KUJAWSKI, W., LEWANDOWSKA, M., J. Food Eng., 82, 2007, p. 618.
7.C.A., CARDONA, O. J. SANCHEZ, Bioresour. Technol., 98, 2007, p. 2415.
8.ROSSILLO-CALLE, F., WALTER, A., Energ. Sustain. Dev., 10, 2006, p. 20.
9.DEMIRBAS A., Energy Source, 27, 2005, p. 327.
10.GRAEME M., WALKER, Bioethanol: Science and Technology of Fuel Alcohol, Graeme M. Walker & Ventus Publishing Aps, 2010, p. 11 – 44.
11.KOPSAHELIS, N., NISIOTOU, A., KOURKOUTAS, Y., PANAS, P., NYCHAS, G.J.E., KANELLAKI, M., Bioresour. Technol., 100, 2009, p. 4854.
12.KOURKOUTAS, Y., BEKATOROU, A., BANAT, I.M., MARCHANT, R., KOUTINAS, A.A., Food. Microbiol., 21, 2004, p. 377.
13.MARIGNETTI, N., FUMI, M.D., SILVA, A., BATTISTOTTI, G., POMINI, M., COLAGRANDE, O., Bitechnol. Technol., 11, 1997, p. 7.
14.WANG, R., KIM, J.H., KIM, B.S., PARK, C.S., YOO S.H., Bioresour. Technol., 102, 2011, p. 6370.
15.NAJAFPOUR, G., YOUNESI, H., ISMAIL, S.K. K., Bioresour Technol., 92, 2004, p. 251.
16.GHORBANI, F., YOUNESI, H., SARI, A. E., NAJAFPOUR, G., Renew. Energ., 36, 2011, p. 503.
17.MARGARITIS, A., KILONZO, P.M., Production of ethanol using immobilised cell bioreactor systems, In: Applications of Cell Immobilisation Biotechnology, V. Nedovic and R. Willaert Eds, Springer, Netherlands, 2005, p. 375 – 405.
18.BALLESTEROS, A., PLOU, F.J., IBORRA, J.L., HALLING, P.J., Proceedings of an International Symposium organized under auspices of the Worldng Party on Applied Biocatalysis of the European Federation of Biotechnology, the University of Cordoba, Spain, and the Spanish Society of Biotechnology Cordoba, Spain, April 19-22, 1998, p. 612
19.ZHENG, C., SUN, X., LI, L., GUAN, N. 2011. Bioresour Technol., 115, 2012, p. 208.

Manuscript received: 6.09.2012